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Analysis of Antitumoral and Antibiotic Drugs in Biological Fluids, Food and Pharmaceuticals through Micellar Liquid Chromatography

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Chapter 1

Introduction to Micellar Liquid Chromatography

1. Introduction

1.1. Role of therapeutic drug monitoring in medical care

Nowadays, the population is subjected to suffer a broad spectrum of diseases. Most of them cause symptoms that can strongly diminish the quality of life of the patient and may have lethal consequences [1]. In order to cure them or palliate their symptoms, many drugs have been developed by the pharmaceutical industry. Physicians have established treatments based on them by selecting the adequate medication and adjusting the dose, frequency and route of administration, as well as the expected duration. Although these therapies usually have a high degree of success, some cases of failure, adverse side effects, weak clinical effects or relapse have been noticed. Therefore, a useful strategy to straighten these situations must be applied [2]. The beneficial effects of a drug are maximized when the plasmatic concentration remains in its therapeutic range. At higher values, adverse effects due to overdose can appear, and under the low value, the drug is ineffective, and the patient may develop tolerance, as well as long-term adverse side effects. Both cases can lead to early interruption of the treatment. However, the blood concentration of a drug depends on diverse features, such as bioavailability, gastrointestinal absorption, drug interactions with other medications or endogenous compounds, metabolization, liver and renal activity, and elimination kinetics, which are related to a large number of personal factors, such as genetics, drug tolerance, immunology, environment, physiology, age, ethnicity, diet, health and lifestyle, among others. These factors strongly vary for each patient, and even for the same patient through time. As a consequence, the prescription of the same dose to diverse patients or at several stages of the therapy for the same patient can lead to different plasmatic concentrations and clinical results, complicating the success of the therapy. The implementation of therapeutic drug monitoring can help to resolve these situations [3,4].

Therapeutic drug monitoring (TDM) is a clinical practice, consisting on the determination of the biological behavior of a prescribed drug in the human body through time. It is carried out through the quantification of the drug and its main metabolites in physiological fluids (mainly plasma, serum and urine) at several times after the ingestion of the pharmaceutical formulation, and at several stages of the treatment. It can be used to establish

the pharmacokinetics for a specific patient and understand the influence of personal characteristics. Besides, this would be useful to explain and predict the clinical effects. The medical staff can use the information provided by TDM to adjust the prescription (dose, frequency and route of administration) for each patient at each stage of the treatment, in order to maximize the beneficial activity and limit the side effects, by maintaining the plasmatic concentration in the therapeutic range. Besides, it can be applied to detect noncompliance or chronic abuse, as well as in medical research, to investigate the influence of the personal factors in the pharmacokinetics. In fact, the main goal of TDM is the improvement of medical assistance for chronic and acute pathologies [4-7].

It is unnecessary to apply TDM for the majority of the medications. It is usually restricted for drugs with well-established therapeutic indications in clinical practice. The main reasons for implementing TDM depend on the situation of the patient and the characteristics of the drug, as indicated as follows [4,5]:

- Patient: abnormal response to the therapy, unexpected toxicity, suspected chronic abuse, lack of adherence, self-medication, taking other medications, changes in liver or renal function, and with altered metabolism (pregnant women, children, elder, and obese, among others).

- Drug: with a narrow therapeutic range, a weak correlation dosage-clinical response, a well-established relationship between the plasmatic concentration and the clinical effects, side effects similar to the symptoms of the disease, the impossibility to monitor the evolution of the patient by physical examination or conventional biochemical analysis, possible associated toxicity, and previously noticed inter- and intra-individual variability in the metabolization and clinical effects.

1.2. Monitorable drugs and related diseases

Epilepsy is characterized by recurrent paroxysmal discharges of brain neurons, provoking a long and persistent seizure without return of consciousness for nearly 30 min. It is a common disease, with a prevalence of 0.7-0.8% [8]. Epilepsy is treated with anticonvulsant

drugs, which must usually be taken throughout all the life. Anticonvulsants have narrow therapeutic ranges, strong interpatient variability and are subjected to adverse drug-drug interactions. The clinical reasons for their monitoring are their quantification at steady state, a variation in the dosage, the coadministration of other medication, changes in the heath stage and after emergency ingestion. The needing of TDM was established for phenobarbital, phenytoin and carbamazepine. These drugs have shown impressive results in comparative studies. They were regarded for a long time as the drugs of the first choice to reduce the incidence and severity of the tonic-clonic seizure. Besides, carbamazepine has also shown activity against bipolar disorders [9].

A cardiac arrhythmia is characterized by a deregulation of the heartbeat, caused by inhibition or abnormalities on the impulse conduction. The causes are heart failure, ischemia, metabolic abnormalities and the ingestion of several drugs of abuse [10]. Antiarrhythmic agents are prescribed to treat both auricular and ventricular arrhythmia and prevent their recurrence. Among them, lidocaine, disopyramide, quinidine, procainamide and its main active metabolite, N-acetyl-procainamide (acecainide), have been found as susceptible to be monitored in clinical practice, especially in patients with slow elimination kinetics, because of their narrow therapeutic range, and tachycardia effects in overdose [11].

Depression is a psychiatric disorder, with several grades of seriousness, affecting 20% of the population. It is caused by a chemical imbalance of several neurotransmitters in the nervous system. Depression is usually treated by combining psychotherapy and the prescription of chemical antidepressants. Some of the most powerful ones are tricyclic antidepressants (TCA), which are nowadays barely used because of their serious side effects, especially in overdose [12]. They act against anxiety, alimentary disorders, hyperactivity, enuresis and severe neuropathy pains. However, at high concentrations, they can produce adverse effects on the cardiovascular system. Imipramine and its main metabolite desipramine (dibenzoazepines) are used in the treatment of depressive affective disorders, especially in severe depression, and against depression with psychotic episodes. These drugs are also beneficial for patients at the depressive phase of bipolar disorder and schizophrenia. However, they are ineffective against, and can even stimulate hypomanic or manic attacks. Amitriptyline and its active metabolite nortriptyline (dibenzocycloheptenes) are administered to treat depression, and to palliate the

severe chronic neurogenic pain, phobia and panic disorders [13]. These compounds require monitoring due to their narrow therapeutic range, strong side effects, high interpatient variability in metabolization and elimination, potential drug-drug interaction, and lack of relationship dosage-response [14].

In current clinical practice, selective serotonin reuptake inhibitors (SSRIs) are considered to be the first-line in the treatment of depressive disorders. They show similar clinical efficiency as the TCAs, but have better-tolerated side effects and are safer in overdose [12]. The main ones are paroxetine, citalopram and fluoxetine. They are prescribed to treat depression, phobias, anxiety, panic, premature ejaculation, as well as obsessive-compulsive, eating, traumatic and premenstrual dysphoric disorders. However, in some cases, they also provoke undesirable side effects. Some occur at the beginning of the therapy, long-term, in cases of abrupt withdrawal (discontinuation effects) and irregular adherence. The most serious is suicidality, especially in adolescents, young adults and elderly patients. SSRIs can induce serotonin syndrome by drug-drug interaction with other antidepressant medication and may become ineffective through time [15-17]. TDM is recommended in SSRI treatments, due to the proven relationship between the plasmatic concentration and the therapeutic and side effects, their non-linear pharmacokinetics, high inter- and intra-patient variability in metabolization, and a large number of contraindications. Besides, it can be useful to detect cases of tolerance or lack of compliance. This is especially cost-effective for young and elderly patients [18].

Acetaminophen (paracetamol) is a widely prescribed mild analgesic (pain reliever) and antipyretic (fever reducer). It is a synthetic non-opiate derivative of p-aminophenol and a major metabolite of the nephropathic analgesic phenacetin. It acts in the brain by inhibiting the biosynthesis of prostaglandins, the inductors of the pain sensation. Acetaminophen is relatively safe at therapeutic doses, but secondary effects can appear by overdose, caused by chronic abuse or in patients suffering anaemia, renal malfunction or gastrointestinal disorders. These situations are relatively frequent, because this drug is often self-prescribed, and included in tablets with other active principles. Paracetamol intoxication causes a stimulation followed by depression of the central nervous system, hypothermia, hyperventilation, cardiovascular failure and liver disorders [19]. Therefore, it should be monitored in suspected cases of dose-related

toxicity, chronic abuse, kidney or liver malfunction, noncompliance and co-ingestion with other drugs [20].

Methylxanthines are a group of alkaloids with a mild stimulant activity of the respiratory, cardiac and central nervous system. They are prescribed as bronchodilators in the treatment of pulmonary disorders, such as chronic obstructive pulmonary diseases (COPD), asthma and apnea. Caffeine and theophylline are the most usually prescribed bronchodilators for teenagers and children and used as second-line for adults. However, they show serious toxicological effects at high concentrations, such as insomnia, nervousness, gastrointestinal intolerance and tachycardia. These drugs must be monitored due to the high number of potential drug-drug interactions, narrow therapeutic range, large inter variability in their metabolization, and the high probability of overdose because they are ubiquitous in the diet and highly addictive. In fact, they are the most consumed psychoactive drugs [21,22], illegally included in products for athletes. Therefore, their monitoring is also required for toxicology and doping control purposes [23].

According to their pharmacological characteristics, the most usually monitored drugs in current Hospital practice are anticonvulsants (phenobarbital, phenytoin and carbamazepine), antiarrhythmics (procainamide, N-acetyl-procainamide, lidocaine, disopyramide and quinidine), tricyclic antidepressants (imipramine and amitriptyline, and their active metabolites desipramine and nortriptyline, respectively), SSRIs (paroxetine, fluoxetine and citalopram), analgesics (acetaminophen), bronchodilators (caffeine and theophylline) [9,11,12,14,18,20,21,22]. In order to correctly carry out TDM, the clinical laboratories require useful, simple, economic, reliable and sensitive analytical methods for the quantification of these drugs in plasma, serum and urine.

1.3. TDM: an analytical challenge

Without doubt, immunoassay can be considered as the technology of choice for therapeutic drug monitoring, due to its selectivity, robustness, automation, high-throughput, less troubleshooting, low price, absence of matrix effect, easy integration into the core

laboratory, simplicity, no needing of qualified staff, easy comparison between laboratories, the ability to perform random-access testing, availability as commercial validated kits, better traceability and affordable maintenance contracts, including 7-days-per-week technical support. However, in the last years, LCMS has been introduced as an alternative method in the clinical laboratory, especially for drugs without adequate antibodies, or when commercial immunoassays are not available or show poor analytical quality. The advantages of LC-MS are: the versatility, the possibility to develop own assays in a short frame of time (then not depending on external manufacturers), the determination of many analytes in a single run (then effective for patient receiving a multidrug therapy), adaptable to the specific needing of the clinical laboratory, absence of interferences by cross-reactivity with metabolites and other substances, and usually higher analytical performances (selectivity, sensitivity, accuracy and precision). Besides, the cost per analysis is reduced if it is intensely used [7,24].

The analysis of urine, plasma and serum by HPLC is problematic, because they contain a large number of endogenous compounds with a wide range of molecular mass, hydrophobicity and charge, such as proteins, lipids, lipoproteins, carbohydrates, hormones, vitamins, uric acid, salts, nutrients, biometabolites, waste products and others. Most of them form molecular aggregates and remain suspended in water [25,26]. Moreover, some of these compounds tend to precipitate in the interparticle voids of the stationary phase, provoking a continuous degradation and increased clogging, which can be noticed by a loss of efficiency and augmentation of the pressure. Besides, endogenous compounds can coelute with the analytes.

Because of the complexity of the biological matrices, a careful sample pretreatment, usually based on extraction and/or protein precipitation, is required. These procedures are multistep, tedious, time-consuming (they represent the main part of the procedure) and need a large amount of sample and reagents [27]. Consequently, the sample preparation has many sources of variance, which lead to variable recovery, then requiring an internal standard. On the other hand, the solutions used in the sample preparation and the mobile phases use a high amount of toxic, flammable and volatile solvents, making them potentially harmful for the environment and laboratory staff, and increasing the expenditure associated with the waste segregation and treatment. These characteristics difficult the analysis of a large number of

samples per day for each operator. Therefore, the cost of the whole procedure is considerable. These are important drawbacks for a clinical laboratory, where a large number of samples must be daily analyzed [28].

Several approaches have been used to streamline the sample preparation as a way to automate the procedure, thus allowing the direct injection of the sample: on-line coupling solid-phase extraction [28], solvation chromatography [29], turbulent flow chromatography [30], column switching [31] and restricted access media [32]. However, these methods require high-cost specific instrumentation and a complex experimental assembly. Moreover, the cleanup/extraction step, although on-line, is already performed, and then recovery problems can also arise. Some authors have proven that MLC can provide a definitive solution to the disadvantages of HPLC [33-36].

1.4. Micellar liquid chromatography to determine drugs in biological samples

Surfactants are amphiphilic compounds, containing a nonpolar hydrocarbon-like chain (tail) bonded to a polar group (head). According to the charge of the polar group, surfactants are classified as anionic, cationic, neutral or zwitterionic. Solved in water, they have a propensity to locate in the air- or oily liquid-water interface, thus altering the interfacial tension and free energy [37]. At concentrations over the critical micellar concentration (CMC), the surfactant monomers tend to aggregate to form a spherical-shaped structure: the normal-phase micelle. The hydrophobic tails stay in the core, whereas the hydrophilic and, for ionic surfactants, charged heads form an outer layer, oriented to the water. The palisade layer is the intermediate region (miscellaneous), and it consists of the inner atoms of the hydrophilic groups and the first few carbon atoms. Therefore, they have nonpolar, polar (and electrostatic for ionic surfactants) and intermediately polar solubilization sites, respectively. The number of monomers per micelle (number of aggregation) remains nearly constant for each surfactant. Therefore, a variation of the quantity of surfactant results in the subsequent modification of the number of micelles, whereas the concentration of the free monomer remains nearly constant and equal to the CMC. The structure of the micelle is supported by the hydrophobic interactions between the carbon-chained tails of the monomers. The polar groups are water-solvated and

allow the suspension of the micelle in the solution, avoiding the fusion of several micelles and the precipitation of the monomers. Micelles are dispersed in water, forming a colloid solution named micellar solution. These solutions are heterogeneous at a microscopical glance, as they contain two differentiated environments: the pseudo micellar phase and the aqueous phase. Therefore, these solutions are able to solubilize compounds with a high range of hydrophobicity, charge and molecular mass [38,39].

Micellar liquid chromatography is a form of RP-HPLC, based on the use of micellar solutions as mobile phases [40]. The anionic surfactant sodium dodecyl sulfate (hydrophobic tail, C12- hydrocarbon chain; hydrophilic head, sulfate; aggregation number, 61; molar volume, 0.246 L/mol) is the surfactant of choice for the determination of drugs in biological samples, mainly because of its ability to denature proteins, low CMC (8.3 mM) and low Krafft point (15 C). Besides, sodium dodecyl sulfate (SDS) is an easily manageable powdered solid, commercially available at elevated purity, harmless, biodegradable, highly soluble in water and relatively inexpensive. Besides, its aqueous solutions hold low viscosity and give a relatively low increase in the system pressure [33,38,41,42].

The columns more usual in MLC contain C18 bonded silica phases. When a micellar solution is used as a mobile phase, the surfactant is incorporated into the stationary phase and strongly modifies its properties. The hydrocarbon tail is associated with the alkyl bonded phase, whereas the sulfate group is oriented to the mobile phase, creating a structure similar to an open micelle. The volume of the pores is reduced, and the surface area increases, resulting in an augmentation of the system pressure. An anionic outer hydrophilic layer appears, which affects the penetration depth of the solutes and provides some ion exchangeability, as in ion-pairing HPLC. On the other hand, the hydrophobicity of the inner alkyl-bonded phase is enhanced [33]. The analyses must be carried out using a saturated stationary phase, in order to keep constant the extent of the modifications induced by the use of a surfactant. The saturation of the column is achieved at SDS concentrations in the mobile phase higher than 10 mM [40]. On the other hand, the presence of the SDS-micelles introduces a new pseudo phase and a secondary equilibrium in the mobile phase. These effects provoke a strong influence in the chromatographic behaviour of the solutes. The analytes can be involved in a larger variety of interactions if compared to hydro-organic RP-HPLC. In the mobile phase, they can remain in

the bulk mobile phase (polar interaction), be associated to the free monomers (miscellaneous), interact with the surface of the micelle (hydrophilic and electrostatic), be inserted in the palisade layer of the micelle (miscellaneous), or penetrate into its inner core (hydrophobic). In the stationary phase, the analytes can interact with the reverse phase (hydrophobic), the polar head of the adsorbed surfactant (hydrophilic and electrostatic) and the silanol groups (electrostatic). A representation of the organization of the molecules of surfactant in a micellar mobile phase with anionic surfactant SDS and reverse-phase C18 is shown in Ref. [38]. The intensity of these interactions depends on the hydrophobicity, steric factor and charge of the analytes. These parameters are influenced by acidic/basic side reactions, and then the pH must be properly adjusted and maintained constant throughout the whole analytical run using a buffer. The retention mechanism has been explained using a three-phase partitioning model, and then, three partition equilibria are considered for the solutes: micelle pseudo phase 4 bulk mobile phase, bulk mobile phase 4 stationary phase and micelle 4 stationary phase [33,40,42,43].

Pure micellar mobile phases have a limited elution power, because of their high hydrophilicity, and then they are unable to elute even moderately polar drugs at working retention times. Besides, the slow transfer between the stationary phase and the micelles results in a peak broadening, and then a decreasing of the efficiency. In order to avoid these drawbacks, several authors have proposed the addition of an organic solvent, such as acetonitrile or short-chained mono alcohols, resulting in hybrid micellar mobile phases. These organic solvents alter the nature of the micelle, and the stationary and mobile phases. Moreover, they influence the retention mechanism, so that the elution power and the peak shape are usually improved. As long as the micelles persist in the mobile phase, the three-phase theory is also applicable to model the retention mechanism, although considering the presence of a new variable. This multiplicity of interactions and environments complexes the retention mechanism and improves the versatility of MLC, and allows the resolution of complex mixtures of drugs with different charges and polarities in a single chromatographic run, even using an isocratic mode [33,35,36,42].

The retention mechanism of the drugs in MLC depends on several experimental factors, such as the column type, concentration of SDS, kind and proportion of organic solvent, and

pH. Therefore, it is desirable to investigate their effects on the main chromatographic parameters of the resulting peaks, such as retention time, efficiency and asymmetry. Indeed, they must be set during the development of the method to resolve the analytes adequately. The stability and reproducibility of the retention mechanism in MLC under an isocratic mode have allowed the prediction of these parameters from the composition of the mobile phase employing a mathematical model, based on the three-phase theory. This can expedite the resolution of complex mixtures [35,40,42].

Because of its high content in salts, SDS-micellar mobile phases are incompatible with mass spectrometry. Micellar liquid chromatography can be coupled to the most common and affordable detectors, such as UV-Visible, fluorescence, phosphorescence, chemiluminescence and electrochemical. In all cases, the sensitivity is enhanced, and the interferences from impurities diminished, because of the micellar environment. Besides, the yielding of chemical or photoinduced derivatization reactions usually increases in an organized medium. Therefore, MLC also contributes to the improvement of the sensitivity and the selectivity at this stage [34,40].

The remarkable properties of SDS-micellar solutions are considerably useful for the sample pretreatment of biological fluids [42]. Hydrophobic and non-water soluble endogenous compounds can be dissolved and do not need to be removed before the injection. Micelles tend to bind proteins and other macromolecules competitively, provoking their denaturation, solubilization and the release of linked drugs. When these compounds are injected, they are free to interact with the stationary phase and can be determined, whereas micelle-bound proteins are harmlessly eluted near the dead time, rather than precipitating into the column. After a simple dilution in a pure micellar medium and filtration, these biological fluids can be directly injected, without the aid of extraction or cleanup steps. Thus, the sample is quantitatively introduced in the chromatographic system [33-36,38]. However, because of the modification of the stationary phase and the presence of salts, special care with the chromatographic instrumentation is required when dealing with micellar mobile phases [34,40].

The use of MLC leads to procedures relatively easy-to-handle, inexpensive, short-time, safe for the laboratory staff, and ecofriendly. This corresponds to the current trend in analytical chemistry, the societal awareness about workplace safety and ecology, and the need by companies and public agencies to reduce costs of analytical services without affecting the quality, imposed by the decrease of the budgets by the economic crisis. The improvement of the analytical throughput, the high versatility (inclusion of new drugs in the portfolio of analytical services) and the amelioration of the practical aspects make more attractive the implementation of MLC for TDM in clinical laboratories. Besides, once the method has been developed, routine analyses can be conducted by not LC-specialized operators, because of their simplicity and similitude, thus allowing the rotation of staff through the area. Also, the cost per clinical results is reduced if the laboratory has a strong workload, thus justifying the acquisition of the equipment from an economic point of view [35,36,43].

Because of its advantages in both sample preparation and chromatographic separation, MLC has been demonstrated as a suitable technique for bioanalytical studies. Indeed, it has been successfully applied for the last years to determine many drugs from different groups and their biometabolites in plasma, serum, urine and gastric fluids, such as anaesthetics, analgesics, antiarrhythmics, antibiotics, anticonvulsants, antidepressants, anti-inflammatory, antiretroviral, antiviral, antitumoral, bacteriostats, bronchodilators, catecholamines, cardiovascular, diuretics, drugs of abuse, hormones, hypnotics, poisons, stimulants, stomach protector and vitamins [33-36,38,44].

1.5. MLC for the determination of monitorable drugs in plasma, serum and urine

The purpose of this review is to provide a survey through the literature about the use of MLC for the quantification of several monitorable drugs in biological fluids: the anticonvulsants phenobarbital, carbamazepine, and phenytoin [45]; the antiarrhythmics procainamide, its main metabolite, N-acetyl procainamide (NAPA) [46], disopyramide, lidocaine and quinidine [47]; the tricyclic antidepressants (TCA) imipramine [48] and amitriptyline [49], and their corresponding biometabolites desipramine and nortriptyline, respectively; and the bronchodilators caffeine and theophylline in serum [50]; the SSRIs

paroxetine, citalopram and fluoxetine in plasma and urine [51]; as well as the analgesic acetaminophen in serum and urine [52]. The first part describes the methodology applied to optimize the analytical procedures (experimental protocol and instrumental conditions), considering both phases of sample pretreatment and chromatographic run, as well as an overview about the validation process. In the second part, we detail and discuss the selected experimental parameters and the validation results. The review also examines the performances, advantages, usefulness and reliability of these methods. A comparison with HPLC procedures to determine the same anticonvulsants [53], antiarrhythmics [54], TCA [55], acetaminophen [56] and bronchodilators [57] in serum; paracetamol in urine [58]; and the SSRIs in plasma and urine [59] has been made. The applicability of MLC for the routine analysis of biological samples in the field of monitoring, as well as in forensic and clinical analysis, is evaluated.

2. Methodology for the optimization of the analytical procedures

In order to develop a method to determine drugs in biological fluids by MLC, the sample preparation and the chromatographic conditions must be optimized by the following criteria: maximal resolution between the drugs without interferences from endogenous compounds, peak shape nearly Gaussian and narrow, minimum analysis time, enough sensitivity to detect the analytes at their therapeutic levels, high recovery, and low variability of the experimental results. Easy experimental procedure and instrumental assembly are also desirable. The method should also be inexpensive, safe for the laboratory staff and ecofriendly. Only mobile phases running under isocratic mode were investigated because of their higher simplicity.

The most important parameters to-be-optimized are: sample dilution ratio, column type, the composition of the mobile phase (surfactant amount, kind and concentration of the organic solvent, and pH), and the detection conditions.

2.1. Structure and physico-chemical parameters of the drugs

The maximum information about the analytes is necessary to dispose of a starting point to develop the analytical method. The structures (Figure-MS-1), acid/basic activity and hydrophobicity are helpful to elucidate the composition of the mobile phase, whereas the electrochemical and spectrophotometric properties must be known to choose the detector and investigate the optimal detection conditions. Besides, the therapeutic ranges and the pharmacological parameters are useful to determine the expected concentrations through the treatment. These parameters can be seen in Table 1 [15,17,22,60-64].

2.2. Selection of the pH

The charge and the polarity, and then the nature of electrostatic and hydrophobic interactions with the stationary phase and the SDS-micelles, depends on the pH for compounds with acidic, alkaline or polar bonds. Therefore, this parameter is crucial for chromatographic behaviour and must be the first to be considered to optimize the composition of the mobile phase. In C18-bonded silica columns, the working range is 2.0-9.5 [65]. However, the extreme values were avoided, and then the study was restrained between 3.0 and 7.5.

The pH must be optimized, considering the desired charge of the solute. If the substances under study do not show any advantage at a specific pH, a neutral value is preferred in order to extend the column lifespan. According to their pKa and structure, acetaminophen, the anticonvulsants and the bronchodilators are neutral, whereas the other drugs are positively monocharged in the whole 3.0-7.5 range. Therefore, no variation of their chromatographic behaviour is expected. This was further confirmed by empirical studies. Finally, the pH was fixed to 7 for all the studied drugs. A phosphate buffer was selected to adjust the pH, as it is effective in the 6e8 range [45-52].

Under these conditions, the drugs are expected to interact with the modified stationary phase and SDS-micelles, and then the retention time and efficiency would decrease at

increasing concentrations of SDS. For these reasons, mobile phases containing more than 0.20 M SDS are not recommended [41]. The mobile phase must contain more than 10 mM, to assure the saturation of the column [40].

2.3. Selection of the organic modifier

The addition of an organic solvent to the mobile phase is a very common strategy in MLC using SDS. The most used are acetonitrile, methanol, ethanol, 1-propanol, 1-butanol and 1-pentanol, in increased order of hydrophobicity. These solvents alter the properties of the mobile phase, the micelles, and the modified stationary phase [42].

The organic solvent interpenetrates the silica-bonded C18-alkyl chains to form a simple monolayer, with the hydroxyl/cyano groups oriented towards the mobile phase. Besides, it partially replaces the surfactant monomers. Consequently, the stationary phase becomes more polar, less rigid, and its negative surface charge diminishes. On the other side, hybrid micellar mobile phases are more hydrophobic. These combined effects facilitate the transfer of the analytes from the micelle and stationary phase towards the mobile phase. Consequently, the elution strength of the mobile phase increases and the partition equilibria are accelerated, thus reducing the retention time, broadness and asymmetry of the chromatographic peaks. These effects are enhanced as the hydrophobicity and the proportion of the organic solvent, and is appreciable even at low quantity. The effect of the organic solvents in the micellization process can be seen in Ref. [42].

The addition of a high proportion of organic solvent causes the disintegration of the micelles and the solubilization of the free monomers, producing a high sub-micellar solution. The maximal concentration compatible with the existence of the micelles is: acetonitrile, methanol and ethanol 30%, 1-propanol, 22%; 1- butanol, 10%; and 1-pentanol, 6%, and then hybrid mobile phases always contain proportions of alcohols under these values [42].

Consequently, the amount of flammable, toxic and volatile organic solvent in mobile phases is significantly lower than typically used in RP-HPLC. Besides, the interaction of the organic solvents with SDS-micelles increases their solubility and reduces their evaporation

rate. Hence, the mobile phase can be preserved for a long period with no significant changes in its composition [43].

The selection of the organic solvent is performed by means of the expected retention of the drug on the column. In SDS-C18 stationary phases, organic solvents with high hydrophobicity are selected for drugs with elevated values of log Po/w and a positive charge.

2.4. Optimization of the SDS/organic solvent concentration

The amount of SDS and organic solvent must be optimized to obtain the maximum resolution and robustness on minimum analysis time, with a Gaussian peak shape. These three parameters are used to express the quality of a chromatographic elution.

The use of a sequential one-by-one strategy is discarded because it requires too much effort and can be difficult due to the occurrence of several local maxima. These two concentrations were better simultaneously optimized using an interpretative strategy. In this case, the experiments are previously designed, and the information is used to fit a model predicting the parameters that define the chromatographic behaviour (retention factor, efficiency, asymmetry, elemental resolution and global resolution) of the analytes and their mixture. This strategy only requires to set up the appropriate experiments and provides accurate results by testing a minimum of mobile phases.

The most important to successfully implement an interpretative strategy is to dispose of reliable and accurate mathematical models describing the chromatographic parameters. The models used to predict the chromatographic behaviour of the studied drugs are described below and are valid using mobile phases running under isocratic mode.

Table 1. Physicochemical and pharmacological parameters of the studied monitorable drugs [15], [17], [22], [60], [61], [62], [63], [64].

Drug	Group	pKa	Log Po/w	Charge at pH 7	Therapeutic range (µg/mL)	Toxic level (µg/mL)	Half-life	Peak plasma	Time to steady state
Phenobarbital	Anticonvulsant	7.4	1.47	0	15 to 40	>40	1.5–3 days	4–12 h	15–25 days
Carbamazepine	Anticonvulsant	7.0	2.45	0	2 to 12	>12	10–26 h	3–12 h	2–10 days
Phenytoin	Anticonvulsant	8.3	2.47	0	10 to 20	>20	7–42 h	3–9 h	7–10 days
Procainamide	Antiarrhythmic	9.2	0.88	+1	4 to 10	>16	2.5–4.5 h	1–2 h	12–18 h
NAPA	Antiarrhythmic	9.1	0.99	+1	6 to 20	>20	6–9 h	1.5–4 h	Not applicable
Disopyramide	Antiarrhythmic	8.4	2.58	+1	2 to 5	>5	5–10 h	2 h	24–48 h
Quinidine	Antiarrhythmic	4.2/8.8	3.44	+1	2 to 5	>6	4–11 h	1 h	30–35 h
Lidocaine	Antiarrhythmic	7.9	2.44	+1	1.5 to 5.0	>5	1.5 h	15 min	6–12 h
Imipramine	TCA	9.5	4.80	+1	0.15 to 0.25	>0.5	9–24 h	3.4 h	2–5 days
Desipramine	TCA	10.2	4.90	+1	0.15 to 0.30	>0.5	12–54 h	3–6 h	6–11 days
Amitriptyline	TCA	9.4	4.92	+1	0.12 to 0.15	>0.5	17–40 h	2 h	3–8 days
Nortriptyline	TCA	9.7	4.51	+1	0.50 to 0.15	>0.5	15–90 h	3 h	4–20 days
Citalopram	SSRI	9.5	3.50	+1	0.02–0.20	>0.5	33 h	2–4 h	7 days
Paroxetine	SSRI	9.9	3.60	+1	0.01 to 0.075	>0.4	12–40 h	5 h	10–14 days
Fluoxetine	SSRI	9.8	4.05	+1	0.150 to 0.500	>0.9	4 – 6 days	4–8 h	30 - 60 days
Paracetamol	Analgesic	9.5	0.46	0	5 to 20	>150	1–3 h	0.5–3 h	10–20 h
Caffeine	Bronchodilator	0.6/14	- 0.07	0	5 to 20	>80	2–10 h	0.5 h	Variable
Theophylline	Bronchodilator	3.5/8.7	- 0.02	0	10 to 20	>20	3–13 h	6.5 h	15–55 h

2.4.1. Modelling of the retention factor

In SDS-micellar mobile phases, the retention factor (k) of the solutes can be predicted using empirical or mechanistic mathematical models. In the first case, the constants of the equations are just experimental ones, and in the second case, they have a direct physicochemical meaning.

Several authors have demonstrated a hyperbolic relationship of the retention factor with SDS and organic solvents. The equation (1) provides adequate values for both polar and moderately hydrophobic compounds [41]:

(1) $1/K = Co + C_1[SDS] + C_2\varphi + C_{12}[SDS]\varphi$

where [SDS] is the concentration of the surfactant in mol/L, φ the proportion (% v/v) of organic solvent, Co an indication of the retention factor without micelles, and C_1, C_2 and C_{12} the effect of each factor and their interaction, respectively. This equation can also be applied for pure micellar mobile phases. For highly hydrophobic compounds, it provided significant errors, and a quadratic term was added, leading to the following equation [66]:

(2) $1/K = Co + C_1[SDS] + C_2\varphi + C_{12}[SDS]\varphi + C_{22}\varphi^2$

The constant C_{22} measures the quadratic effect of the proportion of the organic solvent.

For each drug, the equations are adjusted using the values of retention factor (k) obtained by separately determining each drug using various SDS/organic solvent amounts. The number of tested mobile phases must be at least equal to the number of constants, and an extra experiment is recommended to examine the bias of the theoretical data. Therefore, 5 and 6 assays were performed to fit the equations (1) and (2), respectively, less than required using a sequential approach.

For equation (1), a full factorial design plus the central point ($2^2 + 1$) is desirable. Thus, the five mobile phases should contain a combination between the minimum and the maximum of the considered interval of SDS and organic solvent concentrations, plus the intermediate values [41]. For equation (2), the sixth experiment ought to be at lower values of surfactant and organic solvent. Both models provide deviations under 4% by interpolation [66]. The concentration range usually studied in MLC are: SDS, 0.05-0.15 M; 1-propanol, 2.5-12.5%; 1-butanol, 1-7% and 1-pentanol, 2-6% [38]. The empirical constants are related to partition constants describing the partitioning of the solutes with the three environments (stationary phase, bulk mobile phase and micellar pseudo phase) and the influence of the organic solvent. This has led to the development of a mechanistic model with equations directly based on these constants, allowing a better understanding of the retention mechanism. The equations (1) and (2) can be rewritten as equations (3) and (4), respectively [35]:

$$k = \frac{K_{AS}\dfrac{1}{1 + K_{AD}\varphi}}{1 + K_{AM}\dfrac{1 + K_{MD}\varphi}{1 + K_{AD}\varphi}[M]} \quad (3)$$

$$k = \frac{K_{AS}\dfrac{1}{1 + K_{AD1}\varphi + K_{AD2}\varphi^2}}{1 + K_{AM}\dfrac{1 + K_{MD}\varphi}{1 + K_{AD1}\varphi + K_{AD2}\varphi^2}[M]} \quad (4)$$

K_{AS} is the product of the partition coefficient of the analyte between the stationary and bulk mobile phase times the quotient between the phase ratio (then the retention factor without micelles), whereas K_{AM} is the solute-micelle association constant. K_{AD} and K_{MD} measure the increase of affinity of the solute by the bulk mobile phase and the micelle, respectively, because

of the occurrence of the organic solvent, if compared to a pure micellar solution, and then quantify the subsequent variation of K_{AS} and K_{AM}.

The equation (4) provides important deviations at higher proportions of organic solvents. Therefore, equation (5) was proposed as an alternative. This equation has no empirical equivalent and was built by considering the modification of the stationary phase due to the organic solvent [41]:

$$k = \frac{K_{AS}\dfrac{1 + K_{SD}\varphi}{1 + K_{AD}\varphi}}{1 + K_{AM}\dfrac{1 + K_{MD}\varphi}{1 + K_{AD}\varphi}[M]} \quad (5)$$

where K_{SD} quantifies the increase of the affinity of the solute by the stationary phase, because of the insertion of the organic solvent. This equation provides accurate values of the retention factor in hybrid SDS-micellar mobile phases for analytes in a wide range of hydrophobicity. The adjustment of these equations has to be performed using the same rules as explained for the empirical models.

2.4.2. Modelling of the peak profile

The peak profile is related to the mass transfer between the stationary phase and the bulk mobile phase and micelles, which is rather complex. Its quality is evaluated through efficiency (N) and asymmetry (B/A). The asymmetry is determined by the quotient between the distance (min) from the retention time to the tail (B) and front (A) endpoint time of the peak at the 10% of the peak height, respectively [65]. The efficiency was calculated using the Foley and Dorsey equation, valid for values of B/A < 2.76 [67]:

$$k = \frac{K_{AS}\dfrac{1}{1 + K_{AD1}\varphi + K_{AD2}\varphi^2}}{1 + K_{AM}\dfrac{1 + K_{MD1}\varphi + K_{MD2}\varphi^2}{1 + K_{AD1}\varphi + K_{AD2}\varphi^2}[M]} \quad (6)$$

A good peak shape has high efficiency (low broadness) and asymmetry close to 1.

The most accurate peak-profile predictions are achieved using a distorted Gaussian model. It was assumed that the signal of the chromatogram versus time, when the analyte is eluted, follows a normal curve centered at the retention time, whose standard deviation polynomially depends on the distance time - retention time [68]:

$$h(t) = H_0 e^{-0.5\left(\frac{t-t_R}{s_0+s_1(t-t_R)+s_2(t-t_R)^2+\cdots}\right)^2} \quad (7)$$

H_0 is the height at the retention time, which depends on the sensitivity and the concentration, whereas s_0 quantifies the standard deviation of a typical Gaussian curve, thus describing the width of the central region. The parameters measure the lack of the symmetry: positive values indicate a tailing, while a negative value points to a fronting. Polynomials with higher degree provide more accurate prediction but are more difficult to fit. In general, a one-degree polynomial is enough at asymmetries between 0.4 and 2.5. The values of efficiency and asymmetry can be easily calculated from S_0 and S_1:

$$s_0 = 0.47\frac{\left(\frac{41.7(1+t_R)^2}{N\left(1.25+{}^{B}/_{A}\right)}\right)^{0.5}}{\left(1+\frac{1}{{}^{B}/_{A}}\right)\left(1-\frac{{}^{B}/_{A}-1}{{}^{B}/_{A}+1}\right)} \quad (8)$$

$$s_1 = 0.466\frac{B/A - 1}{B/A + 1} \quad (9)$$

For each drug, the equation (7) must be fitted, taking the empirical values of efficiency and asymmetry obtained using the same experiments performed for the modelling of the retention factor.

The equations used to predict the retention factor and the peak shape of the drugs determined in the same run can be combined to draw simulated chromatogram. Therefore, the operator can visualize the changes in these parameters when the concentration of SDS and organic solvent vary. The reliability of these simulations, assessed for different groups of compounds by comparing the experimental and simulated chromatograms, has been confirmed by the high similarities between them [35].

2.4.3. Modelling of the global resolution

The elementary resolution quantifies the separation degree between two adjacent peaks (R_{ii+1}). It is measured by the valley peak criterion, which considers both retention time and peak shape [69]. The parameter R_{ii+1} ranges from 0 (same retention time) and 1 (the two peaks are separated by a null baseline). Each analyte has associated two R_{ii+1}, one with the solute eluting immediately before and another one with that eluting immediately after (except the first and the last solutes, which have just one).

The global resolution (Z) of a chromatogram evaluates the degree of separation between all the analytes. It is usually calculated as the unnormalized product of the elementary resolutions calculated for each analyte [70]. This criterion can be used with values of elementary resolution previously normalized from 0 to 1. Z ranges from 0 (full overlapping of at least two peaks) and 1 (total separation of all the peaks). Both elementary and global resolutions are better appreciated using simulated chromatograms.

2.4.4. Selection of the SDS/organic solvent concentration

Once the adequate equations have been adjusted, the operator is able to envisage the values of retention time, efficiency, asymmetry and resolution at intermediate SDS/organic solvent concentrations by interpolation. Besides, the operator can visualize this information using simulated chromatograms and 3-D plots of Z v.s. [SDS] and φ.

The mobile phase is primarily selected at the maximal global resolution, but on several occasions, mobile phases with lower Z are accepted if this results in a significant reduction of the analysis time. It is desirable to select the mobile phase situated on a plateau of the graph, in order to minimize the effect of unnoticed small variations of SDS/organic solvent amounts due to random errors arising during its preparation. Anyway, the suitability of the selected mobile phase must be checked by the corresponding simulated chromatogram. Besides, the use of mobile phases that elute all the analytes at retention times more than two or three times the dead time is recommended, in order to avoid coelution with matrix compounds.

The interpretative optimization strategy here described can be easily carried out assisted by computer with the aid of the Michrom software [71], which is able to mimic the methodology followed by experienced chromatographers with reduced time and effort [40].

2.5. Optimization of the detection conditions

Both UV-Visible absorbance (UV) and pulse amperometric (PAD) detection were considered to acquire the signal for the determination of the studied TCAs [48,49] and acetaminophen [52], fluorescence was used to determine the SSRIs [51], and the other monitorable drugs were detected by UV absorbance [45-47,50]. No derivatization step was required. When PAD was used, an inert electrolyte was added to the mobile phase.

The spectrophotometric and electrochemical properties of a solved compound show a high dependence on the surrounding matrix and the chemical environment. For instance, several studies have shown that the maximum absorbance or excitation and emission wavelengths and the absorptivity or fluorescence emission, respectively, increased in a micellar

medium, if compared to a hydro-organic one. The oxidation potential may also change, depending on the affinity of the reduced and oxidized forms for the micelles [34,40]. Therefore, the detection conditions must be empirically optimized by determining the analytes using the selected chromatographic conditions. In this case, the absorption spectrum [45-47,50,52], the intensity at increasing oxidation potentials [48,49,52] or the fluorescence excitation and emission spectra [51], depending on the selected detector, were measured. The optimal wavelengths or the oxidation potential was chosen to maximize the signal-to-noise ratio. Moreover, it is suitable to select a high wavelength or a low oxidation potential to avoid interferences.

2.6. Sample preparation

The studies were conducted using blood and urine samples collected from patients taking the considered drugs. The Review Board approval from the Ethics Committee Samples was obtained, as well as the consent of patients and doctors. Serum or plasma was extracted from blood. The collected aliquots were introduced in sterile vials and stored at - 20 C until analysis [45-52].

The introduction of a filtered sample of a physiological fluid into the chromatographic system is possible, because proteins and other non-soluble macromolecules are solubilized in the micellar mobile phase, and would not precipitate into the column. However, serum, plasma and urine contain suspended molecular aggregates, which may cause the early obstruction of the pores of the filter, and then difficult the obtaining of a reasonable volume of reproducible and representative solution. Besides, when these biological fluids are directly injected, a wideband and several adjacent peaks, produced by the elution of proteins and other endogenous compounds, appear at the beginning of the chromatogram. This can affect the detection of the less retained analytes. Additionally, the injection of a large amount of plasma, serum or urine sample shortens the lifespan of the column and can provoke irreproducible changes in the properties of the stationary phase, due to the absorption of matrix compounds. These effects can be lessened by the dilution of the previous sample filtration, although the sensitivity is also

diminished. Consequently, the optimal dilution ratio was selected considering these two opposite effects [38].

Table 2. Optimized experimental conditions for the sample preparation and the chromatographic detection in MLC and HPLC procedures.

	Dilution solution (dilution ratio)	**Extraction method**	**Detection conditions**		**Reference**	
Drug	**MLC**	**HPLC**	**MLC**	**HPLC**	**MLC**	**HPLC**
Phenobarbital/Carbamazepine/Phenytoin	0.9% NaCl (1/10)	Protein precipitation with CH_3CN	220 nm (UV)	240 nm (UV)	[45]	[53]
NAPA/Procainamide/Disopyramide	0.9% NaCl (1/10)	Extraction with CH_2Cl_2	280 nm (UV)	205 nm (UV)	[46]	[54]
lidocaine/quinidine	0.9% NaCl (1/10)		214 nm (UV)		[47]	
Imipramine/Desipramine	None	Protein precipitation with CH_3CN	+650 mV (PAD) +650 mV (PAD)	MS	[48]	[55]
Amitriptyline/Nortriptyline	Mobile Phase (1/10)				[49]	
Citalopram	Mobile phase (1/5)	Protein precipitation with CH_3CN and on-line solid extraction using 0.2% HCOOH in water/CH_3CN (9:1, v/v) as carrier solution	236/310 nm (FLD)		[51]	[59]
Paroxetine			295/310 nm (FLD)	MS		
Fluoxetine			230/305 nm (FLD)			
Acetaminophen (serum)	Mobile Phase (1/10)	Off-line solid phase extraction	265 nm (UV)	220 nm (UV)	[52]	[56]
Acetaminophen (urine)		Protein precipitation with NH_4OH	+500 mV (PAD)	MS		[58]
Caffeine/Theophylline	Mobile Phase (1/10)	Online solid phase extraction	272 nm (UV)	MS	[50]	[57]

3. Application of MLC for the determination of monitorable drugs in physiological fluids

In this survey, we describe the development and validation of several methods dealing with the determination of monitorable drugs in biological samples: phenobarbital, carbamazepine, and phenytoin (anticonvulsants) in serum [45]; procainamide and its metabolite NAPA (antiarrhythmics) in serum [46]; disopyramide, lidocaine and quinidine (antiarrhythmics) in serum [47]; imipramine and desipramine (TCAs) in serum [48]; amitriptyline and nortriptyline (TCA) in serum [49]; paroxetine, fluoxetine and citalopram (SSRIs) in plasma and urine [51]; acetaminophen (analgesic) in serum and urine [52]; and caffeine and theophylline (bronchodilators) in serum [50]. The groups were designed in order to determine the drugs potentially prescribed together simultaneously.

The operational conditions common to all methods were: injection volume, 20 mL; C18 column (250 4.6 mm, particle size 5 mm, pore size 10 nm); surfactant, SDS; and pH of the mobile phase, 7; except for the SSRIs, where a 150-mm column was used. The other chromatographic conditions (mobile phase composition and detection parameters) and the dilution ratio were optimized for each group as indicated in sections 2.3, 2.4, 2.5 and 2.6, respectively. For the optimization of the mobile phase, only pure micellar solutions, or hybrid micellar solutions of 1-propanol, 1- butanol or 1-pentanol were tested. The flow rate was initially set to 1 mL/min. For each group, the finally selected experimental conditions for both sample preparation and chromatographic analysis steps (with the resulting retention times) are shown in **Tables 2** and **3**, respectively. The validation procedure was carried out, as indicated in Refs. [69], and the obtained results are shown in **Table 4**. A good linearity ($r > 0.992$) was obtained for all the studied drugs. The methods were successfully applied to the analysis of samples from patients or healthy volunteers, and the results were similar to those provided by the reference method [45-52].

The specific strategy followed to establish the chromatographic conditions, and the applications of the methods are described below.

Table 3. Optimized chromatographic conditions and analysis times for MLC and HPLC procedures.

	Mobile phase surfactant and organic modifier	Mobile phase buffer	Stationary phase (temperature)	Mobile phase	Flow-rate (mL/min) and Running mode		Retention time (min)	Analysis time (min)	Ref.	
Drug	**MLC**	**MLC**	**HPLC**	**HPLC**	**MLC**	**HPLC**	**MLC**	**HPLC**	**MLC**	**HPLC**
Phenobarbital/Carbamazepine/Phenytoin	0.05 M SDS/7% 1-butanol	0.01 M Na_2HPO_4	C18 (room)	7 mM acetate buffer (pH 7)/ CH_3CN (78:22, v/v)	1 (isocratic)	2 (isocratic)	3.8/5.7/9.5	30	[45]	[53]
NAPA/Procainamide	0.05 M SDS/ 1% 1-butanol	0.01 M Na_2HPO_4	Cyano (40 °C)	CH_3CN/CH_3OH/ phosphate buffer (pH 7)/triethylamine (60/7/32.3/0.7, v/v)	1 (isocratic)	2 (isocratic)	6.5/10.5	6	[46]	[54]
Disopyramide/Lidocaine/ Quinidine	0.15 M SDS/7% 1-butanol	0.01 M Na_2HPO_4			1 (isocratic)		3.9/6.2/9.8		[47]	
Imipramine/Desipramine	0.15 M SDS/6% 1-pentanol	0.01 M Na_2HPO_4 + 0.001 M NaCl	C18 (room)	0.1% HCOOH in water/0.1% HCOOH in CH_3CN	1.5 (isocratic)	1 (gradient)	12.5/14.5	2	[48]	[55]
Amitriptyline/Nortriptyline	0.15 M SDS/6% 1-pentanol	0.01 M Na_2HPO_4 + 0.001 M NaCl			1.5 (isocratic)		12.0/13.5		[49]	
Citalopram/Paroxetine/Fluoxetine	0.075 M SDS/6% 1-butanol	0.01 M Na_2HPO_4	C18 (30 °C)	0.2% HCOOH in water/CH_3CN (70/30, v/v)	1 (isocratic)	0.2 (isocratic)	10.2/14.2/17.2	11	[51]	[59]
Acetaminophen (serum)	0.02 M SDS	0.01 M NaH_2PO_4+0.001 M KCl	C18 monolithic (23 ± 1 °C)	0.1 M KH_2PO_4 in water (pH 2.41)/CH_3CN (95/5 v/v)	1 (isocratic)	9 (isocratic)	4.3	5	[52]	[56]
Acetaminophen (urine)			Phenyl (room)	0.1% HCOOH in water		0.2 (isocratic)				[58]
Caffeine/Theophylline	0.05 M SDS/2.5% 1-propanol	0.01 M KH_2PO_4	C18 (room)	0.1% HCOOH in CH_3CN/0.1% HCOOH in water (20/80 v/v)	1 (isocratic)	4 (isocratic)	3.6/4.9	3	[50]	[57]

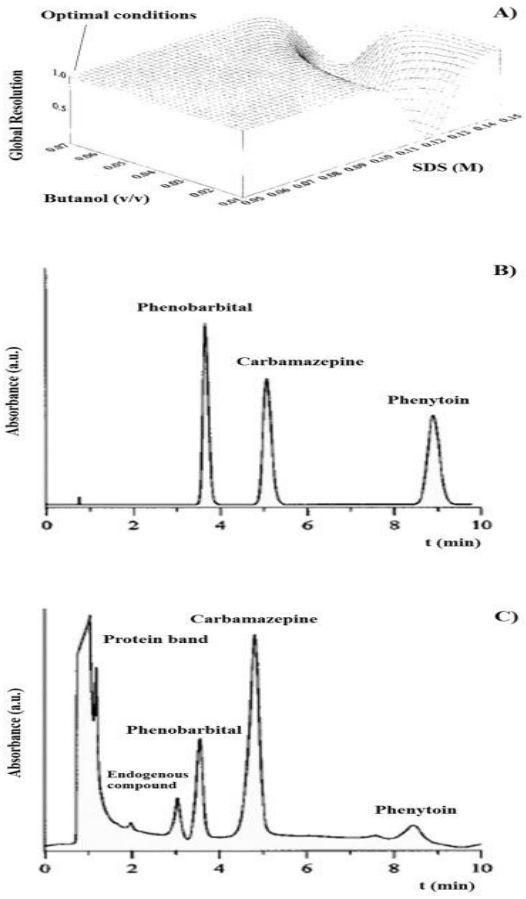
3.1. Determination of the anticonvulsants: phenobarbital, carbamazepine and phenytoin in serum

According to their moderate-high hydrophobicity (log Po/w: 1.47-2.47) and null charge, a moderate interaction between the drugs and the column is expected. Therefore, the addition of alcohol was judged necessary. After several empirical studies, 1- butanol was finally selected.

The equation (4) was selected to model the retention factor of the drugs. The tested SDS (M)/1-butanol (%) concentrations were: 0.05/1; 0.05/7; 0.10/4; 0.15/2 and 0.15/7. The resulting plot Z vs. [SDS] (0.05-0.15 M) and [1-butanol] (1-7%) can be seen in Fig. 1A. The optimal SDS/1-butanol concentrations were 0.05 M/7%, with a Z = 0.997 and

an analysis time of 10 min. A simulated chromatogram and another one obtained by the analysis of a serum sample from a patient treated with a ternary mixture can be seen in Fig. 1B and 1C, respectively. The error in the predicted retention factors was <1.9%.

Fig. 1. A) 3-D plot of the simulated global resolution vs. SDS and 1-butanol concentration for the simultaneous determination of the three studied anticonvulsants, chromatograms B) simulated and C) obtained from the analysis of a serum sample from a patient containing 20 μg/mL phenobarbital, 7 μg/mL carbamazepine and 12 μg/mL phenytoin.



3.2. *Determination of the antiarrhythmics procainamide, NAPA, disopyramide, lidocaine and quinidine in serum [46,47]*

The elution of the slightly polar (log Po/w: 0.88 to 0.99) non-cationic procainamide and NAPA was only possible using mobile phases containing 1-propanol

or low amounts of 1-butanol. How- ever, under these conditions, the hydrophobic (log Po/w: 2.44 to 3.44) and positively monocharged disopyramide, lidocaine and quinidine were eluted too late. These drugs required 1-pentanol or high amounts of 1-butanol to obtain adequate retention times.

The use of hybrid mobile phases containing 1-butanol was envisaged to elute the two groups of antiarrhythmics. In both cases, the equation (5) was used to model the retention factor. The tested mobile phases were the same as those described in section 3.1. The optimal SDS/1-butanol concentrations were 0.05/1 and 0.15/7 to resolve procainamide/NAPA and disopyramide, lidocaine and quinidine, respectively. A comparison of the simulated chromatogram and those obtained by analysis of spiked serum samples, for both groups, are shown in Fig. 2. The deviation of the predicted retention factors was <3%.

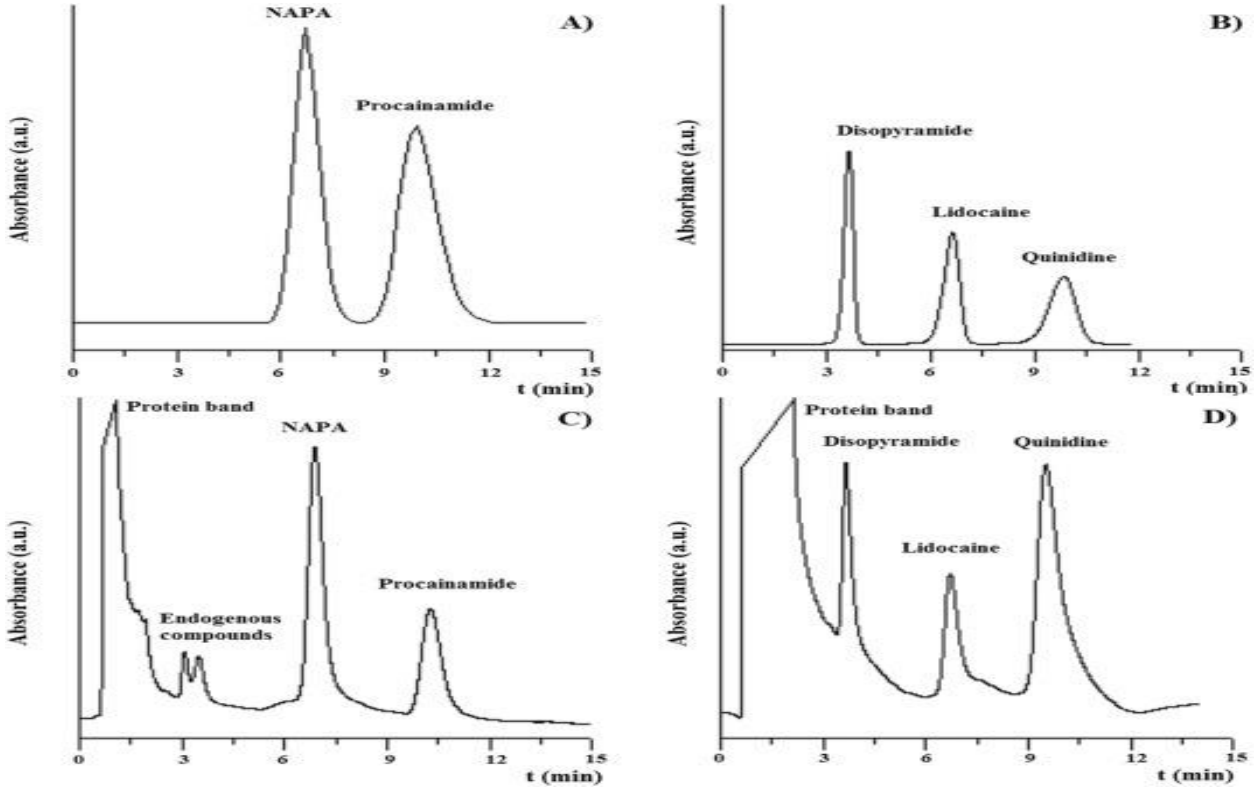


Fig. 2. Chromatograms simulated (A and B) and experimentally obtained (C and D) by the analysis of serum samples spiked with the following mixtures: A and C) 2.5 μg/mL procainamide, 6 μg/mL NAPA, and B and D) 2.0 μg/mL disopyramide, 1.0 μg/mL lidocaine and 2.0 μg/mL quinidine.

Table 4. Validation parameters obtained using the MLC [45], [46], [47], [48], [49], [50], [51], [52] and HPLC [53], [54], [55], [56], [57], [58], [59] methods for the analysis of monitorable drugs.

Drug	Linear range (µg/mL)		LOD (ng/mL)		LOQ (ng/mL)		Intra-assay Accuracy (%) (n = 5)		Repeatability (%) (n = 5)		Intermediate precision (%)
	MLC	HPLC	MLC	HPLC	MLC	HPLC	MLC	HPLC	MLC	HPLC	MLC
Phenobarbital	15–40	0.5–150	50	0.20	150	0.58	98.9–100.3	96.7–100.7	<1.1	<4.3	<2.1[a]
Carbamazepine	2–12	0.5–100	10	0.22	35	0.66	98.6–100.5	96.3–96.9	<1.1	<6.7	<1.7[a]
Phenytoin	10–20	2–100	10	1.05	35	3.13	98.9–100.6	89.9–99.2	0.0	<7.1	<1.9[a]
Procainamide	1.25–20	0.2–30	84	0.07	280	0.2	96.5–108.0	98.5–103	<1.3	<6.2	<1.9[a]
NAPA	3–36	0.2–30	52	0.07	170	0.2	100.6–101.7	98.5–103	<1.0	<6.2	<1.4[a]
Disopyramide	0.5–8	0.2–30	59	0.07	200	0.2	97.5–105	98.5–103	<2.9	<6.2	<2.8[a]
Lidocaine	0.5–10	0.2–30	61	0.07	200	0.2	92–110	98.5–103	<4.2	<6.2	<3.3[a]
Quinidine	0.5–8	0.2–30	68	0.07	230	0.2	102.5–105	98.5–103	<1.4	<6.2	<2.6[a]
Imipramine	0.05–1	0.06–1	0.35	2.5	3	19	97.2–100.7	122	<1.8	<6.0	<2.2[b]
Desipramine	0.05–1	0.06–1	0.24	5.4	1	14	99.8–100.2	97	<0.8	<4.6	<1.6[b]
Amitriptyline	0.12–0.25	0.06–1	0.25	4.0	0.9	10	101.2–103.2	118	<3.8	<6.8	<7.3[b]
Nortriptyline	0.05–0.15	0.06–1	0.31	5.5	1.0	21	95.1–102.5	118	<5.5	<9.2	<5.4[b]
Citalopram	0.02–0.5	0.001–0.05	7	0.06	20	0.19	92.8–96.8	84.8–103.3	<5.1	<5.3	<4.9[b]
Paroxetine	0.02–0.5	0.001–0.5	7	0.08	20	0.26	93.5–103.2	93.3–98.5	<9.3	<3.8	<5.4[b]
Fluoxetine	0.02–0.5	0.001–0.5	7	0.10	20	0.33	91.3–95.3	85.4–93.9	<4.5	<9.7	<6.8[b]
Acetaminophen (serum - PAD)	0.05–5	1.25–100	0.83	450	4.4	1250	97.5–105	85.6–111.5	<9.2	<21.6	<4.1[b]
Acetaminophen (serum - UV)	0.05–50		16.1		40		98–106		<15.7		<3.0[b]
Acetaminophen (urine-PAD)	0.5 to 5	1.5–180	0.74	100	5.8	300	98.0–106.7	100	<13.3	<2.1	<2.2[b]
Acetaminophen (urine-UV)	0.5 to 50		13.8		45		100.4–108		<11.1		<4.9[b]
Caffeine	0.05–50	5–120	3	1000	30	3000	95.4–99.5	93.0–103	<1.74	<12.4	<1.0[a]
Theophylline	0.05–50	5–120	3	500	30	1500	98–98.5	95.3–103	<4.1	<11.7	<0.9[a]

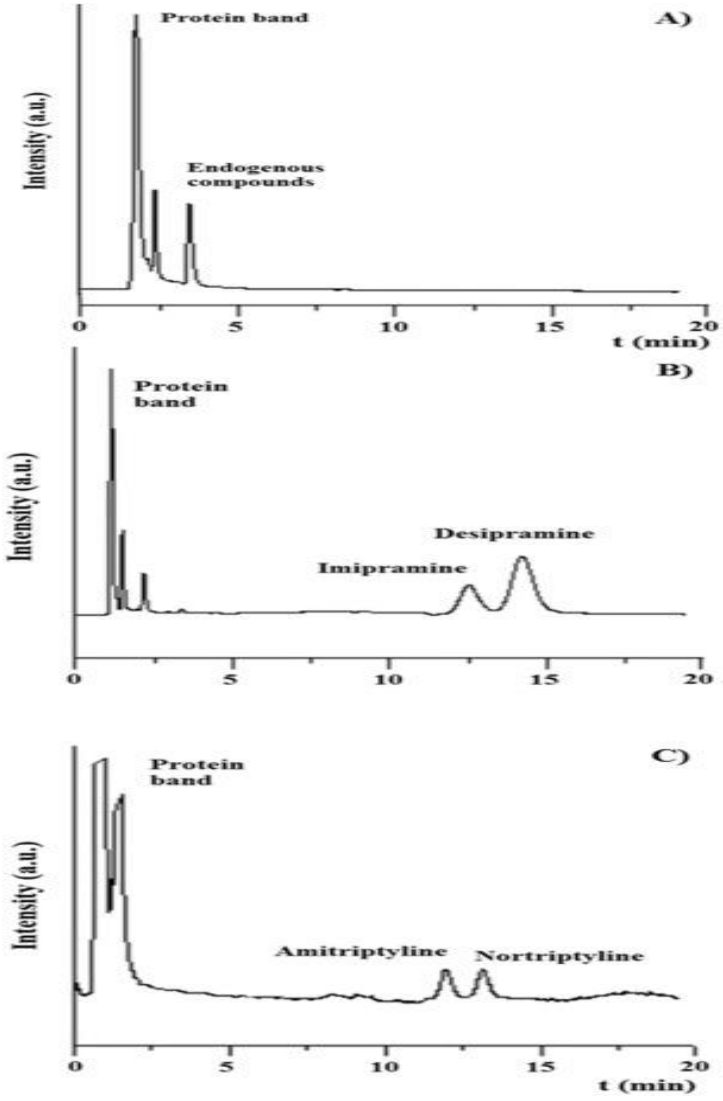
3.3. Determination of the tricyclic antidepressants imipramine, desipramine, amitriptyline and nortriptyline in serum [48,49]

As their therapeutic range attain low concentrations, these four drugs were detected by electrochemical (amperometry) detection, which holds a higher sensitivity. Hence, 0.001 M NaCl was added to the mobile phase as an ion strength buffer. The studied drugs are monoprotonated and quite hydrophobic (log Po/w: 4.51 to 4.92), and then the largest alcohol, 1-pentanol, was selected as organic modifier.

The assayed SDS/1-pentanol concentrations were: 0.05/2; 0.05/ 6; 0.10/4; 0.15/2 and 0.15/6. For both groups, a good resolution was obtained for all the combinations. Therefore, the most eluent conditions were directly taken without adjusting the equations. Besides, the flow rate was augmented to 1.5 mL/min to shorten the retention time, and the resolution was not affected. For imipramine and desipramine, the sample was not diluted in order to reach the sufficient sensitivity to cover the therapeutic range adequately [48]. Serum samples, blank (Fig. 3A), and spiked with 30 ng/mL of imipramine/desipramine (Fig. 3B) or amitriptyline/nortriptyline (Fig. 3C) were analyzed under the optimal conditions by MLC-PAD to ensure the correct identification of each drug.

The MLC-PAD-procedure was used to conduct pharmacokinetic studies in healthy volunteers. The measured values of the half-life of imipramine and desipramine (19.8 ± 2.9 and 22.1 ± 3.2 h, respectively) were nearly in accordance with those found in the bibliography (see Table 1). The peak serum concentrations of amitriptyline and nortriptyline were reached at 4 and 10 h, respectively.

Fig. 3. Analysis of serum samples: A) blank; and spiked with 30 ng/mL of B) imipramine and desipramine, and C) amitriptyline and nortriptyline.



3.4. Determination of citalopram, paroxetine and fluoxetine in plasma and urine [51]

These compounds show natural fluorescence, and then this detection mode was selected, according to its higher sensitivity and specificity. Because of their moderately high hydrophobicity (log Po/w: 3.5-4.1) and positive charge, a strong interaction between the SSRIs and the stationary phase was expected. Therefore, 1- butanol was used as an organic modifier to obtain adequate retention times.

The SDS/1-butanol amount was optimized as detailed in section 2.4, using the equation (3). The model was built using the experimental results obtained at the following SDS/1-butanol concentrations: 0.05/2; 0.05/6; 0.01/4; 0.15/2 and 0.15/6. The error in the prediction of the retention factors was <2.4%. The maximal resolution was attained at 0.075 M SDS/6% 1-butanol. No significant differences were found between the results (chromatograms, experimental conditions and validation parameters) obtained for both plasma and urine matrices. Fig. 4 shows the chromatograms obtained by the analysis of plasma (A, B) and urine (C, D) samples, blank (A, C) and spiked with the studied SSRIs (B, D).

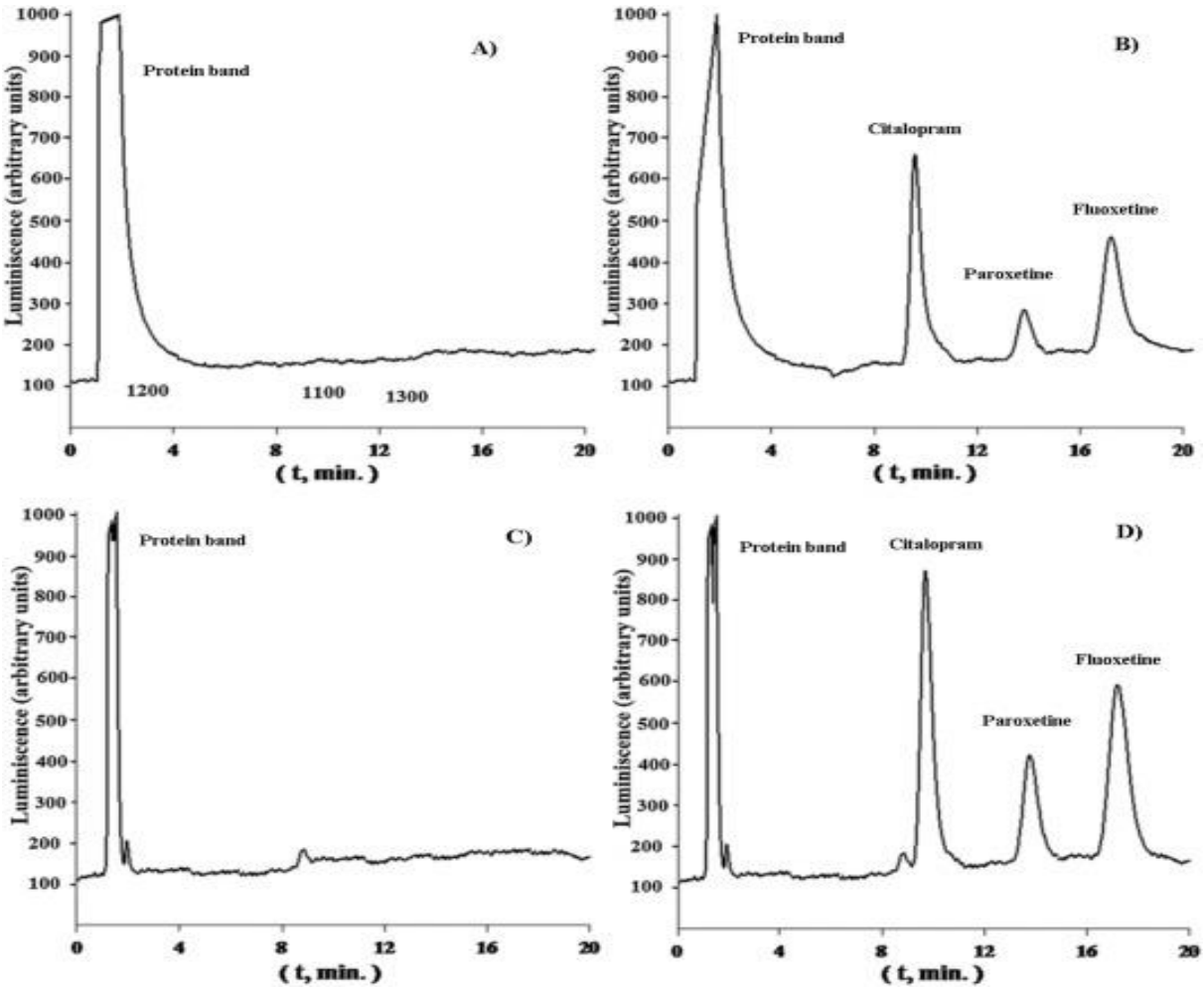


Fig. 4. Chromatograms obtained by the analysis of plasma (A, B) and urine (C, D), blank (A, C) and spiked at with of 60 ng/mL citalopram and fluoxetine, and 30 ng/mL paroxetine (B, D).

3.5. Determination of acetaminophen in serum and urine [52]

Acetaminophen is hydrophilic (log Po/w = 0.46) and, under common analytical conditions, neutral. Therefore, using hybrid mobile phases, this substance ought to appear at too short intervals, and then only pure micellar solutions were tested. Both UV and pulsed amperometric detectors were used because of the relatively high therapeutic range of the drug. Consequently, the mobile phase was buffered using 0.001 M KCl.

The chromatographic behaviour of acetaminophen was studied in the 0.02e0.10 M SDS range. The optimal mobile phase was directly selected as the one with 0.02 M, in order to avoid overlapping with the protein band.

The validation was separately performed for both matrices and both detectors. Blank and spiked samples of serum and urine were analyzed using UV (Figure-SM-2A and B, respectively) and PAD (Figure-SM-2C and D, respectively), to verify the lack of interferences. Because of its higher sensitivity, amperometry was preferred for the applications. Samples of serum (Figure-SM-2E) and urine (Figure-SM-2F) spiked at the corresponding LOQ were analyzed by MLC-PAD using the optimal conditions.

The selectivity was confirmed by determining a large number of drugs usually co-administered with paracetamol. None of them provide a peak near the retention time of the analyte. The method was applied to study the pharmacokinetics of acetaminophen in healthy volunteers. It was found that the maximum concentration in serum and urine was reached at 2 h and 3 h, respectively. Paracetamol was totally eliminated after 10 h, and its half-life was 2.2 h. These values are consistent with those found in the bibliography (See Table 1).

3.6. Determination of theophylline and caffeine in serum [50]

These two drugs are quite polar (log Po/w: 0.07 to 0.02) and neutral, and then would have a weak interaction with the stationary phase. Using hybrid mobile phases containing 1-butanol and 1- pentanol, the analytes were eluted too close to the dead time. Only 1-propanol was able to provide useful retention times and then was selected as

organic modifier. The retention factor was modelled using equation (5).

The chromatographic parameters (k, N and B/A) were experimentally measured using mobile phases containing the following SDS (M)/1-propanol (%) concentrations: 0.05/2.5, 0.05/12.5, 0.10/ 7.5; 0.15/2.5 and 0.15/12.5. A complete resolution was achieved, except at higher proportions of 1-propanol, and the analysis time was quite low in all cases. Finally, the lesser eluent mobile phase (0.05 M SDS/2.5% 1-propanol) was selected, to avoid overlapping with the protein band. A chromatogram obtained by the analysis of a blank and a spiked serum sample can be seen in Fig.-SM-3A and B, respectively.

3.7. Description and discussion of the common results [45-52]

Empirically, all the studied analytes showed a decreasing retention time and efficiency at higher concentrations of SDS. That means they bind to the SDS-micelles. Indeed, the retention times decreased and the efficiencies augmented using alcohols at increasing concentrations and with a larger carbon chain, as usual in RP-HPLC. Besides, the order of the elution power of the mobile phases required to determine each drug mixture (TCAs > SSRIs > disopyramide/lidocaine/quinidine > anticonvulsants > procainamide/NAPA > bronchodilators > acetaminophen), matches that of log Po/w (TCAs > SSRIs > disopyramide/lidocaine/quinidine > anticonvulsants > procainamide/NAPA > acetaminophen > bronchodilators), except for the more hydrophilic drugs.

The analysis of a blank matrix produces quite clean chromatograms, despite the complexity of these physiological samples. A wide and highly intense band and several peaks were observed from the dead time to up to nearly 3.5 min (Figs. 3A, 4A and C, SM- 2A, SM-2B, SM-2C, SM-2D and SM-3A). Besides, no significant peaks were detected at working retention times, thus facilitating the recognition and quantification of the drugs. Therefore, it was confirmed that the proteins and other endogenous compounds strongly interact with SDS-micelles and then were barely retained. The same result was obtained in spiked samples (Fig. 2C,D, 3B,C,4B,D, SM-2E, SM-2F and SM-

3B) and in those collected from healthy volunteers and patients after oral administration of the medication (Fig. 1C). The intensity of the protein band and the number of endogenous compounds in the chromatograms were higher in urine than in serum or plasma, as usual in MLC [72]. In all cases, the drugs were unambiguously identified without interferences, assessing the selectivity of the procedures.

The drugs were eluted in only <20 min using a mobile phase running under isocratic mode. Therefore, the stabilization time between the two injections, required in gradient, is not needed, thus reducing the effective analysis time and the production of waste.

For all the described analytical methods, excellent results were obtained in the validation studies in terms of sensitivity, calibration range, linearity (r2 > 0.992), accuracy (91.3-110.0%), and precision (<15.7%). Hence, these methods cover the therapeutic range of the drugs and provide concentrations close to the true value with low variability.

3.8. Comparison with HPLC methods

The analytical methods here described were compared to RP-HPLC-procedures, about the determination of the here-studied anticonvulsants [53], antiarrhythmics [54], antidepressants [55] and bronchodilators [57] in serum, paracetamol in serum [56] and urine [58], as well as SSRIs in plasma and urine [59]. The sample preparation, chromatographic conditions and validation results can be seen in Tables 2-4, respectively.

The main feature of the MLC-assays, compared to those based on HPLC, was, undoubtedly, the strong simplification of the sample preparation, which can be performed in 2-3 min. HPLC procedures use time-consuming and cumbersome protein precipitation and extraction steps, or, complex and expensive on-line extraction systems for sample purification, unlike in MLC. Besides, HPLC procedures required internal standard, whereas in MLC, it was not necessary. The advantages of HPLC were the possibility to determine the five antiarrhythmics and the four TCAs in the same run, respectively, and

the minor duration of the chromatographic analysis, except for the anticonvulsants. However, in all cases, the global analysis time remains higher than for MLC.

The linear range and the sensitivity are higher for HPLC than for MLC procedures. However, these differences are not significant for the clinical applications, because both sets of methods cover the corresponding therapeutic ranges. The accuracy was similar, except for the TCAs and acetaminophen in serum, where MLC shows better results. The variability was lower for MLC, except for acetaminophen in serum. With a global glance, the quality of the quantitative results obtained by MLC was higher than HPLC, despite the use of simpler instrumentation. This may be due to the minimization of the participation of the operator, the number of steps, and the quantitative introduction of the sample in the chromatographic system, reducing the probability of analyte loss (either by operational error, physico-chemical changes or low yielding in the extraction), and then the potential sources of variance.

In MLC, the dilution solution and the mobile phase contain a less proportion of toxic, flammable and volatile organic solvent (<12.5%) than hydro-organic RP-HPLC (up to 67%). Therefore, the operator would be barely exposed to toxic chemicals, and the waste contains a minimum quantity of pollutants. This fits the current trend in analytical chemistry [73].

MLC allows the processing of a larger number of samples per day than HPLC, which is extremely useful in laboratories where many samples must be analyzed. Besides, HPLC-based procedures use more expensive instrumentation, due to the extraction systems and the higher amount of chemical and reagents. This is especially applicable to the methods described in Refs. [55, 57-59], which use mass spectrometry. Therefore, MLC permits the reduction of the price per analysis, which is reasonably interesting in the current context of economic crisis.

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Chapter 2

Objectives

The work presented in this report is part of a research line developed by the group of "Bioanalytical Chemistry" of the Department of Physical Analytical Chemistry at Jaume I University, code 029 of the Office for Cooperation in Research and Technological Development (OCIT). This line has the purpose of development of new methods of analysis in the fields of clinical chemistry and analysis toxicological and forensic, using micellar liquid chromatography (MLC) and capillary electrophoresis. It has been funded by several research projects from different areas: state (Ministry of Education and Science), autonomous (Generalitat Valenciana) and university (Jaume I). The study exposed in this thesis has been funding through the UJI-B2018-20 and AICO/2017/063 project.

The MLC is a real alternative to liquid chromatography in the reverse phase classical (RPLC) that uses mobile phase aquo-organic. Among its remarkable features are its great versatility, due to the variety of interactions that are established between the solutes, the aqueous phase, the micelles and the phase stationary. Also, its elution characteristics allow the analysis of solutes in a wide range of polarities, from a single injection and isocratic elution. Attended that the retention is highly reproducible, it can be modeled with great accuracy to predict retention changes by varying the composition of the mobile phase, facilitating this way the optimization of the conditions of separation. It should be noted, that the concentration of organic solvent is much smaller than that used in classic RPLC. For this reason, mobile phases in MLC are less toxic, flammable and polluting. On the other hand, the MLC provides a simple solution to the problem of the direct injection from physiological fluids or food samples. Maybe this is one of the main advantages, speeding up analytical procedures.

Since the MLC was first proposed by Armstrong in 1981, numerous published articles aimed at the development of technique and knowledge of its retention mechanisms. This type of studies is essential for improve the possibilities of separation systems and establish the bases to develop methods of analysis of compounds of different nature. Based on this type of studies, the main objective of the memory presented has been the development and validation of procedures for the separation and determination of different classes of antibiotics in several matrices (urine, pharmaceutical products and food) through MLC.

Due to the data obtained from a new method have to be reliable, they must be validated following a series of well-defined criteria. This is another goal of the present Doctoral Thesis. Most of the methods developed have been validated considering the following parameters:

selectivity, linearity, detection limits and quantification, accuracy, precision, recovery and robustness. For this purpose and according to the type of matrix studied, four different validation guides have been used: the regulations of the Administration of Drugs and Foods (FDA), norms of the Conference International Harmonization (ICH), and European Medicines Agency Guidelines (EMA), European Commission (2002/657/EC).

Antibiotics or antibacterial agents are low-weight chemicals molecular, synthetic or produced by microorganisms, that have the capacity, in small concentrations, to inhibit growth and even to destroy certain microorganisms His main applications are in medicine, veterinary medicine and agriculture. In humans, they are used in the treatment of urinary, respiratory and infections others of a systemic type. Its use for the treatment of livestock diseases by to human consumption, as well as its use as additives in industrial farms, have given that its potential presence in food of animal origin must consider yourself residues of antibiotics in food of animal origin can cause allergic reactions in hypersensitive individuals, but above all, the administration of low levels of antibiotics can lead to resistant bacteria, which can reach the be human through the aforementioned foods. In addition to these immediate adverse effects, There are also long-term effects that are not yet known. For all these reasons, it is more than justified the development and validation of analytical methods for this type of compounds.

The group of antibiotics and antitumoral includes compounds with very different structures, many of them with great pharmacological interest. They have appeared, for this reason, a very high number of articles that show analytical procedures through various techniques, be very used classic RPLC. The availability of new tools that make it possible the improvement of the procedures developed by our research group, and the interest for investigating the determination of new generation antibiotics and antitumoral, led to the proposal of one new PhD thesis on the determination of these drugs.

To reach the general objectives, the following specific objectives are proposed, which are common to all applied studies:

- Study of the physicochemical parameters of the analytes (pKa, hydrophobicity, solubility, absorptivity and electrochemical properties).

- Establish the general chromatographic conditions (stationary phase, injection volume, flow, elution mode, sodium dodecyl sulfate as surfactant ...), as well as the dilution coefficient of the initial sample in a micellar solution.

- Determination of the optimal composition of the mobile phase (concentration of sodium dodecyl sulfate, nature and proportion of organic contamination), for the resolution of the mixture of analytes without overlapping with other matrix compounds, in the minimum analysis time.
- Optimization of detection conditions.
- Selection of the appropriate validation guide, depending on the nature of the analytes and the sample.
- Determination of the statistical parameters required by the validation guide: selectivity, linearity, linear interval, sensitivity, precision, precision, dilution test, cross-contamination, matrix effect, robustness and stability.
- Check that the values obtained are below the levels tolerated by the guideline and that the concentration levels at which the drugs studied in clinical cases can be found are covered. Otherwise, make the appropriate changes in the experimental conditions and repeat the validation.
- Application of the implemented methods to clinical running. Determination of analytes in samples of biological fluids taken from patients.

Chapter 3

Advances on Melamine Determination by Micellar Liquid Chromatography

ABSTRACT

Melamine is a toxic triazine which has been recently proven as a threat to human health. It can be ingested by several methods, being the most important the unethical adulteration of protein-rich foodstuff for economic reasons. This review presents several analytical methods, taken from the literature studies, devoted to the determination of melamine in milk, dietetic supplements, drinking and wastewater, swine kidney, plasma, and urine, using micellar liquid chromatography (MLC). We consider that the control of these samples is crucial to prevent and manage melamine intoxication. This technique has been demonstrated as an excellent tool to determine organic compounds in these matrices. We detail the optimization strategy and the obtained results in the different steps of method development, such as sample pretreatment, chromatographic separation, and validation process. The similarities and differences of the procedures have been described and discussed, as well as their advantages. The main ones were the possibility of direct injection and the efficient chromatographic elution, in spite of the complexity of the samples. Besides, it was found that the MLC procedures were fast, easy-to-handle, inexpensive, eco-friendly, safe, and useful for routine analysis. Therefore, they represent an excellent alternative to evaluate the melamine concentration in that kind of samples reliably.

1. Introduction

Melamine: A global threat

Melamine, also known as cyanuramide or triaminotriazine (1,3,5-triazine-2,4,6-triamine, C3H6N6, MW = 126 g/mol), is a small hydrophilic (log Po/w = -1.4) nitrogen-rich (66.6% w/w) biodegradable molecule belonging to the family of the heterocyclic organic compounds. It is a trimer of cyanamide and has three amino groups in its structure. It is a colorless to white powdered or crystalline solid and sublimates at 350° C. It is water-soluble and weakly basic with a pKa (melamine-H+) = 5.0. It is highly stable in the main situations and decomposes under strong acidic and basic conditions as by intense heating. Melamine analogues (ammeline, ammelide, and cyanuric acid) are formed by successive substitution of amino to hydroxy groups by hydrolysis. Therefore, they can be found in raw melamine as impurities of the melamine synthesis process. Melamine can self-assembled and with analogues in high molecular weight structures by intermolecular networks of hydrogen bonds and π–π aromatic ring stacking.[1,2]

Melamine is a non-expensive industrial chemical. It is mainly used as a starting material in the manufacture of synthetic resins, mostly as melamine-formaldehyde polymer, which is used in the production of a large variety of molded plastic objects, coating materials, filters, laminates, permanent press fabrics, wrinkle-free textiles, tarnish inhibitors, paper and textile finishers, tableware, paints, adhesives, and as additive in flame retardant formulations.[2–9] In agricultural backgrounds, melamine can be found in fertilizer mixtures, and as a decomposition product of cyromazine, an insecticide approved for use in many countries on animals and fields.[2,10] The population is exposed to melamine from a wide number of sources including food and environmental ones. Melamine can be found at a low level in food by migration from package [4,11,12] or from the breakdown of cyromazine.[10] It can also be found in wastewater, [13,14] from industry using this chemical as prime material or from agricultural water from an area where cyromazine has been used. If this water is inadequately purified, melamine can finally pass into the water supply system.[15]

Recently, deliberate illegal melamine adulteration was detected in different protein-rich products destined for humans, like milk and milk derivatives [5,6,16–19] and animals, as pet

food[4,5,7,9,19–21] and feed for farm animals.[22] Because of its high amount of nitrogen, melamine causes an incorrectly high reading in the quantification of proteins based on the nitrogen amount (the Kjeldahl nitrogen determination method), that increases the apparent nutritional value and then the final price of the product.[16,23]

The pharmacokinetics and toxicology of melamine in mammalians are nowadays well known.[2] Melamine is rapidly absorbed orally after ingestion.[24] Melamine flows through the gastrointestinal system toward the bloodstream, from which it could be absorbed by other organs. However, melamine is unable to pass through cellular membranes.[25] Melamine is

not metabolized and does not accumulate in tissues or organs. Therefore, it is quantitatively removed from the blood by the kidney and eliminated mainly unchanged in the urine in a few days. [13,26,27] Melamine has low acute toxicity by oral ingestion. It irritates the digestive tract due to the formation of melamine and melamine/analogue crystals, resulting in nausea, vomiting, diarrhea, hypertension, and edema.[2] However, the toxicity of melamine is enhanced in the urinary system, as it can react with uric acid or cyanuric acid to form calculi which can precipitate in the kidney, urethra, or bladder. The accumulation of these crystals can cause oliguria, nephrolithiasis, urolithiasis, or bladder cancer.[2,6,28] In 2007, a large number of cat and dog intoxications in the United States were related to the intake of protein-rich pet food manufactured from gluten and other cereal-based ingredients strongly contaminated with melamine.[4,5,7,9,19–21] One year later, in China, the ingestion of milk and milk-based infant formula highly contaminated with melamine (up to 3300 mg/L) resulted in a major outbreak of renal diseases in newborns and young children, and even several deaths were confirmed.[5,6,16,17–19]

It was the first outbreak of kidney failures related to melamine intoxication in humans. In both cases, melamine–uric acid stones were found in the kidney, urethra, and bladder of the patients. This event causes the launching of international sanitary alert about this toxic compound.[18,25]

Due to its toxicity, US Food and Drug Administration (FDA) has set the following maximum residue limits (MRLs) for the presence of melamine in foodstuff: 2.5 mg/kg in adult’s food[6,21,25] and 1 mg/kg in infant formula.[5,6] Moreover, the World Health

Organization (WHO) has established a tolerable daily intake (TDI) of 0.2 mg/kg body.[25] Therefore, the determination of melamine in milk products, dietetic supplements, and flesh must be performed for safety health reasons. Milk and supplements are taken because of their high nutritional value and as protein and mineral sources. Children formula must be carefully controlled because they are important for their growth and represent the main ingredient of infant alimentation.[5,16] Flesh samples should also be analyzed. Although not directly adulterated, melamine residues can occur, if the farm animal has been nourished with contaminated animal feed.[22] WHO also recommends the control of the level of melamine in wastewater to evaluate the danger for the environment and in drinking water for health safety reasons.[13,25] The occurrence of melamine in blood, urine, and kidney in humans and farm animals is an evidence of a strong timely or a continuous intoxication, and then to assess if they have been taken adulterated food. It can also be useful in clinical studies to determine the pharmacokinetics and toxicology of melamine.[13,27,29,30] Then, because of the different types of sources of melamine and its effect on the human health, the development of economic, easy, and rapid analytical methods to reliably quantify melamine in these samples is of the utmost importance.

2. Analytical approaches for melamine in biological, food and environmental matrices

The impact and awareness of melamine threat in our society can be inferred by the high number of analytical procedures developed for its quantification in a relatively short period (2008–2015).[2,31,32] It has been determined in a wide range of matrices, like food for humans: yoghurt, fish,[33] cereal gluten,[34] rice,[3,34] and soy[34] concentrates, beverages,[11] dairy products,[33,35,36] dietetic supplements,[24,34] eggs,[8,26], poultry meat,[8] royal jelly,[37] milk and milk-derived products, [5,16,19,30,33,38–48] bakery flour and goods,[42] and water in contact with kitchen and tableware made of melamine resin;[12] animal feeds: farm animal feeds,[38,49] pet food,[4,9,34,50] cereal flours,[7] fish feed;[33] biological samples: muscle tissue,[8,13] urine and plasma of pigs,[51] goat blood,[45] hen plasma,[26] human urine,[30,52] chicken body fluids,[17] plasma,[24,29,53] urine and feces[53] of rats, and kidney[9,13,20,24,53] and other organs, such as liver, spleen, bladder, and brain[24,26,53] of several animals; and environmental samples: crop,[13] waste

water,[10,13–15] and soil[10,13] samples. Melamine studies have been undertaken using a large variety of techniques, such as electrochemistry,[30] nuclear magnetic resonance (NMR),[47] immunoassay analysis (ELISA),[4,17] Raman spectroscopy,[40] direct mass spectrometry (MS),[5,16] and several kinds of sensors.[2,31,32] Because of the complexity of the matrices, higher signal-to-noise signals have been obtained using separative techniques, like electrophoresis,[33,39] thin layer chromatography,[41] gas chromatography-mass spectrometry,[10,17,19,30,34,38] and liquid chromatography. This last one can be considered, undoubtedly, the technique of choice for the quantification of melamine.[2,31,32] Indeed, many methods have been developed using different branches of HPLC, like ion exchange,[50] hydrophilic interaction,[3,8,13,20,24,29,36,44,45,46,49] turbulent flow,[35] ultraperformance liquid chromatography (UPLC)[42,43,53] and packed reverse phase, either by using ion-pairing,[11,26,37,38,42] or hydro-organic,[4,7,9,10,12,14,15,48,51,52] coupled to UV–visible absorbance diode array (DAD),[3,4,7,8,10–12,14,15,26,37,38,46,50,51] fluorescence[12,48] or MS[4,9,13,15,20,24,29,35,36,42–45,49,52,53] detection. This last instrumentation has the problem of easy contamination and high cost, considering both acquisition and maintenance, then only a few laboratories can afford it.

The determination of melamine in food, biological, and environmental samples by HPLC requires a preliminary sample processing to separate the analytes from the matrix to avoid the introduction of proteins, lipids, and other insoluble substances, which may precipitate in the chromatographic system and damage the stationary phase.[31,32] These sample pretreatments use tedious, time-consuming and multistep extraction, preconcentration and purification steps, such as leaching,[3] matrix precipitation,[8,24,29,35,42–44,46,48,53] and liquid [10,12,13,20,26,36–38,42,43,48–50,52,53] or solid [8,10,11,15,24,26,36–38,42,45,50,51] phase extraction, thus introducing additional sources of variance. In several cases, even various consecutive treatments are required. This improves the probability of loss of analyte, either by error operator, apparatus malfunction, or physicochemical change, thus reducing the recovery and increasing the uncertainty of the results. These effects can be partially corrected by the addition of an internal standard [3,13,20,35,42–44,49,52]. Besides, they require specific instrumentation, and/or large volumes of toxic chemicals, which is harmful to the laboratory staff and the environment.[31,32] Online solid phase extraction–HPLC has been proposed to improve the automation degree, but it requires expensive and

complex experimental assembly.[46] Recently, the use of micellar liquid chromatography (MLC) has been proposed as an excellent alternative for the quantification of melamine in a wide variety of matrices, such as milk,[54] dietetic supplements,[55] swine kidney,[56] waste and drinking water,[57] as well as human plasma and urine,[58] avoiding the above-described drawbacks.

3. Basic description of micellar liquid chromatography

Surfactants are amphiphilic compounds containing a hydrophobic chain (tail) bonded to a polar group (head), which can be cationic, anionic, neutral, or zwitterionic. In aqueous solutions, the surfactant monomers join to form a spherical shaped configuration: the normal-phase micelle, at concentrations over the critical micellar concentration (CMC). The nonpolar tails stay in the core to be isolated from water, whereas the polar groups are oriented to the water and configure an outer layer and are water solvated. The palisade layer is located in the intermediate region. For each surfactant, the micelles contain nearly the same number of monomers (aggregation number), and then an increase of the monomers in solution provokes an augmentation of the number of micelles. The hydrophobic interaction between the carbon chains sustains the structure of the micelle, whereas the polar groups are water solvated and interact with the aqueous environment, allowing the dispersion of the micelles throughout the solution. Therefore, micellar solutions are colloidal and microscopically heterogeneous, being composed of two distinct media: the amphiphilic micellar aggregates (micellar pseudo phase) and the surrounding bulk water or aqueous organic solvent that contains surfactant monomers in a concentration approximately equal to the CMC.[59,60] Micelles have several sites of solubilization: hydrophobic (core), hydrophilic (outer layer), miscellaneous (palisade layer), and, eventually, electrostatic (polar head for charged surfactants). Because of these multiplicities of environments, micellar solutions are able to interact with compounds in a large variety of charge, hydrophobicity and molecular mass, and consequently provoke their solubilization.[61,62]

Micellar liquid chromatography is a well-established branch of reverse phase (RP)–HPLC, which use micellar solutions as mobile phases, instead of hydro-organic ones.[63] The

most used columns are those containing an octadecyl (C18)-bonded silica phase.[64] The micelles and the free surfactant monomers strongly affect the properties of both stationary and mobile phases. The monomers are adsorbed on the stationary phase, with the polar group oriented to the water, creating a structure similar to an open micelle. A polar and eventually charged outer layer appears on the stationary phase, while the inner layer turns more hydrophobic. The volume of the pores is reduced and the surface area increases resulting in an augmentation in the system pressure. Besides, the silanol groups are partially blocked facilitating the study of basic compounds. The stationary phase environment is independent of the micelle concentration, as the number of adsorbed monomers remains constant beyond the saturation point. In the mobile phase, the presence of the pseudo micellar phase introduces a new environment available for the solutes and then a secondary equilibrium. Besides, the solutes can be involved in a larger variety of interactions. These effects provoke a strong influence on the chromatographic behavior of the solutes, and complexes the retention mechanism, if compared to hydro-organic HPLC. The retention mechanism has been accurately described by the three-partitioning theory. It proposes that the analyte has available three differentiated environments and then considers three partition equilibria for the solutes: modified stationary phase ↔ bulk mobile phase; modified stationary phase ↔ micellar pseudo phase; and bulk mobile phase ↔ micellar pseudo phase. The distribution of the analyte is influenced by the hydrophobicity, charge and steric factor of the solutes, stationary phase; and micelles. As these parameters depend on the acid/basic side reactions, a buffer must be added to adjust the pH to the adequate value and maintain in constant during the whole chromatographic run.[62,65–68]

Pure micellar mobile phases hold a limited elution power because they are essentially polar. In addition, they provide relatively lower efficiencies due to the slow mass transfer between the micelle and the stationary phase. To avoid these drawbacks, a common strategy in MLC is the decrease of the polarity of the mobile phase by the addition of a water-soluble organic solvent, such as acetonitrile, methanol, ethanol, 1-butanol, or 1-pentanol (in increasing order of hydrophobicity). The solvent monomers adsorbed on the stationary phase with the hydroxy/cyano groups oriented toward the mobile phase and then increase its polarity. These combined effects thermodynamically and kinetically impulse the transfer of the analyte from the stationary phase toward the mobile phase. Consequently, the retention, broadness and

asymmetry of the peaks decrease. However, the organic solvent also may alter the characteristics, such as the structure, CMC, and aggregation number, of the micelle.[68] Beyond a specific proportion, micelles even disaggregate, as surfactant monomers remain preferably solubilized, to produce a high sub-micellar solution. This limits the maximum proportion of organic solvent useful in hybrid mobile phases. The influence of the organic solvent is enhanced as its hydrophobicity and its proportion increase and is noticeable even at low values. As long as the micelles persist in the mobile phase, the three-phase theory is also used to model the retention mechanism by considering that the organic solvent changes the value of the equilibrium constants.[62,66,69]

The retention mechanism in MLC depends on several experimental factors, such as type of column, pH, nature, and quantity of surfactant and alcohol, which must be set to provide the maximal resolution between the analytes and other compounds of the matrix at the minimum time with a good peak shape. Therefore, it is desirable to investigate the effect of these chromatographic conditions over the retention time, efficiency, asymmetry, and resolution of the peaks. The reproducibility and stability of the retention mechanism in MLC with isocratic mode have permitted the development of a mechanistic and empirical mathematical model, based on the three-partitioning theory, which permits the prediction of the chromatographic behavior from the composition of the surfactant and the organic solvent. This can expedite the resolution of complex mixtures.[67–69]

The remarkable characteristics of micellar solutions are significantly helpful for the sample preparation step for clinical,[70] environmental,[71] as well as for liquid[72] and solid[73] food analysis. Most of the hydrophobic and other non-water-soluble compounds are solubilized. Besides, micelles tend to bind proteins and other macromolecules provoking their denaturation. Therefore, they are barely retained and harmless eluted near the dead time, rather than precipitating into the column. After dilution in a micellar solution and filtration, the sample can be directly injected without extraction and cleanup steps. In this manner, the experimental procedure is strongly expedited, and less instrumentation and chemicals are used.[65]

4. Method Validation

4.1. Development and optimization of the analytical methods

Several analytical procedures based on MLC to quantify melamine in several matrices with quite different physical properties and chemical composition, such as milk[54], dietetic supplements[55], water,[57] body fluids,[58] and swine kidney,[56] have been previously described. These methods were optimized by selecting the main experimental conditions (sample preparation, column type, pH, kind and amount of surfactant, organic solvent and absorbance wavelength) to obtain a resolved peak of melamine, a normal-shaped peak with high efficiency, short analysis time, sufficient sensitivity to reach expected levels, high recovery, and low variability of the experimental results. Although these methods aim to determine the same compound, the optimization strategy was adapted to each matrix. Here, we summarize the development of these analytical procedures.

4.2. Sample preparation

As previously indicated, the use of micellar mobile phases allows the quantitative injection of liquid samples. For solid samples, a previous solid/liquid extraction is mandatory to recover the analyte in a solution. Despite the different composition of the studied matrices, the use of micellar solvents and mobile phases allows the treatment of dissimilar samples in a similar method. In general terms, liquid samples (milk and dietetic supplements, water, biological fluids, and water) were diluted in a micellar solution of 0.05 M sodium dodecyl sulfate (SDS) solution phosphate-buffered at pH 3, filtered and directly injected into the chromatographic system.[54,55,57,58] The powdered milk and dietetic supplements were reconstituted in water as recommended by the supplier and then processed as liquid samples.[54,55] Conversely, swine kidney (1 g) was solid/liquid extracted by ultrasonication with 2 mL methanol and then diluted with 3 mL of the same micellar solution.[56]

The filtration is essential to avoid the introduction of particles and nonsoluble compounds in the chromatographic system, which can provoke column clogging, stationary phase deterioration, and increase of the pressure. The dilution in micellar media is needed to

previously denature proteins and solubilize them together with other nonsoluble compounds.[70,73] The surfactant, buffer, and pH were set at the same values as in the mobile phase (Sections Selection of the surfactant and Selection of the pH).

The dilution ratio was optimized on the basis of avoiding the early blocking of the filter membrane, and then obtaining a solution enough representative of the whole sample, without excessively diminishing the sensitivity. The results are shown in Table 1.

Table 1. Optimized conditions for sample treatment and chromatographic run.

Matrix	Dilution ratio(v/v)	[SDS] (M) in mobile phase	[1-propanol] (%, v/v) in mobile phase	Melamine retention time (min)	References
Milk	1/10	0.05	7.5	9.3	55
Dietetic Suplements	1/10	0.15	0	8.1	56
Swine Kidney	1/5	0.11	7.5	6.0	57
Plasma	1/5	0.2	0	6.3	58
Urine	1/5	0.2	0	6.3	58

Waste and drinking water do not need the previous dilution because they contain a low amount of particles.[57] Conversely, milk and dietetic supplements have a huge amount of suspended substances, such as proteins, fats, and oligosaccharides, so that a 10-fold dilution is required before the filtration.[54,55] Plasma and urine contain less concentration of proteins and fats than milk, then a fivefold dilution was enough.[58] Swine kidney analysis requires a different approach because it is a solid matrix with a high content of proteins, fats, cholesterol, vitamins, electrolytes, and secondary metabolites. Moreover, melamine can be found in both

the inner and outer layer of the particles. Therefore, the solid/liquid extraction with an organic solvent and ultrasonication was mandatory to recover melamine, together with other soluble substances and suspended particles, in an injectable solution. This extract was finally diluted in the micellar solution to a final ratio of 1/5 to facilitate the filtration and work with a nonvolatile solvent close to the mobile phase (Table 1).[54–58]

4.3. Chromatographic conditions

In all cases, the stationary phase was in a column with a hydrophobic C18 coating (reverse phase): 150 x 4.6 mm2 i.d., pore size 100 Å, and particle size 5 μm; the most widely used column in MLC to resolve basic compounds.[64] The mobile phase runs at a constant flow rate of 1 mL/min, and 20 μL of each processed solution was injected using an autosampler device.

Absorbance was selected for detection. The spectrophotometric properties of a solute are enhanced in a micellar environment, resulting in an increase of the wavelength and the absorptivity.[67] Therefore, the optimal wavelength was performed by taking the absorption spectrum of melamine in the 200–300 nm range, during the chromatographic analysis of a standard solution by MLC. Therefore, the optimization was performed at the same conditions as applied to the real samples. As the maximum signal-to-noise ratio was found at 210 nm, the detection of absorption wavelength was set at this value.[54]

4.3.1. Selection of the surfactant

The surfactants more used in MLC are SDS, cetyltrimethylammonium bromide (CTAB), polyoxyethylene 23 dodecyl ether (Brij-35R), and *p*-octyl benzene polyoxyethylene 9.5 alcohol (Triton™ X-100). These surfactants are described in Table 2.[62] The ionic surfactants are provided as salts, which totally dissociate in water. The Krafft point (for ionic surfactants) and the cloudy point (for nonionic surfactants) are the temperatures at which the

solubility of the surfactant equals the CMC and the surfactant forms a separate cloudy phase, respectively.

Sodium dodecyl sulfate was selected for the analysis because of its practical performances: it is an easily manageable powdered solid, harmless, biodegradable, relatively inexpensive, with adequate CMC, Krafft point under laboratory temperature, highly soluble in water, low viscosity of aqueous solutions, and a relatively low increase in system pressure and ability to denature and dissolve proteins. In fact, SDS can be considered as the surfactant of choice for MLC, and it has been selected in at least 90% of the analytical reports.[62,66]

In a C18 stationary phase, the hydrocarbon tail is bonded by hydrophobic interaction with the immobilized hydrocarbon chain, whereas the sulfate group is oriented outward. Therefore, an anionic outer hydrophilic layer appears, which affects the penetration depth of the solutes and provides some ion exchangeability, as in ion-pairing HPLC. Otherwise, the hydrophobicity of the inner alkyl-bonded phase is enhanced.[66]

The quantity of surfactant adsorbed on the stationary phase has important implications regarding the retention behavior of the solutes. Several studies have shown that this value rapidly increases with the concentration of SDS in the mobile phase and remains constant beyond 10 mM (saturation point).[69] MLC analysis must be conducted using saturated stationary phase to keep constant the extent of the modifications induced by the use of a surfactant. Otherwise, the use of excessively high concentrations of SDS is not recommended because of the high viscosity of the mobile phase, the excessive diminishing of the efficiency, and the increase of the background noise in the detector.[62] Therefore, the working range was restricted from 50 mM (several times the CMC) to 200 mM.[61,62]

Table 2. Main parameters (in aqueous solution) of the surfactants most used in MLC.

	SDS	CTAB	Brij-35	Triton X-100
Charge formula	Anionic	Cationic	Nonionic	Nonionic
Hydrophobic tail	Linear hydrocarbon	Linear hydrocarbon	Linear hydrocarbon	Branched hydrocarbon–phenyl
Polar head	Sulphate	Quaternary amine	Polyethylene oxide chain	Polyethylene oxide chain
Molecular weight (g/mol)	288.4	364.5	1225	625
CMC (mM)	8.1	0.83	0.06	0.3
Aggregation number at 25°C	62	90	41	140
Partial specific volume (L/mol)	0.246	0.364	1.12	0.743
Krafft point (°C)	16	26	-	-
Cloudy point at 1–6% solution (°C)	-	-	100	64

SDS: sodium dodecyl sulfate; CTAB: cetyltrimethylammonium bromide.

4.3.2. Selection of the pH

Because of the basic activity of melamine, the pH must be optimized and fixed to avoid the oscillation of analyte properties during the elution. In C18-bonded silica columns, the working range pH is from 2.0 to 9.5. However, the use of even slightly basic and extreme acidic solutions is not recommended because it causes a slow and continuous degradation of the stationary phase.[75] Average values of pH (around 5.0) were also discarded as the two forms of melamine coexist. Therefore, the study was restricted to 3 and 7. A phosphate buffer was selected to fix the pH as it works in the 2–3 and 6–8 range.

According to its low value of Po/w = -1.4, melamine is a quite polar compound. Therefore, it would barely interact with an SDS-modified C18 stationary phase by means of hydrophobic interaction. Adequate retention would be only acquired by the electrostatic interaction between a positively charged melamine and the negative charge of the modified stationary phase.[76] This was confirmed experimentally as melamine elutes at the dead time (≈ 1.0 min) at pH 7, while, at pH 3, a workable retention time (<20 min) was obtained. Consequently, the optimal mobile phase pH was set to 3.[54]

4.3.3. Selection of the organic solvent

Because of its polarity, melamine elutes at a useful retention time using pure aqueous mobile phases.[54] The addition of an organic solvent was investigated to regulate the retention time and improve the efficiency. Finally, 1-propanol was selected because of its average hydrophobicity: 1-butanol and 1-pentanol are too hydrophobic, and acetonitrile, methanol, and ethanol require a higher proportion to give the same effect as 1-propanol.[62,67]

The presence of 1-propanol affects the micellization process. The selected alcohol solvates the sulfate group and reduces its electrostatic repulsion between the monomers, facilitating the formation of the micelle and diminishing the CMC.[62] Otherwise, the interaction of the organic solvents with SDS micelles increases their solubility and reduces their evaporation rate.[65] The maximal concentration of 1-propanol compatible with the existence of the micelles is 15%. Therefore, the studies were conducted between 2.5 and 12.5% of 1-propanol.[61,62]

4.3.4. Optimization of the SDS/1-propanol concentrations

The amount of SDS and 1-propanol must be optimized to elute melamine without interferences at the minimum analysis time. The possible interfering compounds are not the same for the studied matrices, according to their different chemical composition. Therefore, the challenge to establish the optimal concentration of SDS/1-propanol was separately undertaken.[54–58]

In all cases, hybrid mobile phases containing SDS/1-propanol and pure SDS micellar mobile phases were tested. From the preliminary studies, it was found that the elution strength and the efficiency increase at higher proportions of 1-propanol as expected in MLC. Otherwise, the retention time and efficiency decreased at higher values of SDS concentration. This behavior points to an attractive interaction between melamine and the SDS micelles, probably by electrostatics.[62,68]

The optimization was separately performed for each matrix. A large number of peaks were detected near melamine peak in milk, dietetic supplements, swine kidney, wastewater, and urine. For each matrix, the SDS/1-propanol amounts were selected on the basis of maximizing the resolution between melamine and the interfering compounds eluting immediately before (P1) and after (P2) melamine (these compounds were not identified and were different for each matrix). An interpretative strategy was applied with the aid of a mathematical model based on several mechanistic equations, which relate the chromatographic behavior of a solute to the SDS concentration and 1-propanol proportion in the mobile phase, by maintaining the other chromatographic parameters at a constant value. This chemometric model is based on the three-phase partitioning theory.[69] Two separate mechanistic models were constructed for hybrid and pure mobile phases, respectively, and then the data obtained for these mobile phases were independently processed. The retention factor (*k*) and the peak shape were modeled by Eqs. (1) and (2), respectively:[69]

$$k = \frac{K_{AS}\dfrac{1}{1+K_{AD}\varphi}}{1+K_{AM}\dfrac{1+K_{MD}\varphi}{1+K_{AD}\varphi}[M]} \quad (1)$$

$$h(t) = H_0 e^{-0.5\left(\frac{t-t_R}{s_0+s_1(t-t_R)}\right)^2} \quad (2)$$

The symbol *k* means the retention factor; K_{AS}, the partition constant of the analyte between the modified column and the bulk mobile phase times the volume phase ratio; K_{AM}, the partition constant of the analyte between the micelle and the bulk mobile phase; K_{AS} and K_{MD} refer to the modification of K_{AS} and K_{AM}, respectively, induced by the organic solvent; $h(t)$, the height of the detector signal due to the elution of the compound; and H_0, the maximal peak height. H_0 was taken as default value as it depends on the concentration and the sensitivity.[69] The constant s_0 quantifies the broadness of the Gaussian curve and $s1$, the distortion of the normal model.[69] These parameters can be calculated as indicated in.[77]

For each compound (melamine, P1, and P2), these two equations were adjusted using the MICHROM software,[78] taking the experimental values of *k*, N, and B/A obtained by the following mobile phases:

– Hybrid mobile phases: SDS (M)/1-propanol (%, v/v): 0.05/2, 0.05/12.5, 0.1/7.5, 0.15/2.5, and 0.15/12.5%. They were selected by a full factorial design plus the central value:

the combination of the maximal and minimal concentration recommended for SDS and 1-propanol in MLC and the intermediate concentrations.[61,62]

– Pure micellar mobile phases: SDS (M): 0.05, 0.10, 0.15, and 0.20 M. Four values between the minimal and maximal working concentrations of MLC.[61,62]

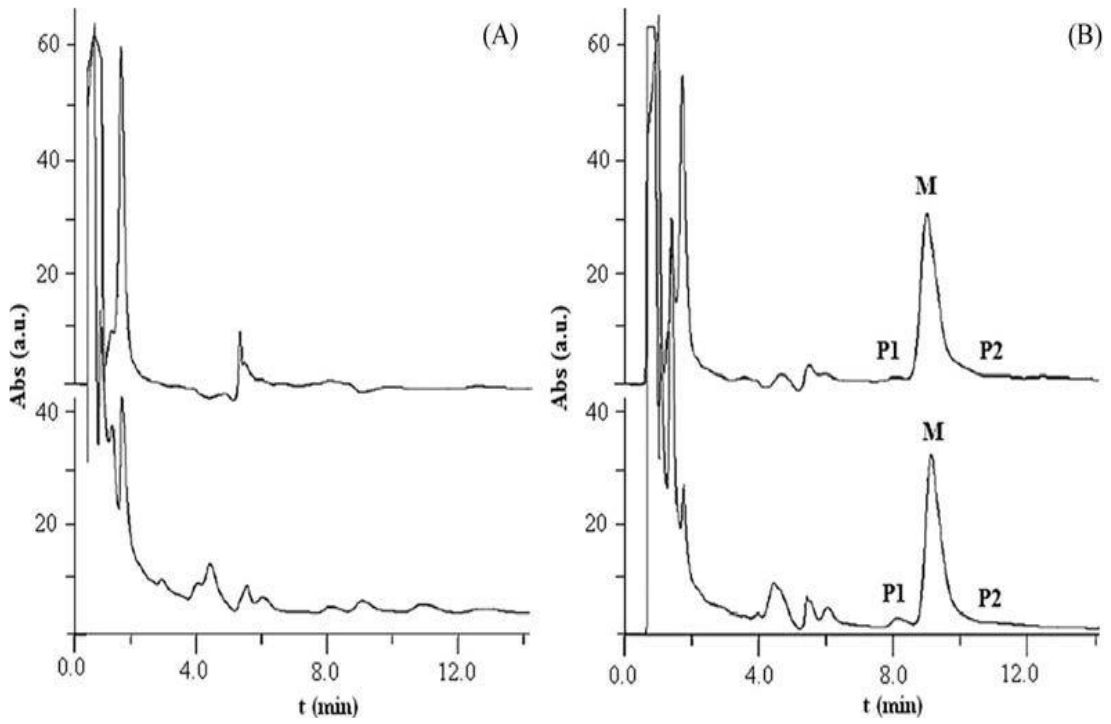
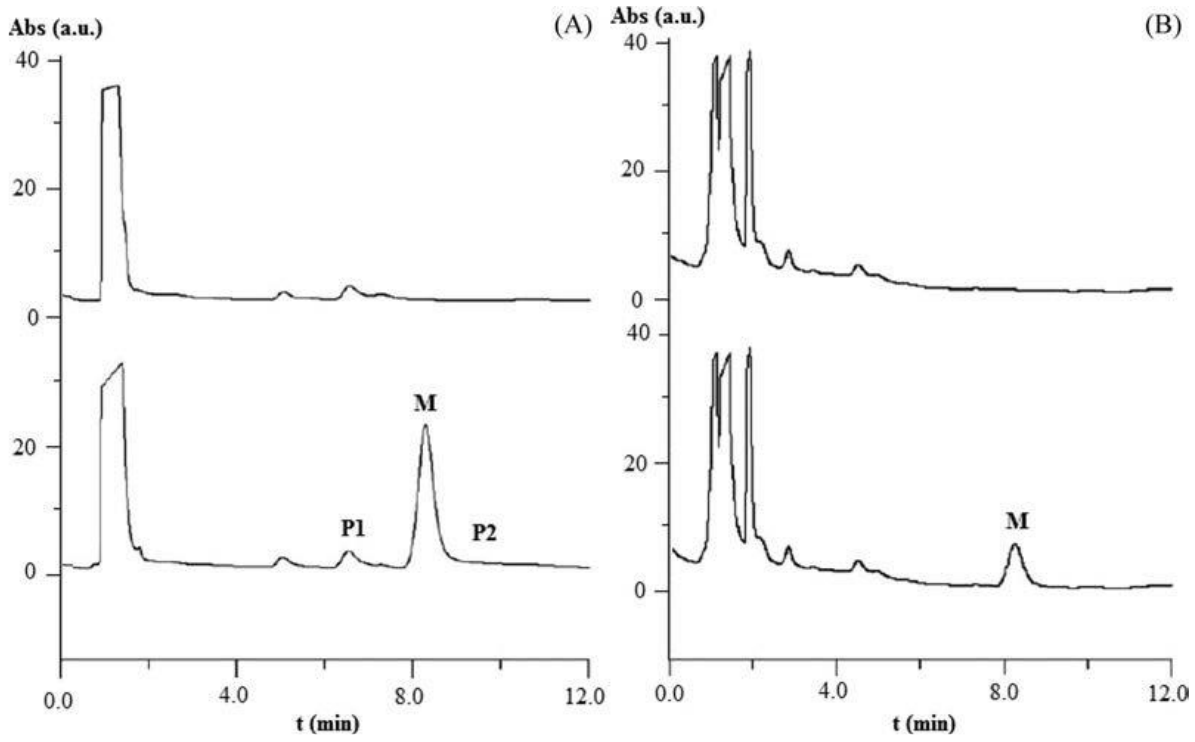


Figure 1. Chromatograms of (a) blank milk and (b) milk samples—2 mg/L melamine spiked. The top chromatograms correspond to Nutriben continuación" (Alter Group, Seville, Spain) and "Blemil Plus 2A" (Laboratorios Ordesa, Sant Boi de Llobregat, Spain). Both were powdered milk for infants supplied by a local chemist.

Figure 2. Chromatograms corresponding to the following powdered dietetic supplements provided by a local chemist: (top) PediaSure Vanilla (Abbott Laboratories Spain, Madrid) for infants and (bottom) Nutrinovex Endurance (Grupo Farmacéutico de Levante, Castelló, Spain) for adults. In both cases (a) and (b) refer to blank matrix and 2-mg/L melamine spiked sample.



Once fitted, the equations are able to predict the chromatographic behavior of these substances at intermediate values by interpolation. The theoretical values of *k*, *N*, and B/A obtained for the three analytes, at each SDS/1-propanol concentration, were combined to calculate the individual (r_{ij}) and global resolution (*Z*), following the valley-peak and the unnormalized product, respectively.[69] Besides, the equations can be used to draw simulated chromatograms, and then the analyst can visualize the theoretical changes of retention time and peak shape at slight variations of SDS and 1-propanol concentration. The optimal conditions were selected on the basis of obtaining the maximum resolution at an analysis time as short as possible but far enough from the front of the chromatogram. The results can be seen in Table 1. The error in the prediction of the retention factor was <4%. The chromatograms obtained by the analysis of melamine-spiked milk, dietetic supplements, swine kidney, urine, and wastewater samples can be seen in Figures 1–5, respectively. In all figures, P1 and P2 represent the peaks appearing immediately before and after melamine, respectively. The obtained

chromatograms show dissimilar shape because of the differences in the optimal mobile phase and the chemical composition of the matrix.

Unlike the other studied samples, plasma matrix (Figure 6) and drinking water chromatograms show a clean baseline without any peak at >4 min at any SDS/1-propanol combination. Then all the studied mobile phases can be used without overlapping risk. For plasma samples, the same chromatographic conditions as urine were suggested to allow the simultaneous analysis of both body fluids by clinical laboratory for bioanalytical purposes.[58] Drinking water was analyzed using the same mobile phase as wastewater, due to the similitude of both matrices, to enable the study of aqueous samples using the same chromatographic program.[57]

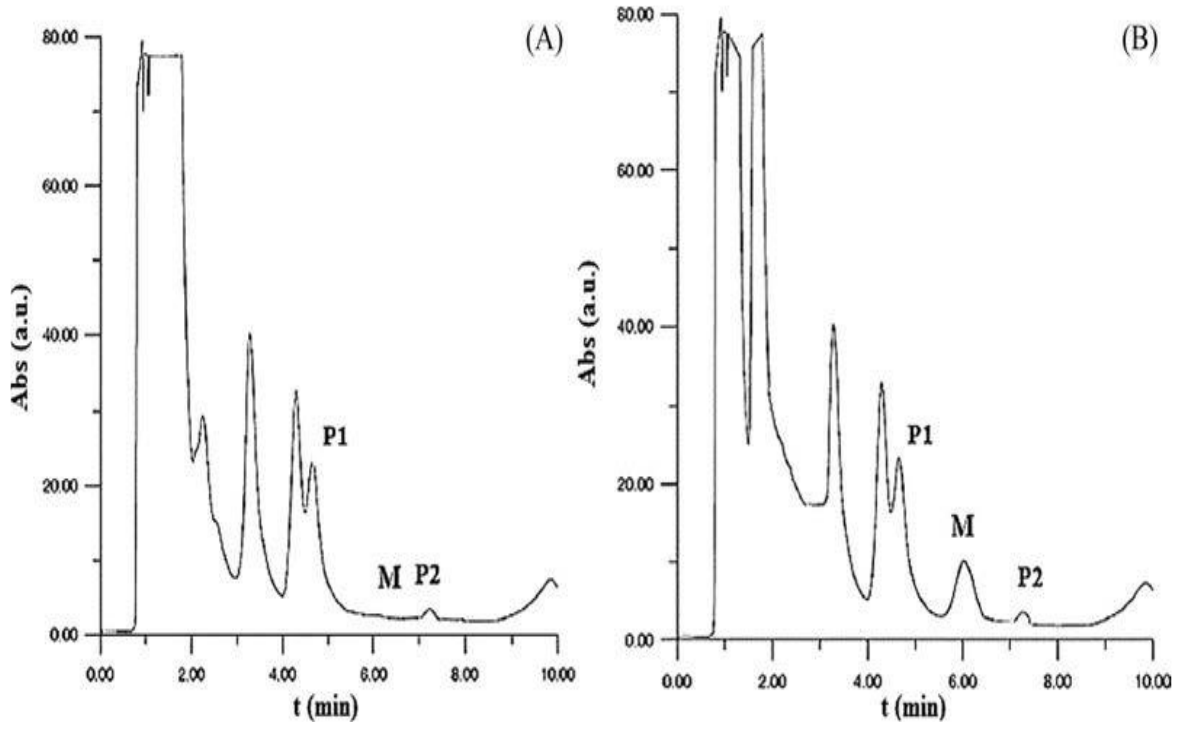


Figure 3. Chromatogram obtained by the analysis of swine kidney sample: (a) blank and (b) spiked with 0.3 mg/kg (M) of melamine.

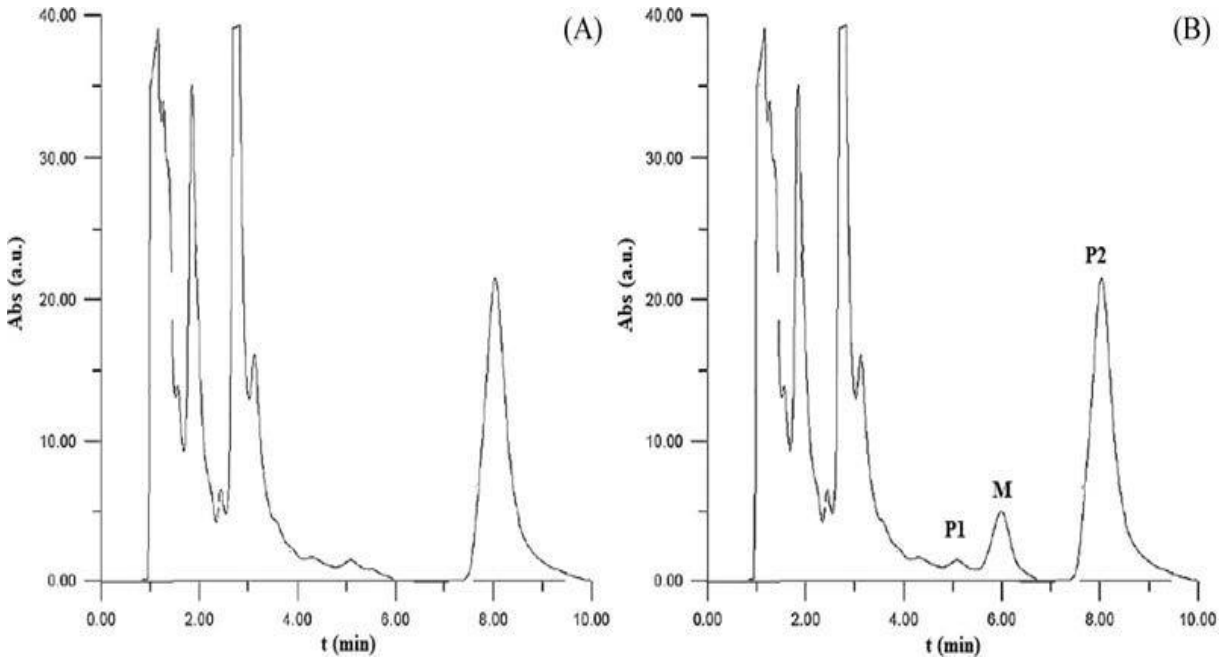


Figure 4. Chromatograms corresponding to the analysis of urine: (a) blank and (b) spiked with 2.5 mg/L melamine.

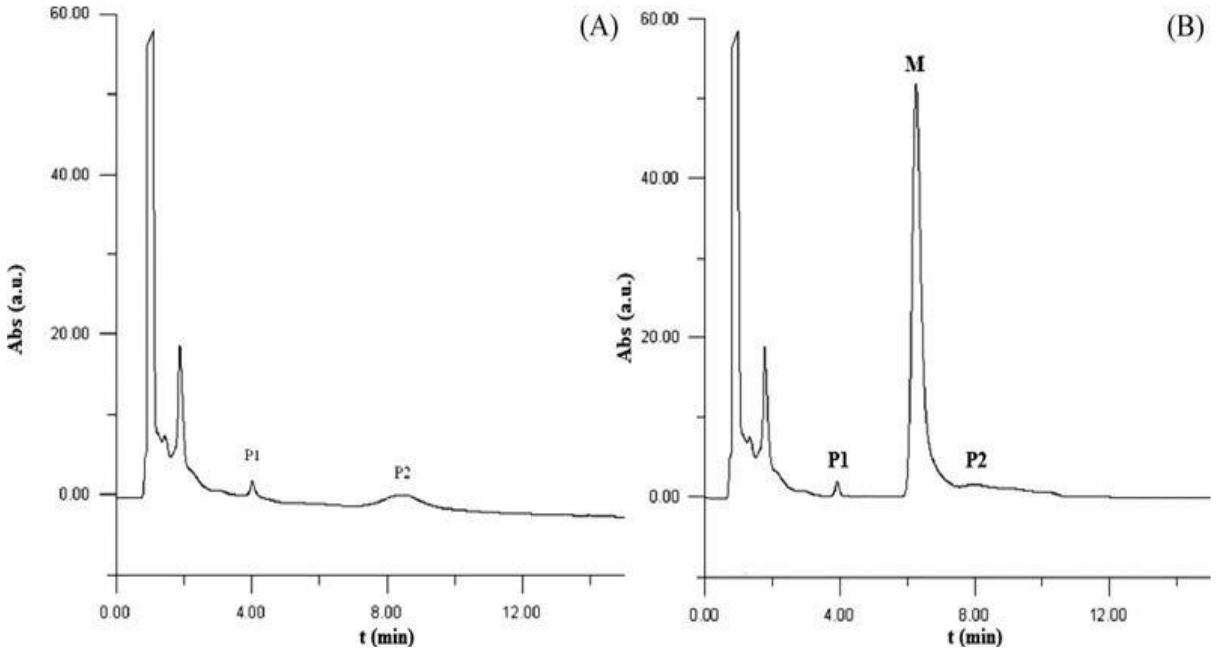
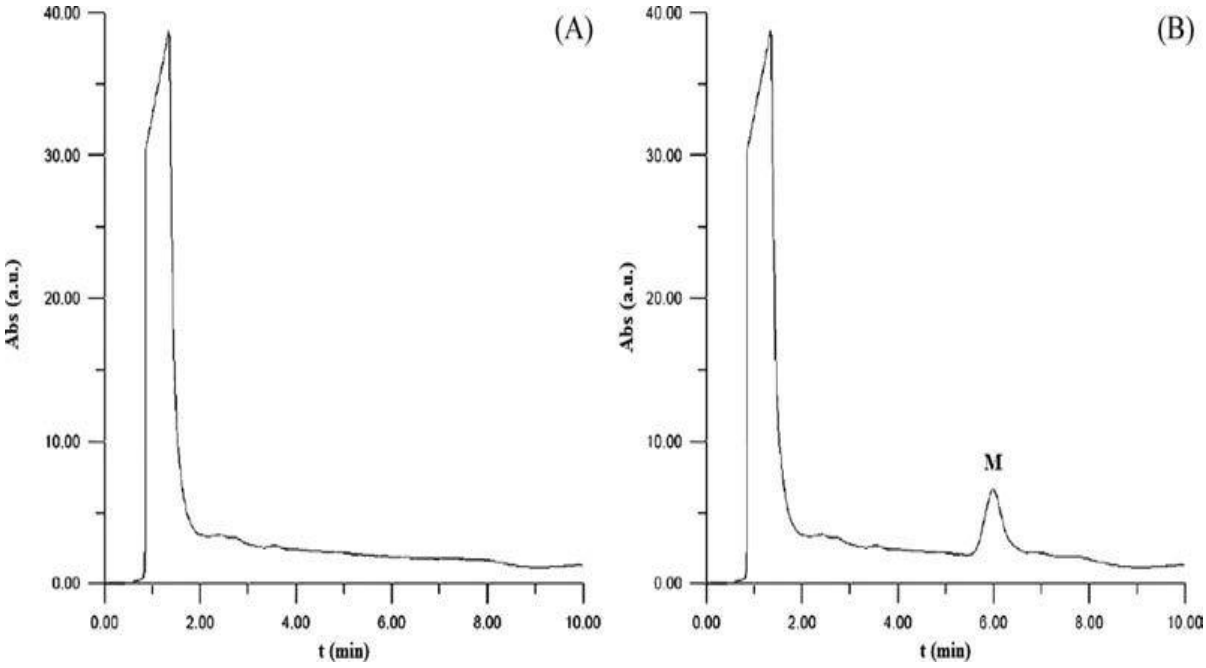


Figure 5. Chromatograms obtained by the analysis of output wastewater from a chemical company which produces Nylon and other organic compounds: (a) blank and (b) after spiking by 1 mg/L of melamine.

Figure 6. Chromatograms obtained by the analysis of plasma: (a) blank and (b) spiked with 2.5 mg/L of melamine.



4.4. Validation of the procedures

The presented analytical procedures were separately and successfully validated, as part of their development, following the FDA (body fluids, water and swine kidney)[79] and EU Decision 2002/957/EC (milk and dietetic supplement)[80] guidelines. The studied validation parameters were selectivity, sensitivity, the limit of detection (LOD), the limit of quantification (LOQ), calibration range, linearity, intra- and interday accuracy and precision, and robustness. A detailed description of each validation parameter [74] can be found in Table 3 (calibration and sensitivity), 4 (accuracy and precision), and 5 (robustness), respectively. The validation was performed using spiked samples, and the concentration values refer to the quantity in the unprocessed sample, not in the injected solution.

Table 3. Comparison of the sensitivity and linearity for melamine detection of the considered analytical methods (melamine concentration in mg/L or mg/kg, for liquid or solid matrices, respectively).

Matrix	LOD	LOQ	Calibration Range	Linearity (r^2)	References
Milk	0.05	0.2	0.20-100	0.9990	[54]
Dietetic Supplements	0.09	0.2	0.20-100	0.9996	[55]
Swine Kidney	0.1	0.3	0.3-30	0.9997	[56]
Water	0.013	0.03	0.03-5	0.9998	[57]
Plasma	0.05	0.25	0.25-25	0.9995	[58]
Urine	0.05	0.25	0.25-25	0.9996	

5. Discussion and comparison of the analytical procedures

In the studied matrices, melamine was successfully identified, the peak area was measured without overlapping, and reliably quantified thus assessing the reliability of the procedures and their applicability to real and commercial samples. The similarities and the differences of the described methods, in terms of sample treatment, chromatographic conditions, and validation results, are deeply discussed. In general, we can see that similar points are related to the analyte, while the different parameters are those depending on the composition of the matrices.

5.1. Chromatographic conditions and selectivity

The main chromatographic conditions, such as stationary phase, surfactant, pH, and absorbance wavelength, are the same in all works because they have been uniquely selected for their ability to provide an adequate resolution, retention time and sensitivity. The SDS/1-propanol concentrations were different because they were optimized considering the specific substances of the matrix. The retention time of melamine was relatively short and similar in all cases (6.0–9.3 min; Table 1).[54–58]

In all cases, no compounds appear at the window time of melamine. For plasma and drinking water, no other peaks were observed beyond >4 min, and then no confusion in the identification of melamine was possible. For the other matrices, the optimized chromatographic conditions allow avoiding coelution between melamine and the two nearest compounds P1 and P2. In milk, these compounds elute at -1.2 min and +1.7 min; in dietetic supplements, at -2 min and +1 min; in urine, at -0.9 min and þ2.0 min; in wastewater, -2.2 min and +1.9 min; and in swine kidney, -1.5 min and +1.1 min, taking as reference the retention time of melamine. In

four cases, the peaks were eluted far enough from melamine and no overlapping was detected. Therefore, a similar adequate specificity was obtained for the studied matrices.

5.2. Calibration range

The minimal level of the calibration curve was taken by visual appreciation, whereas the maximal concentration level was established as the higher concentration expected in this kind of sample. Therefore, the studied calibration interval for each matrix was selected as the working range of practical applications, and adequate linearity (r2 >0.9990) was found (Table 3).[54–58]

The method for analysis of water was developed to cover low concentrations (0.03–5 mg/L). Indeed, contaminated wastewater from industry or crops is normally diluted in irrigation ditches, rivers, and barrages, resulting in microgram per liter levels. Otherwise, drinking water is not expected to contain melamine, as it is supposed to have undergone several

strong purification processes.[57] For milk and dietetic supplements, the calibration range covers a wide interval of concentration levels (0.2–100 mg/L). Indeed, we expect moderate/

elevated concentrations of melamine in adulterated samples because melamine is deliberately added to increase the price of the product.[54,55] In fact, milk samples with a high concentration of melamine (up to 3300 mg/L) were found during China incident in 2008.[5] The upper calibration range was inferior for swine kidney, plasma, and urine, as the expected concentration in a practical case is significantly lower than in foodstuff, as, after ingestion, melamine is rapidly absorbed, distributed, and eliminated, without bioaccumulation. This prevents melamine from getting a too high concentration in body tissues and fluids. A high level is only obtained either immediately after the intoxication or by continuous ingestion of adulterated food.[13,27]

5.3. Sensitivity

The sensitivity (Table 3) was sufficient to detect melamine at the expected concentration in each kind of sample. As the measured absorbance wavelength and the injected volume were the same for all procedures, the differences in sensitivity were due to the sample pretreatment and the chemical composition. In addition, they are able to detect noncompliant

samples, which is extremely useful for quality control purposes.[54–58]

The limits of detection and quantification were lower for water samples because no dilution was applied in the experimental procedure.[57] The other matrices show similar values

of sensitivity. Indeed, urine, plasma, and swine kidney use a lower dilution ratio than milk and dietetic supplements, but the biological fluids and the methanolic extract of swine kidney contain a higher amount of solubilized compounds. These compounds are not removed by the filtration and produce a higher noise in the baseline.

5.4. Accuracy, precision and robustness

The values of accuracy, precision (Table 4), and robustness (Table 5) were similar for all the methods. Indeed, the sample pretreatment is comparable and the matrix effects are negligible because melamine is effectively separated from the matrix compounds during the chromatographic run. Besides, they fit the requirements of the validation guidelines. Therefore, the methods provide reliable quantitative values of melamine concentration, and their performances barely change through time and with slight variations of the experimental running conditions.[54–58]

Table 4. Comparison of the accuracy (calculated concentration/true concentration, %), precision (R.S.D., %), and recovery (%) for melamine quantification for each studied matrix.

		Intraday		**Interday**		
Matrix	**Evaluated Levels**	**Accuracy**	**Precicion**	**Accuracy**	**Precicion**	**References**
Milk	9	85.0-106	< 7.6	90.2-105.9	< 9.7	[54]
Dietetic Supplements	4	85.8-114.3	< 12.4	95.0-101.3	< 10.5	[55]
Swine Kidney	4	91.7-103.6	< 7.2	91.0-103.3	< 7.6	[56]
Water	4	87.8-104.1	< 14.8	93.3-101.6	< 13.4	[57]
Plasma	4	85.7-103.7	< 10.2	94.8-103.6	< 9.1	[58]
Urine	4	92.2-103.8	< 9.3	93.6-103.5	< 9.1	

Table 5. Variation (RSD, %) of retention time and peak area at slight changes in the main chromatographic conditions.

	SDS		1-Propanol		pH		Flow Rate		References
Matrix	t_R	A	t_R	A	t_R	A	t_R	A	
Milk	7.4	2.7	1.3	4.5	1.8	5.0	5.2	2.0	[54]
Dietetic Supplements	4.8	2.3	-	-	7.2	5.0	4.7	1.6	[55]
Swine Kidney	1.6	4.1	2.7	5.0	2.0	3.5	5.2	4.2	[56]
Water	1.7	2.9	1.8	4.0	1.2	9.0	5.1	5.1	[57]
Plasma	2.7	4.6	-	-	0.22	7.1	4.9	7.1	[58]
Urine	2.7	6.7	-	-		4.6	5.0	4.5	

SDS: sodium dodecyl sulfate. n = 3.

5.5. Advantages of the procedures

The here-presented procedures show significant benefits over those based on hydro-organic HPLC, which disadvantages in the sample preparation have been described in Section "Basic description of micellar liquid chromatography".[2,31,32]

The main feature of these MLC assays is, undoubtedly, the strong simplification of the sample preparation, which can be performed in 2–3 min. Effectively, the strong solubilization power of micellar solutions allows the direct injection of the samples, even from the diverse origin, without risk for the column, after a simple dilution and filtration. Therefore, tedious and time-consuming extraction or cleanup steps or complex and expensive online systems for sample purification, normally used in hydro-organic RP–HPLC, are avoided. As the sample is quantitatively introduced in the chromatographic system, the addition of an internal standard

is not needed. The participation of the operator, the number of steps, the complexity of the experimental handling, and then the potential sources of variance are minimized. This reduces the probability of analyte loss, either by side reactions, low yielding in the cleanup step, or

operational or instrumental error, thus increasing the sample throughput and the reproducibility of the method.

The use of micellar solutions also improves the separation performances. Proteins and other molecules are barely retained because of their strong affinity with the SDS micelles. Besides, the modification of the stationary phase, due to the adsorption of the anionic surfactant, improves the retention of the protonated melamine, and then its elution can be delayed until most of the possible interfering compounds have been removed. The use of an isocratic mode, instead of a gradient, allows the successive injection without stabilization time, thus reducing the effective duration of the chromatographic run and the production of waste.

These MLC procedures hold important practical advantages. The used solutions contain innocuous and biodegradable reagents (SDS, phosphate buffer, and salts), and only a small amount of toxic, flammable, and volatile organic solvent: <2 mL of methanol per sample in the dilution solution and <7.5% of 1-propanol in the mobile phase. These values are significantly less than usually required in hydro-organic RP–HPLC. In addition, the alcohol becomes less dangerous in micellar solutions, as its volatility decreases. Therefore, the operator is barely exposed to dangerous chemicals, and the produced waste contains a minimum proportion of pollutants. This fits the current trend in analytical chemistry. Besides, the work and expenditure related to waste segregation and treatment are reduced.[81]

A large number of samples per day can be processed using these procedures due to the short global analysis time and easy experimental work. Therefore, they are particularly useful in laboratories receiving many samples which must be quickly analyzed, such as clinical, food safety, and environmental ones. Besides, the methods only require basic apparatus and instrumentation, and a small number of inexpensive reagents, and then the analysis of the samples can be performed at a reasonable price, which is interesting in the current context of economic crisis.

6. Conclusions

In the present review, we prove the applicability of MLC for the determination of melamine in several kinds of samples, which must be controlled to evaluate and manage the impact of this compound on the consumer's health: milk, dietetic supplements, swine kidney, plasma, urine, as well as drinking, and wastewater. The strategy for the development and validation of the analytical assays, as well as the finally obtained results, has been described. We have also outlined and explained the similarities and differences between them. Besides, the advantage of using MLC over hydro-organic RP–HPLC has been discussed.

The main feature of the procedures is their simplicity, so that it consists of a one-step assay based on dilution and direct injection, in spite of the diversity and complexity of the studied matrices. Only the solid sample had to be liquid extracted before the dilution. The experimental and chromatographic conditions were the same for all methods, except the dilution ratio and the SDS/1-propanol concentration, which was adapted to the chemical composition of each sample. In all cases, melamine was resolved from the endogenous compounds of the matrix in <12 min with an adequate peak shape and sufficient sensitivity. In general, the procedures and their corresponding performances were found quite similar, and the differences were because of the dissimilar chemical composition of the sample. The methods were validated in spiked samples, following the requirements of official validation guidelines, and then successfully applied to practical cases. No significant differences in terms of selectivity, linearity, accuracy, precision, and robustness were found between the studied matrices. However, the water analysis method shows higher sensitivity and its calibration range cover a lower concentration interval, while these parameters were similar for the other matrices.

According to the validation results, the methods are able to reliably detect and quantify melamine at the permitted levels for dairy products, as well as to detect slight melamine intoxication through the biological fluids or industrial spills in environmental water. Besides, the here-described analytical procedures were relatively inexpensive, easy-to-handle, rapid, safe, eco-friendly, accessible, efficient, reliable, and useful for routine analysis. According to the practical aspects and the validation results, MLC can be considered as an attractive and alternative to be implemented by analytical laboratories devoted to the global preventing and managing of the melamine menace.

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Chapter 4

Development and Validation of a Micellar Liquid Chromatographic Method to determine three Antitumoral TKIs in Plasma

ABSTRACT

- Aim: A micellar liquid chromatographic method to determine several antitumoral drugs (pazopanib, dabrafenib and regorafenib) in plasma was developed and validated by the guidelines of the EMA.
- Experimental: Plasma samples were directly injected, after a 1/5-dilution in a micellar solution. The drugs were resolved in <18 min using a C18 column. The mobile phase was an aqueous solution of 0.12 M SDS – 2% 1-pentanol, buffered at pH 7. The detection was performed by absorbance at 260 nm.
- Results: The values of the main validation parameters were: LOD (0.1–1 mg/l), calibration range (0.2–2 to 80 mg/l), accuracy (-12.5 to +11.7%) and precision (<11.9%).
- Conclusion: The procedure was conducted by minimum cost, effort, manipulation, time and quantity of hazardous chemicals. The method was useful to determine the drugs at their respective target concentrations, and was found useful for clinical analysis.

1. Introduction

Cancer is a group of diseases involving uncontrolled cell proliferation. The development of oncogenic malignancies is caused by genetic modifications, which induces numerous abnormal biological processes. Tyrosine kinases are involved in the control of several biological processes and have been recognized as hot spots of oncogenic transformation. The tyrosine kinase inhibitors (TKIs) are a potent group of antineoplastic agents that specifically target tyrosine kinases that participate in the abnormal growth of tumor cells. TKIs have minimal side effects compared with cytotoxic chemotherapeutic agents and are often synergistic in combination with radiotherapy and/or chemotherapy [1–3]. Within this group of drugs that are getting satisfactory results, we found pazopanib (PAZO) [4], regorafenib (REGORA) [5] and dabrafenib (DABRA) [6]. The main chemical and pharmacological parameters can be seen in Table 1 [7–11] and the structures in Figure 1.

Pazopanib (Votrient®) is an angiogenesis agent and multi-TKI, which is able to block a large number of receptors: PDGFR-α and -β, VEGFR-1, VEGFR-2, VEGFR-3, Kit, FGFR-1 and -3, cFms, Itk and Lck. PAZO is administered to patients suffering from advanced soft tissue sarcoma or advanced renal cell carcinoma, who have already been treated by other chemotherapeutic agents [7,12,13].

Regorafenib (Stivarga®, BAY 73-4506) is a multikinase inhibitor for RET, VEGFR-1, VEGFR-2, VEGFR-3, KIT, PDGFR-α, PDGFR-β, FGFR-1, FGFR-2, TIE-2, DDR2, Trk2A, Eph2A, RAF-1, BRAF, BRAFV600E, SAPK2, PTK5 and Ab. On 27 September 2012, the US FDA approved REGORA for the treatment of patients with metastatic colorectal cancer [7,14,15]

Dabrafenib (TAFINLAR®) is a TKI prescribed as monotherapy for unresectable or metastatic melanoma with BRAF V600E or V600k mutation. At high concentrations, DABRA is also able to inhibit wild-type BRAF, CRAF, SIK1, NEK11 and LIMK1 kinases [7,16].

Separation techniques, especially HPLC, are nowadays preferred for bioanalysis for clinical purposes [17]. Literature reveals a few chromatographic methods to determine PAZO [18,19], REGORA [20,21] and DABRA [22,23] in plasma. However, these methods require large and complicated sample preparations, with several extraction and purification steps because of the extremely complex chemical composition of plasma. Besides, they use specific

instrumentation, a large amount of toxic solvents and the analysis results at a high price. In our knowledge, no HPLC method has been developed for the simultaneous analysis of these compounds. The availability of an easy, rapid and reliable HPLC method to determine the plasma concentration of these TKI analytes at values over their Ctrough–Cmax (Table 1) would be very useful to monitor and control the therapeutic treatments [17].

Micellar liquid chromatography (MLC), using hybrid mobile phases of the anionic surfactant SDS, is an alternative to the determination of drugs in biological fluids. Indeed, the micellar solutions have different solubilization sites: bulk water, polar; the surface of the micelle, electrostatic and polar; the core of the micelle, hydrophobic; and miscellaneous, at the palisade layer between these two zones. Micelles and monomers tend to bind proteins and other macromolecules competitively releasing bound drugs. Therefore, they are denatured and solubilized, and then, in the column, they are harmlessly eluted at the front of the chromatogram, instead of precipitating. This allows the direct injection of the sample. The environment in the column is strongly modified. The monomer settles on the C18 stationary phase, with the polar group facing outward. This increases the hydrophobicity of the mobile phase and generates an outer anionic layer. The mobile phase contains free monomers, at the critical micellar concentration, the corresponding organic solvent and the micelles. The solute can partition between three phases (modified stationary phase, bulk mobile phase and micellar pseudo phase). This high multiplicity of interactions and equilibria complexes the retention mechanism and improves the stability and versatility of the technique, enabling its modeling by chemometrics. Besides, the use of hybrid micellar mobile phases makes a method more innocuous, nonflammable, biodegradable and inexpensive than those based on hydro-organic ones [24,25].

The aim of the work was the development of an analytical method to quantify PAZO, DABRA and REGORA in plasma. The use of MLC was explored. The method should be able to reliably quantify the drugs at the clinical concentrations, and be useful for routine analysis in a clinical laboratory. Especially, the sample preparation must be easy to handle, and the use of hazardous chemicals must be limited. In order to test the analytical performances, the procedure must be validated by the guidelines of the EMA [26]. Finally, its reliability should be demonstrated by analyzing samples of plasma from cancer patients following a therapy

based on these drugs, provided by a local hospital.

Table 1. Physicochemical and pharmacological parameters of the studied drugs [7-11].

Drug	**Pazopanib**	**Dabrafenib**	**Regorafenib**
Developer	GlaxoSmithKline (London, UK)	GlaxoSmithKline (London, UK)	Bayer (Leverkusen, Germany)
Log Po/w	3.6	5.5	4.5
pKa (basic)	2.1/6.4	2.2/6.6	2.3
pKa (acidic)	10.2	-----	12
Charge at pH 7	0	0	0
Bioavailability	14-39 %	95 %	69-83 %
Usual treatment	800 mg once daily for 22 days	150 mg each 12 h, until disease progression or unacceptable toxicity	160 mg once daily for 21 days
Time to peak plasma	2 - 4 h	2 h	4 h
Cmax	58.1 mg/L	1.5 mg/L	2.5 mg/L
C trough	20.5 mg/L	0.03 mg/L	3.9 mg/L
Half life	35 h	8 h	28 h
Route of elimination (unchanged)	82.2 % feces 4 % urine	71 % feces	47 % feces 2 % urine

2. Experimental

2.1. Standards & reagents

Powdered standards of PAZO hydrochloride (purity >98.0%), DABRA mesylate (>98.0%) and REGORA (>98.0%) were purchased from LGM Pharma (TN, USA). SDS (>98.0%), 1-pentanol and DMSO (HPLC grade) were supplied by Scharlab (Barcelona, Spain). Sodium dihydrogen phosphate monohydrate (>98.0%) and hydrochloric acid (37%) were bought to Panreac (Barcelona, Spain). Sodium hydroxide (>98%) comes from Riedel–deHaën (Hannover, Germany). Ultrapure water was generated in the laboratory from deionized water, supplied by the university as tap water, using an ultrapure water generator device, Simplicity

UV (Millipore S.A.S., Molsheim, France). This ultrapure water was used to prepare all solutions and mobile phases.

2.2 Preparation of solutions & mobile phases

Micellar solutions were prepared by weighting the appropriate quantity of SDS and NaH2PO4.H2O and solving them in ultrapure water using an automatic stirrer. Afterward, the pH was adjusted to the desired value by adding drops of HCl or NaOH solutions. Furthermore, the adequate volume of organic solvent was added and the flask was filled up with ultrapure water. Finally, the solutions were vigorously shaken, ultrasonicated for 15 min to ensure the total solubilization and filtered through a 0.45 μm nylon membrane filters (Micron Separations, MA, USA) situated on a Büchner funnel, with the aid of a vacuum pump. They were stored in amber bottles.

Individual stock solutions of the studied antitumor drugs (200 mg/l) were prepared in DMSO. Working solutions (individual and mixtures) were prepared from stock solutions by successive dilution in a micellar solution of 0.05 M SDS buffered at 7 with 0.01 M phosphate salt.

2.3. Chromatographic conditions

The chromatographic system was an HP1100 (Agilent Technologies, CA, USA) equipped with an isocratic pump, an autosampler, a degasser, a 20-μl loop, a Kromasil C18 column (150 × 4.6 mm; 5 μm particle size; 10 nm pore size) and a UV-absorbance diode array detector. The software Agilent ChemStation (Rev. A.10.01) was used to control the system and to register the signal. The dead time (t0), retention time (tR) and peak area (A) were taken from each chromatogram using the same software.

The mobile phase was an aqueous solution of 0.12 M SDS – 2% 1-pentanol, buffered at pH 7 with 0.01 M phosphate buffer, running at 1 ml/min under isocratic mode without controlling the temperature. The detection absorbance wavelength was set to 260 nm. The chromatographic vials and the column remained at room temperature throughout the whole analysis. The special care required with the chromatographic instrumentation when dealing

with micellar mobile phases is described in [27].

2.4. Sample collection & preparation

Samples of plasma from cancer patients and healthy volunteers, were provided by a local hospital, after consent of patients and doctors at the time of blood collection. The blood was collected by the clinical staff using a DB SST tube (SBD Vacutainer Systems, Plymouth, UK), and centrifuged at 4°C at 3000 rpm for 10 min (3000 x g), to obtain the noncellular fraction. The samples were frozen and stored at -20°C from collection to analysis. For confidentiality reasons, our laboratory did not receive any personal or clinical information about the patients. The samples were only used for this study and later destroyed. No additional analyses were carried out, and no information was shared with other institutions. The healthy volunteers do not take these medications. Therefore, their plasma did not contain the analytes and was taken as 'blank' plasma samples.

Patient and blank samples were processed in the same way. Before the analysis, the samples were thawed for 30 min at room temperature. Afterward, they were 1/5 diluted using a micellar solution of 0.05 M SDS buffered at pH 7 with 0.01 M phosphate salt, vigorously shaken, filtered through a 0.45-μm nylon membrane filter (micron separations) with the aid of a 3-ml syringe, and directly injected. For spiked samples, the appropriate amount of the standard solution of drugs was added before dilution. The processed samples were destroyed after analysis.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

The anionic surfactant, SDS is considered as the most adequate for the analysis of drugs in biological fluids. Indeed, it shows a proven ability to denature and solubilize plasma proteins

and other nonwater-soluble macromolecules as well as other interesting practical advantages (purity, solubility, low viscosity of the solutions and low Kraft point). Therefore, it was selected for this study. Therefore, a C18 column was chosen because SDS monomers are easily adsorbed on C18 hydrocarbon chain, with a saturation point of only 10 mM. Isocratic mode was chosen for its higher simplicity [24,28]. According to the hydrophobicity (log Po/w 3.6–5.5) and charge of the drugs (neutral), they would be strongly retained on the modified stationary phase, and then the addition of 1-pentanol was envisaged to increase the hydrophobicity of the mobile phase, thus resulting in a faster elution and improving the peak shape [24].

The following experimental parameters were optimized: mobile phase composition (pH, and concentration of SDS and 1-pentanol) and the detection conditions. The chromatographic analyses were carried out using a working standard solution containing 1 mg/l of each TKI.

3.2. Selection of pH

The effect of the pH on the retention was evaluated, inside the working range of the column (1.5–9.5). However, the study was restrained to acidic (3 and 5) and neutral (7) pH, in order to limit the damage to the stationary phase. At pH 3, no peaks were observed, whereas at 5, strongly distorted peaks were observed. At pH 7, the peaks show a normal shape. Therefore, the optimal pH was set to this value.

3.3. Effect of SDS & 1-pentanol amount on the retention

The influence of SDS and 1-pentanol (qualitative and quantitative) on the retention factor of the three antitumoral drugs was investigated to model the retention factor and the global resolution, using an interpretative strategy. The experimental design was a face-centered composite design, taking as -1 and +1 the minimal and maximal concentrations recommended in MLC for both chemicals. Therefore, the analytes were injected in nine micellar mobile phases, containing the following concentrations of SDS (M)/1-pentanol (%): 0.05/2; 0.05/4;

0.05/6; 0.1/2; 0.01/4; 0.01/6; 0.15/2; 0.15/4 and 0.15/6. The experimental dead time was 1.00 min. For each one, the retention factor (k) of the TKIs was calculated.

In the nine mobile phases, PAZO was the less retained, followed by DABRA and REGORA. The three drugs showed a binding behavior with the micelles, as the retention time diminished at increasing concentrations of SDS. This is probably due to the hydrophobic interaction between the drug and the inner core of the micelle. Otherwise, the augmentation of the proportion of 1-pentanol results in an earlier elution of the three compounds, as expected in MLC [28].

3.4. Modeling of the retention factor

A chemometric model was proposed to model the retention factor, in order to diminish the time and effort required for the optimization. Eq. (1) has been proposed for moderately hydrophobic compounds, to predict the retention factor depending on the concentration of SDS and 1-pentanol (φ) in the hybrid micellar mobile phase, running under isocratic mode [29]:

$$1/k = C_0 + C_1[SDS] + C_2\varphi + C_{12}[SDS]\varphi$$

[SDS] and φ are in normalized unities (-1 to +1) from (0.05 - 0.15 M) and (2 - 6 %), respectively, in order to better compare the constants. The constant C_0 indicates the retention factor at average values of each chemical, while C_1, C_2 and C_{12}, quantify the effect of each factor and their interaction. For each drug, the equation was adjusted using the values of retention factor obtained in the experimental design (5 freedom degrees), by curve-fitting non-linear least-square regression [30]. The accuracy of the mathematical model was proven

experimentally by the values of the multiple regression coefficient (R2), and results are showed for pazopanib, dabrafenib and regorafenib and respectively:

$1/k = (0.531\pm0.024)+(0.28\pm0.03)[SDS]+(0.13\pm0.03)\varphi+(0.14\pm0.04)[SDS]\varphi \quad R^2 = 0.96$

$1/k = (0.1556\pm0.0016)+(0.0401\pm0.0021)[SDS]+(0.0376\pm0.0021)\varphi+(0.004\pm0.003)[SDS]\varphi$

$R^2 = 996$

$1/k=(0.101\pm0.003)+(0.041\pm0.004)[SDS]+(0.043\pm0.004)\varphi+(0.013\pm0.005)[SDS]\varphi$

$R^2= 0.990$

Figure 1 shows the theoretical retention times of the three drugs at concentrations of SDS and 1-pentanol ranging from 0.05-0.15 M and 2-6 %, respectively. The order of retention times (regorafenib > dabrafenib > pazopanib) was maintained in the whole interval of surfactant and alcohol concentrations.

The effect of each factor and the interaction on the retention factor was inferred from the values of the constants. For the three drugs, the significance of the constants was < 7 %, and then all of them were included in the model. Besides, they have a positive value, that means an increase of the concentrations of SDS and 1-pentanol effectively reduce the retention. These deductions are similar to those extracted from the unprocessed results of the experimental design. Besides, the effect of SDS is enhanced at higher amounts of 1-pentanol, and vice versa. This may be caused by the decrease of the CMC and the aggregation number, and then the augmentation of the total number of micelles, caused by the introduction of the organic modifier [25]. For pazopanib and dabrafenib, SDS has a higher influence than 1-pentanol, unlike for regorafenib. The interaction was the less relevant.

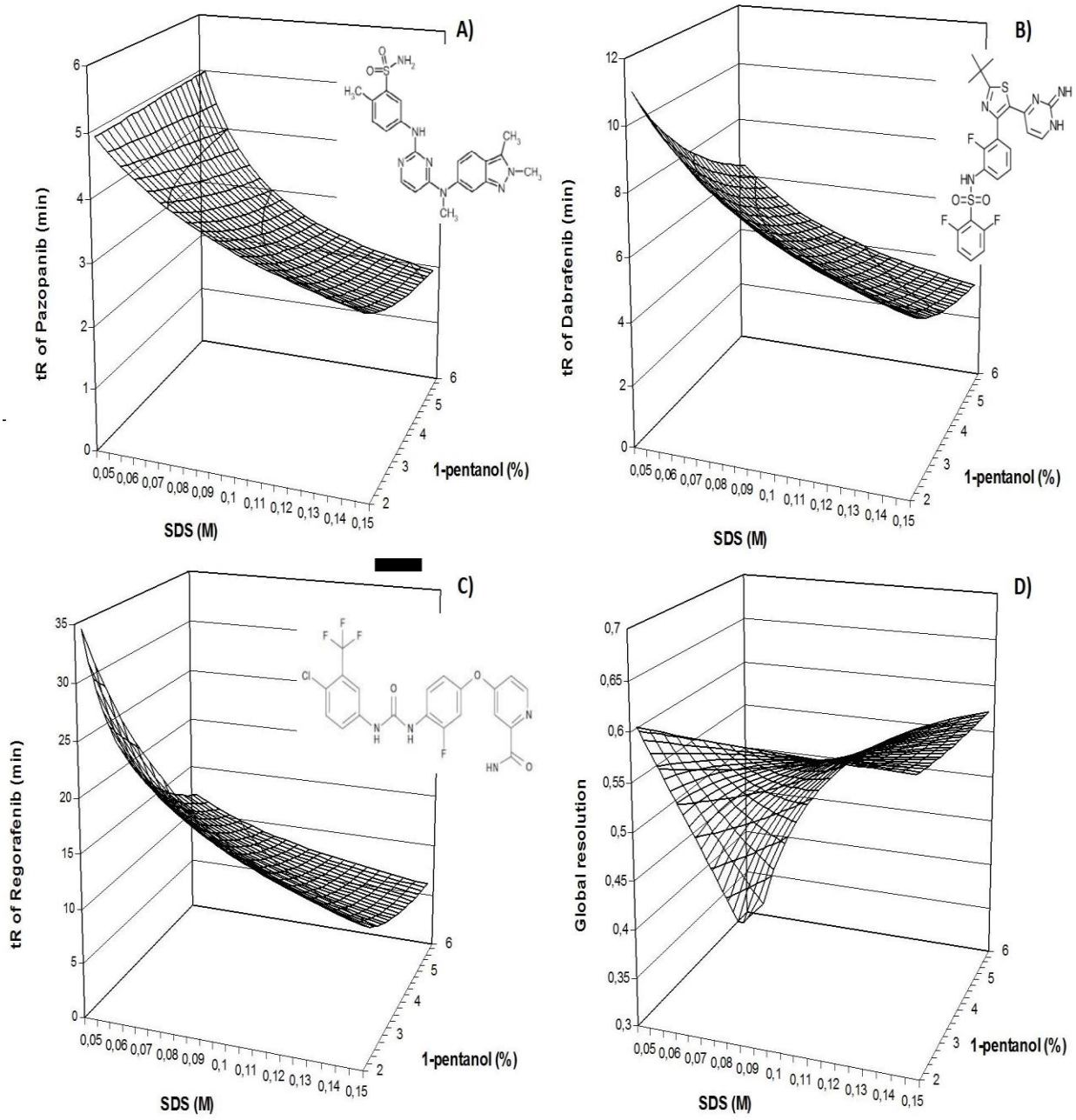

Figure 1. Predicted retention time of A) pazopanib, B) dabrafenib and C) regorafenib, and D) global resolution, at several SDS/1-pentanol concentration. The structure of each drug is also shown.

3.5. Optimization of the SDS/1-pentanol concentration

The optimal concentrations of SDS and 1-pentanol were established by surface response methodology, in order to maximize the global resolution (*Z*) and minimize the analysis time (retention time of the most retained drug).

The elementary resolutions at any combination of SDS/1-pentanol were calculated between two consecutive peaks as the modified selectivity, from the equations adjusted in 3.4. The paired peaks were (pazobanib: dabrafenib) and (dabrafenib: regorafenib), as the elution order was maintained:

$$\alpha'(PAZO/DABRA)=1-k(PAZO)/k(DABRA)=1-(0.1556+0.0401\,[SDS]+0.0376\varphi+0.004[SDS]\varphi)/(0.531+0.28[SDS]+0.13\varphi+0.14[SDS]\varphi)$$

$$\alpha'(DABRA/REGORA)\ 1-\ (0.101+0.041[SDS]+0.043\varphi+0.013[SDS]\varphi)/\ (0.1556+0.0401\,[SDS]+\ 0.0376\varphi+0.004[SDS]\varphi)$$

The global resolution was modelled as the minimal elementary resolution (Figure 1D). At proportions of 1-pentanol of 2 %, the maximal resolutions were reached, and remained acceptable (0.45-0.64) for the entire interval of SDS. The optimal SDS concentration was fixed to 0.12 M, where the global analysis time was acceptable (15.6 min) and the less retained compound was eluted far enough (3.2 min) to prevent overlapping with the front of the chromatogram when plasma samples are injected. Thereafter, a mixture of the three drugs was analyzed using a mobile phase of 0.12 M SDS - 2 % 1-pentanol buffered at pH 7 with 0.01 M phosphate salt. The peaks showed adequate shape, and the experimental retention times (min) were: PAZO, 3.4; DABRA, 8.8 and REGORA, 15.9. The error in the prediction of the retention times was < 6.5 %. The experimental modified selectivity was 0.47.

3.6. Detection conditions

A working solution containing the three drugs was analyzed under the optimized conditions, and the absorbance spectra were measured between 200 and 800 nm at their retention time. The three drugs showed a high absorptivity at 260 nm. Therefore, the detection was performed at this value, and no wavelength changes were required.

3.7. General comments about the procedure

The developed method shows several practical features, because of the specific properties of micellar solutions.

The foremost advantage of the method is the simplification and shortening of the sample preparation. Indeed, the ability of SDS-micellar solutions to solubilize the proteins has eradicated the needing of extraction or cleanup intermediate steps. Therefore, plasma can be quantitatively injected into the column, thus minimizing the sources of variance and limiting the loss of the analytes, and then an internal standard is not compulsory. The dilution decreases the sensitivity, but it is necessary to reduce the number of proteins and other macromolecules in the chromatographic system.

The analysis has a scarcely harmful effect on the environment and the health of the laboratory staff. The pure micellar solution (used in the sample pretreatment) contains only innocuous and biodegradable chemicals, and the hybrid micellar solution (mobile phases) additionally includes a lower amount (<2%) of volatile, toxic and flammable organic solvent, less than usually required in RP-HPLC (up to 100 %). Besides, its volatility is reduced by the interaction with SDS. Therefore, the handling and waste of toxic chemicals are minimal.

The method is adapted to the analysis of a large number of samples per day, as the successive analysis of the samples is facilitated and the global analysis time is significantly reduced. A single operator can successively process many samples, due to the short duration of the sample treatment (2 - 3 min).

The use of this method makes the analyses of individual plasma samples relatively inexpensive. The cost and the amount of the consumed chemicals are relatively low, and only basic laboratory materials and instrumentation are used. Otherwise, the characteristics of the chromatographic conditions (pH of the mobile phase, sample dilution and isocratic mode) have been selected to enlarge the lifespan of the stationary phase.

4. Method validation

The developed procedure was in-lab fully validated by the Guideline on Bioanalytical Method Validation, published by the EMA [26]. The studied parameters were: selectivity, calibration curve, lower limit of quantification (LLOQ), within-run and between-run accuracy and precision, carryover, dilution integrity, matrix effect and stability [26]. The detection limit [31]and the robustness [32] were as well investigated. The validation was carried out using blank plasma samples from healthy volunteers fortified with the studied TKIs, unless specified.

4.1. Selectivity

Blank samples of plasma were taken from six healthy individuals, who do not take any medication: three males and three females, and for each gender, at three different age frames: 20, 40 and 50. Each sample was analyzed before and after (Figure 2A) spiking with 5 mg/l of each analyte. The retention times (min)/capacity factor were: PAZO, 3.4/2.4; DABRA, 8.8/7.8; and REGORA, 15.9/14.9. The selectivity values were: αDABRA/PAZO = 3.3 and

αREGORA/DABRA = 1.9. The resolution parameters were: RsPAZO–DABRA: 5; and RsREGORA–DABRA: 5.8. These values indicate the high quality of the chromatographic resolution.

In the chromatograms obtained by the analysis of blank plasma, only a high and broad front of the chromatogram (dead time to 2.5 min) and a quite stable baseline without peaks (>2.5 min) were observed. Indeed, as the matrix compounds are strongly associated with the micelles, they barely interact with the modified stationary phase, and are quickly eluted. In addition, the dilution has contributed reducing the size of the protein band.

The spiked samples provide chromatograms with a similar protein band and baseline, and without other peaks, except those corresponding to the analytes. These ones were eluted at similar retention time (<2%) and showed the same absorbance spectra, as those obtained by injecting a standard micellar solution (see the 'Optimization of the SDS/1-pentanol concentration' section). The less retained drug (PAZO) was eluted after the front of the chromatogram and then no overlapping was observed. Therefore, the procedure was selective enough to identify these three drugs in plasma unequivocally.

4.2. Calibration range & sensitivity

For calibration purposes, samples of plasma were spiked at increasing concentrations of the three drugs (up to 80 mg/l) and analyzed by triplicate. For each drug, the peak area was related to the corresponding concentration by a first-grade equation, using the least-square linear regression method. The lowest LOQ was the smallest level which can be measured (using the calibration curve) with accuracy and precision within the requirements of the guide, while the highest LOQ was established at the maximal concentrations usually reached in the clinical practice. Levels under LLOQ were removed from the definitive calibration curve. The LOD was determined by the 3-s criterion [31]. The values of these calculated parameters can be seen

in Table 2. A chromatogram obtained by the analysis of a plasma sample spiked at the LLOQ for each drug is shown in Figure 2B.

An adequate linearity (r^2 >0.9999) was obtained along the studied range for the three drugs. Besides, the LLOQs were below their target concentration.

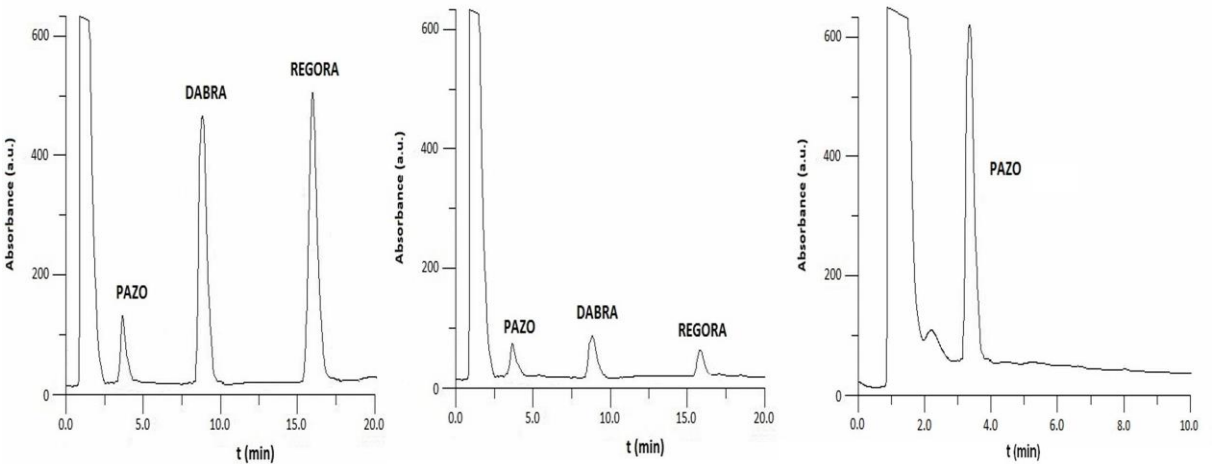

Figure 2. Chromatogram obtained by the analysis of plasma samples spiked with each drug at A) 5 mg/L and B) their corresponding LOQ, and C) from patient 5.

4.3. Accuracy & precision

These parameters were studied in the matrix at four concentrations for the three drugs: LLOQ, nearly three times the LLOQ, 20 and 60 mg/l. The within-run measurements were performed out by six successive injections of a spiked sample. The accuracy was calculated as the average relative error of the found concentration, whereas the precision was the RSD of the peak areas. The between-run values were determined to evaluate their consistency over time. The within run experiment was repeated in 5 days over a 2-month period, using renewed spiked samples each time. The accuracy was determined as the average of the five within-run errors, while the

precision was the RSD between the five average peak areas (each one calculated from the six analysis). The results can be seen in Table 3.

The values of error (12.6–11.7%) and variability (<11.9%), were inside the requirements of the guide (20% for LLOQ and 15% for upper levels). These results were reached by the minimization of the sources of variance and the quantitative injection of the sample. Therefore, the analytical method provides reliable quantitative values for the three drugs throughout the studied calibration range.

Table 3. Intra- and inter-day accuracy and precision for the studied drugs.

		Intra-day[a]		**Inter-day[b]**	
Drug	Concentration (mg/L)	Accuracy (ε, %)	Precision (RSD, %)	Accuracy (ε, %)	Precision (RSD, %)
Pazopanib	2	+4.2	11.9	+6.1	8.1
	5	-2.1	7.7	-4.1	6.9
	20	+3.8	0.3	1.7	4.1
	60	-2.2	0.4	-2.3	1.9
Dabrafenib	0.5	-1.9	6.2	-4.5	5.3
	2	-2.0	3.6	-2.6	4.7
	20	+2.2	0.3	+1.2	1.3
	60	-6.1	2.3	-2.2	4.0
Regorafenib	0.2	+11.7	8.3	+11.2	8.8
	0.5	-12.6	10.8	-12.5	5.3
	20	-8.5	2.7	-4.8	2.1
	60	+5.8	0.5	+6.8	0.7

[a]n=6; [b]n = 5

4.4. Dilution integrity

The within- and between-run accuracy and precision were determined at 50 mg/l for each drug, using the protocol indicated in the previous section, introducing a 1/10 dilution in a solution of 0.05 M SDS buffered at pH 3.

The values of intraday accuracy and precision were similar to those shown in Table 3, indicating that the introduction of the dilution step does not significantly affect the quantitative information, and then can be a useful strategy to analyze samples contaminated at levels over the high LOQ.

4.5. Carry-over effect

The carry-over effect was evaluated, in order to evaluate the possibility of cross-contamination in an analytical run. A plasma sample spiked at 80 mg/L of each TKI, and a blank sample was processed and successively analyzed. No peak was observed at the window time of the analytes in the chromatogram obtained from the blank sample. Consequently, the transfer of drugs to the following analyzed sample can be considered insignificant, at concentrations under the high LOQ.

4.6. Matrix effects

The possible interference of endogenous compounds on the quantitative results was investigated at two levels for each drug: PAZO, 5 and 60 mg/l, DABRA, 2 and 60 mg/l and REGORA, 0.5 and 60 mg/l. A standard micellar solution and plasma-spiked samples were 1/5-diluted and analyzed. For the three drugs, the peak areas were similar in both cases, indicating that the matrix effect can be neglected. Indeed, after the dilution, the micellar solution is predominant, and then the chemical environment of the drugs is quite similar, in spite of the original matrix. Besides, the micelles bind the most important matrix compounds (proteins and

macromolecules), weakening their interaction with the drugs [24].

4.7. Stability

The stability of the drugs was evaluated at the same concentrations as in the previous section in working solutions and in plasma matrix (freeze and thaw, and long-term freeze), at their usual storage conditions. The possible degradation was considered by the diminishing of the peak area of each analyte in the chromatogram obtained by the analysis of the samples and the appearance of other peaks, corresponding to decomposition products. A stored solution must be discarded if the concentration varies more than 15% of the initial concentration.

The working solutions were kept at 4°C in the fridge. Once a day, the working solutions were thawed, 1/5-diluted, analyzed and stored another time. This was repeated for two months. No variation of the peak area was observed, and then the working solutions can be used for this period without introducing a systematic error in the method.

Spiked plasma samples were kept at the usual storage conditions of biological fluids for clinical purposes (-20°C in a freezer for 15 days). Two situations were considered:

- Freeze-and-thaw stability: a spiked sample for each matrix and concentration was stored. Every 12 h, the sample was thawed, an aliquot analyzed, and replaced in the freezer. No variation of the peak area was observed in the considered period. Therefore, the same sample can be used for the clinical and analytical studies each day for a minimum of 2 weeks;

- Long-term freeze stability: fifteen samples (for each matrix and concentration) were spiked and kept. Each day, a sample (for each category) was thawed, analyzed and discarded. The concentration of the drugs was the same in all the measures, and this storage protocol can be applied for the quantification of these drugs in biological samples extracted from patients.

When many samples arrive at the laboratory and must be successively analyzed, a processed sample may remain a long time in the autosampler tray before injection. Therefore,

the short-term stability of the drugs in the processed matrix at room temperature must be evaluated. A sample (for each concentration and matrix) was processed, placed in the autosampler tray and successively injected for 1 day. The time between two successive injections was the duration of a single chromatographic run (18 min), and then the sample was injected 80-times. No variation of the peak area was observed for the three drugs. Therefore, a maximum of 80 samples can be processed and analyzed in 1 day.

Table 2. Calibration curves and sensitivity of the method (concentrations in mg/L)

n = 7

TKI	Slope	y-intercept	r^2	LOD	LLOQ-HLOQ
Pazopanib	12.14±0.11	-2±2	0.9990	1.0	2-80
Dabrafenib	68.8 ± 0.3	0.0±0.8	0.9990	0.2	0.5-80
Regorafenib	98.6±1.3	-1.1±1.5	0.997	0.1	0.2-80

4.8. Robustness

The effect of small oscillations of the experimental factors on the chromatographic response (retention and sensitivity) was evaluated. The studied minimal and maximal values were that we consider that can be reached in the preparation of the mobile phase and the normal use of the instrumentation: SDS concentration, 0.11–0.13 mM; 1-pentanol, 1.8–2.2%, v/v; phosphate salt, 0.008–0.012 M; pH, 6.8–7.2; flow rate, 0.95–1.05 ml/min; injection volume 18–22 μl and detection wavelength 255–265 nm.

A working standard solution containing 1 mg/l of the three antitumor drugs was analyzed by stating each experimental parameter as indicated in the Youden's approach [32], to calculate the difference in retention time and peak area caused by the variation of each parameter. The relative differences are shown in Table 4.

For the three drugs, the detection wavelength and the injection volume make an appreciable influence in peak area, because of the variation of the coefficient of extinction and quantity of sample, respectively. Retention time was significantly affected by the variation of

flow. Although this parameter does not alter the retention, it has a direct influence on dead time. Besides, for PAZO and REGORA, retention time was excessively affected by the considered variation of 1-pentanol, probably by its role in the retention mechanism. DABRA peak area was influenced by SDS and pH. The effect of the other factors was insignificant.

The most influential parameters (detection wavelength, injection volume and flow rate) are adjusted by the instrumentation and remain constant, and then will not cause analytical troubles. However, the operator must pay special attention to the manipulation of 1-pentanol, SDS and buffer when preparing the mobile phase. Considering this, the developed procedure is robust enough to be unaffected by slight changes in the main experimental conditions, that can arise from random errors. This proves the system suitability of future chromatographic analysis.

Table 3. Intra- and inter-day accuracy and precision for the studied drugs.

an=6; bn = 5

Drug	Concentration (mg/L)	Intra-day[a]		Inter-day[b]	
		Accuracy (ε, %)	Precision (RSD, %)	Accuracy (ε, %)	Precision (RSD, %)
Pazopanib	2	+4.2	11.9	+6.1	8.1
	5	-2.1	7.7	-4.1	6.9
	20	+3.8	0.3	1.7	4.1
	60	-2.2	0.4	-2.3	1.9
Dabrafenib	0.5	-1.9	6.2	-4.5	5.3
	2	-2.0	3.6	-2.6	4.7
	20	+2.2	0.3	+1.2	1.3
	60	-6.1	2.3	-2.2	4.0
Regorafenib	0.2	+11.7	8.3	+11.2	8.8
	0.5	-12.6	10.8	-12.5	5.3
	20	-8.5	2.7	-4.8	2.1
	60	+5.8	0.5	+6.8	0.7

Table 4. Robustness of the method. Difference between upper and lower level of each factor divided by the optimal value (%)

Drug	Instrumental response	λ	[SDS]	[1-pentanol]	pH	Flow	Injection Volume
Pazopanib	Retention time	+4.4	+8.7	-18.6	-8.5	-18.1	-1.8
	Peak area	+34.1	+7.1	+10.6	+1.4	-4.2	+12.2
Dabrafenib	Retention time	+1.4	+5.0	-6.8	-8.4	-15.8	-9.7
	Peak area	-57.0	-14.7	+3.4	+19.4	+6.4	+37.7
Regorafenib	Retention time	+3.0	-4.1	-10.1	-0.0	-10.4	-4.4
	Peak area	+26.5	+5.0	+23.1	+1.2	-1.7	+18.5

5. Analysis of incurred samples

The developed method was employed for the quantification of PAZO, DABRA and REGORA in plasma from cancer patients taking these medications by medical prescription (five for each one), provided by a local hospital. These samples were frozen from collection to delivery. A blank sample and a blank plasma sample spiked at low QC (PAZO, 5 mg/l; DABRA, 2 mg/l; REGORA, 0.5 mg/l), medium QC 20 and high QC 60 mg/l were analyzed, in order to verify the keeping of the analytical quality of the measurements. All the samples were processed and analyzed in a single batch in the same analytical run (Table 5). The chromatogram obtained by the analysis of the biological matrices from patient 5 is shown in Figure 2C.

The blanks and the QC samples provide adequate values (all under 15% error), and the antitumor drugs were quantified without interferences. Therefore, the method can be applied in a realistic clinical analysis.

Table 5. Concentrations (mg/L) of the studied TKIs in plasma samples from cancer patients taking this medication (blanks and QCs are included in the analytical run).

Patient	PAZO	DABRA	REGORA
Blank	Under LOD	Under LOD	Under LOD
1	23.4		
2	12.8		
3	48.4		
4	6.9		
5	25.7		
Low QC	1.8	5.3	0.54
6		2.1	
7		0.7	
8		Under LOQ	
9		Under LOQ	
10		1.6	
Medium QC	18.7	21.4	19.1
11			2.7
12		<0.2	4.1
13			3.2
14			4.5
15			2.9
High QC	64.8	57.4	63.1

6. Conclusion

MLC has been proven as an interesting alternative to determine PAZO, DABRA and REGORA in plasma. The sample preparation was a simple dilution and direct injection, thus avoiding time-consuming and cumbersome extraction steps. This contributes to shorten the global analysis time and to improve the reproducibility. The effect of the main components of

the mobile phase (SDS and 1-pentanol) on the retention in a C18 column was quantified using an experimental design. Therefore, the retention time and the resolution were modeled, which expedited the optimization of the mobile phase from few assays. The drugs were eluted in <18 min using a hybrid micellar mobile phase running under isocratic mode, without interferences from endogenous compounds. The method was validated by the guidelines of the EMA in terms of selectivity, linearity, calibration range, sensitivity, carry-over effect, accuracy, precision, dilution integrity, matrix effect, stability and robustness. The results were in agreement with the requirements of the guideline, thus ensuring the reliability of the quantitative data at the concentration levels that can be found in clinical samples. The procedure also holds practical

advantages, like being half automated, getting completed in short time, safe for the laboratory staff, ecofriendly, inexpensive, available, able to analyze many samples per day and applicable to routine analysis. The outstanding achievements of the procedure are possible because of the specific properties of micellar solutions. Therefore, it can be implemented in clinical laboratories to monitor these drugs in biological samples.

7. Future perspective

The current trend in analytical chemistry is the development of inexpensive, simple, automated and eco-friendly analytical procedures. Therefore, the implementation of these kinds of methods will increase in the future in routine analysis for clinical purposes. An interesting approach is the substitution of current analytical methods by other ones, based on direct injection, and using a lower quantity of toxic chemicals. MLC can play a major role in this process.

The application of the here-described method may be enlarged to determine other drugs for therapeutic drug monitoring, by varying the separation and the detection conditions. Besides, it can also be applied to other biological fluids (urine, cerebrospinal, gastric, saliva, sweat, etc.), and further to solid tissues (feces, organs and muscles). In this case, a solid–liquid extraction

would be necessary. The chromatographic conditions will be similar, as the micellar environment reduces the matrix effect.

A modification of MLC, based on the use of pure mixed micellar mobile phases (using a biodegradable and safe nonionic surfactant), instead of hybrid ones (using toxic, flammable and volatile organic solvent), has been recently proposed and has attracted huge interest. This new technique can be applied to this method, in order to totally remove the use of hazardous chemicals and then totally fulfill the requirements of 'green' chemistry.

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Chapter 5

Development of a Method to Determine Axitinib, Lapatinib and Afatinib in Plasma by Micellar Liquid Chromatography and Validation by the European Medicines Agency Guidelines

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ABSTRACT

A method based on micellar liquid chromatography to quantify the tyrosine kinase inhibitors axitinib, lapatinib and afatinib in plasma is reported. The sample pretreatment was a simple 1/5-dilution in a pure micellar solution, filtration and direct injection, without requiring extraction or purification steps. The three drugs were resolved from the matrix in 17 min, using an aqueous solution of 0.07 M sodium dodecyl sulfate – 6.0% 1-pentanol, buffered at pH 7 with 0.01 M phosphate salt as mobile phase, running under isocratic mode at 1 mL/min through a C18 column. The detection was performed by absorbance at 260 nm. An accurate mathematical relationship was established between the retention factor of each drug and the surfactant/organic solvent concentration in the mobile phase, achieved with a limited number of experiments, in order to optimize these factors. Binding behavior of the analytes face to the micelles was found out. The method was successfully validated by the guidelines of the European Medicines Agency in terms of: selectivity, linearity ($r2 > 0.9995$), calibration range (0.5 to 10 mg/L), limit of detection (0.2 mg/L), carry-over effect, accuracy (− 8.1 to + 6.9%), precision (< 13.8%), dilution integrity, matrix effect, stability and robustness. The procedure was found reliable, practical, economic, accessible, short-time, easy-to-handle, inexpensive, environmental-friendly, safe, useful for the analysis of many samples per day. Finally, the method was applied to the analysis of incurred, using quality control samples in the same analytical run, with adequate results. Therefore, it can be implementable for routine analysis in clinical laboratories.

Chapter 5. Development of a method to determine Axitinib, Lapatinib and Afatinib in Plasma by Micellar Liquid Chromatography and Validation by the European Medicines Agency

1. Introduction

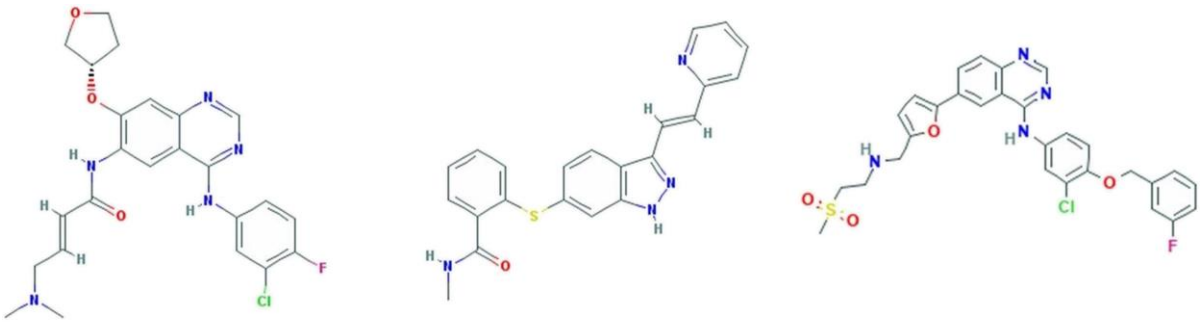
For the last years, many tyrosine kinase inhibitor drugs (TKIs) have been developed against several oncogenic diseases, in the frame of targeted therapies, with encouraging clinical results. These small molecules act by blocking specific receptors of tyrosine kinase proteins that are involved in several signal transduction pathways related to tumor cell proliferation and growth, as well as in angiogenesis and suppression of apoptosis [1], [2].

Afatinib (Gilotrif®) is an irreversible blocker of several epidermal growth factor receptors (EGFR or ErbB), like HER1 (ErbB1), HER2 (ErbB2), and HER4 (ErbB4). This small molecule was developed by Boehringer Ingelheim (Ingelheim am Rhein, Germany) and was approved by FDA in 2013 as first-line treatment against metastatic non-small cell lung cancer (NSCLC) [3], [4]. Axitinib (Inlyta®) is a selective and potent inhibitor of the vascular endothelial growth factor receptors (VEGFR) tyrosine kinase 1, 2, and 3. It was developed by Pfizer (New York, NY, USA) and approved in 2012 by the FDA against metastatic renal cell carcinoma (mRCC) after the failure of a previous systemic therapeutic. It has also shown promising results to treat kidney cell cancer, metastatic melanoma, thyroid cancer, and advanced non-small cell lung cancer [3], [5], [6]. Lapatinib (Tykerb®) is a strong inhibitor of the human epidermal growth factor receptor type 2 (HER2/ERbB2) and epidermal growth factor receptor (HER1/EGFR/ERbB1). It was developed by GlaxoSmithKline (Brentford, UK) and approved in 2007 by the FDA to treat advanced metastatic breast cancer in combination to other chemotherapeutic agents. Its prescription against other solid tumors and metastatic pancreatic cancer is currently under study [3], [7], [8]. The three drugs are orally administered as tablets [3]. Their main pharmacological and chemical properties are described in Table 1 [3], [6], [9], [10], [11], [12], [13], and the structures can be seen in Fig. 1 [13].

Table 1. Physicochemical and pharmacological parameters of the studied drugs [3], [6], [9], [10], [11], [12], [13].

Drug	Afatinib	Axitinib	Lapatinib
Chemical group	4-Anilinoquinazoline	Indazole derivative	4-Anilinoquinazoline
Log Po/w	3.8	5.0	5.2
pKa (basic)	8.8	4.8	3.8/7.2
Charge at pH 7	+ 1	+ 1	+ 1
Bioavailability	Unknown	Variable, ≈ 58%	Unknown
Usual treatment	40 mg once daily	5 mg twice daily	1250–1500 mg once daily
Time to steady state	8 days	15 days	6–7 days
Time to peak plasma	2–5 h	2.5–5.1 h	4 h
Half life	37 h	2.5–6.0 h	14.2 h
Route of elimination	85% feces 4% urine	41% feces 23% urine	14% feces 10% urine

Fig. 1. Structures of the studied antitumoral TKI drugs.



Afatinib **Axitinib** **Lapatinib**

TKI-based therapies show a significant inter- and intra-patient variability clinical response at the same dosage. Indeed, the pharmacokinetics of the TKI depends on many factors: genetics, physiology, pathology, and habits, which affects the bioavailability, metabolism and elimination kinetics. Besides, the TKI have long-term therapies with poor tolerability, undesirable side effects at therapeutic drug exposure, association between plasmatic concentration and efficacy/toxicity, drug-drug interactions, and development of

resistance [7], [8], [9], [10], [11], [12], [13], [14], [15], [16]. In this context, TDM may be a valuable tool for effective medical supervision and to ensure the optimal response by the individualization of the treatment. Firstly, it can be used to verify the adherence of the patient. In cases of failure, weak clinical effects, severe toxicity or suspected any factor altering the pharmacodynamics of the drug, the determination of the plasmatic concentration may assist the clinician to properly modify the treatment, by adjusting the dosage or changing the prescribed drug. Therefore, this strategy would improve patient survival and quality of life [1], [9], [10], [14], [17], [18]. In order to support pharmacological studies in daily clinical practice and in oncology research, a reliable and convenient bioanalytical method to measure the concentration of afatinib, axitinib and lapatinib in plasma is required.

We have previously demonstrated the suitability of micellar liquid chromatography for the rapid analysis of TKIs in plasma, using a hybrid micellar mobile phase and sodium dodecyl sulfate (SDS) as a surfactant [19], [20]. Indeed, SDS-micelles and SDS monomers bind to proteins, fats and other biopolymers, provoking their denaturation and solubilization, and the releasing of linked drugs. Therefore, these macromolecules are eluted near the dead time, rather than precipitating in the column, and do not interact with the analytes. Otherwise, small hydrophobic solutes are also solubilized. This avoids the needing of extraction and cleanup intermediate steps to remove harmful compounds and interfering compounds, or recover the analytes, which strongly expedite the experimental manipulation. Therefore, plasma samples can be directly injected, after a simple dilution and filtration, in the column [17], [18]. Otherwise, the use of micellar mobile phases also offers benefits for the chromatographic resolution step. The variety of interactions that occurs in the column complicates the retention mechanism and provides a high versatility to MLC, allowing the resolution of mixtures of solutes with different charges and hydrophobicity values using an isocratic elution. The retention factor is highly stable and reproducible, and can be modeled from the composition of the mobile phase, using chemometrics [21], [22].

The aim of the work was the development of an analytical method to quantify afatinib, axitinib and lapatinib in plasma by micellar liquid chromatography. The method should provide reliable results and exhibit practical advantages to be used in routine clinical practice, like easy-to-handle, use a low amount of hazardous chemicals, inexpensive and able to process many samples in a short time. In order to test the analytical performances, the procedure must be validated by the guidelines of the European Medicines Agency [23]. Finally, its reliability should be demonstrated by analyzing samples of plasma from cancer patients following a therapy based on these drugs. The effect of the composition of the mobile phase on the retention should also be investigated.

2. Experimental

2.1. Standard and chemicals

Solid standards of afatinib free base (purity > 99%), axitinib free base (> 99%) and lapatinib free base (> 99%) were bought from LC laboratories (Woburn, MA, USA). Sodium dodecyl sulfate (> 98.0%), 1-pentanol and dimethyl sulfoxide (DMSO) (HPLC grade) were purchased from Scharlab (Barcelona, Spain). Sodium hydroxide (> 98%) was supplied by from Riedel-deHaën (Hannover, Germany). Sodium dihydrogen phosphate monohydrate (> 98.0%) and hydrochloric acid (37%) came from Panreac (Barcelona, Spain). Ultrapure water was in-lab obtained by the purification of deionized water, provided as tap water by the University, using an ultrapure water generator device Simplicity UV (Millipore S.A.S., Molsheim, France). All aqueous solutions were prepared using this water.

2.2. Preparation of solutions and mobile phases

Individual stock solutions of afatinib, axitinib and lapatinib (100 mg/L) were prepared by solving the appropriate mass of the solid standard in dimethyl sulfoxide. The working

solutions were prepared by dilution in a micellar solution of 0.05 M SDS buffered at pH 7 with 0.01 M phosphate salt.

The micellar solution (either for dilution or as mobile phase) was prepared as follows: the adequate amount of SDS and NaH2PO4·H2O were weighed and solved in ultrapure water, then, the pH was adjusted by adding drops of HCl or NaOH solutions, afterwards, the appropriate volume of the organic solvent was added, and finally the volumetric flask was filled up with ultrapure water. The solution was ultrasonicated to achieve solubilization and filtered through a 0.45-μm Nylon membrane filters (Micron Separations, Westboro, MA, USA), with the aid of a vacuum pump. The solutions were stored in the dark at room temperature in amber bottles.

2.3. Chromatographic conditions

A HP1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with an isocratic pump, an autosampler, a degasser, a 20-μL loop and a UV-Absorbance diode array detector (DAD) was used. The control of the instrumentation, the registration and visualization of the signal and the measurement of the chromatographic parameters (dead time, t_0; retention time, t_R and peak area, A) was performed through the software Agilent ChemStation (Rev. A.10.01). The special manipulation of the instrumentation required when working with micellar mobile phases can be seen in [19].

The stationary phase was coated on a Kromasil C18 column (AkzoNobel, Amsterdam, The Netherlands), with the following characteristics: 150 × 4.6 mm; 5 μm particle size; 10 nm pore size. The mobile phase was an aqueous solution of 0.07 M SDS – 6.0% v/v 1-pentanol, buffered at pH 7 with 0.01 M phosphate salt, running under isocratic mode at 1 mL/min. Absorbance detection wavelength was set at 260 nm. All the injected solutions, either standard or processed plasma samples, were filtered through a 0.45-μm Nylon membrane filter (Micron Separations) with the aid of a 3-mL syringe before placing in the chromatographic vial.

2.4. Sample collection and preparation

Plasma samples from healthy volunteers and cancer patients were supplied by a local Hospital, after the consent of doctors and patients. The blood was collected using a DB SST tube (BD Vacutainer Systems, Plymouth, UK), and centrifuged at + 4 °C at 3000 rpm for 10 min (3000 × g), to obtain the non-cellular fraction. Then, the samples were frozen and stored at − 20 °C. Furthermore, they were sent to the laboratory, where they were kept at − 20 °C until analysis. The "healthy volunteers" did not take any medication and then their plasma was free of drugs. The matrix-matched blank was made of equivalent volumes of plasma taken from six healthy volunteers: three men and three women, and in each group, one in his twenties, another in his thirties and the last one in his fifties.

For confidentiality reasons, the Hospital did not provide any personal or clinical information about the patients, and the plasma samples were sent unlabeled. The laboratory did not share any information with other institutions (except that included in this paper). The plasma samples were stored for further studies.

The blank and patient samples were treated alike. They were thawed for 30 min at room temperature on the same day of the analysis. Then, an aliquot was 1/5 diluted in a solution of 0.05 M buffered at 7 with 0.01 M phosphate salt, filtered and directly injected. For spiked samples, the adequate volume of a standard solution of the analyte was added before the dilution. The processed plasma samples were discarded after the analysis.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

The main chromatographic conditions were taken from previous studies about the analysis of tyrosine kinase inhibitor drugs in plasma: flow-rate, 1 mL/min; elution mode, isocratic; stationary phase, C18; surfactant, SDS; and pH, 7 [19], [20]. Under these conditions, the stationary phase is saturated with SDS-monomers adsorbed onto its surface, with the sulfate group oriented outwards. The mobile phase contains SDS-micelles, which number is proportional to the concentration of SDS, and SDS monomers (8 mM).

We expect strong retention of the TKIs in the column, considering their positive mono-charge and moderate hydrophobicity (log Po/w 3.8–5.2). Therefore, the addition of a hydrophobic monoalcohol, such as 1-pentanol, was considered as mandatory to obtain useful retention times, and, additionally, increase the efficiency.

The optimized experimental parameters were the concentration of SDS and 1-pentanol, and the detection absorbance wavelength. The study was performed using working standard solutions of 1 mg/L of each analyte.

3.1.1. Influence of SDS/1-pentanol concentration on the retention

The qualitative effects of the SDS and 1-pentanol concentration in the mobile phase on the retention of afatinib, axitinib and lapatinib were investigated. The experimental value of the retention factor (k) was calculated for each analyte at nine mobile phases. Assayed concentrations of SDS (M) and 1-pentanol (%, v/v) were selected using a face centered composite design plus the central point, taking as − 1 and + 1 scores the minimum and maximum concentration of SDS and 1-pentanol recommended in hybrid micellar mobile phases, 0.05–0.15 M and 2–6% v/v, respectively: 0.05/2; 0.05/4; 0.05/6; 0.10/2; 0.10/4; 0.10/6; 0.15/2; 0.15/4 and 0.15/6. The experimental dead time was 1.0 min.

The elution order was the same, regardless of the tested mobile phase: t_R(axitinib) < t_R(lapatinib) < t_R(afatinib). For the three drugs, the retention time diminished at increasing concentration of SDS and 1-pentanol. Therefore, the TKIs exhibit a binding behavior to the micelles, probably by both electrostatic and hydrophobic interaction.

3.1.2. Modeling of the retention factor

For each drug, the retention factor was modeled depending on the concentration of SDS and 1-pentanol, in order to evaluate the quantitative influence of these both chemicals on the retention, using the following equation:

$$1/k = Co + C_1[SDS] + C_2\,\varphi + C_{12}[SDS]\,\varphi$$

where [SDS] and φ are the concentrations of surfactant and organic solvent in unities normalized from the minimum and maximum tested concentrations to − 1 and + 1, in order to easily compare the constants. The parameter C_0 indicates the inverse of the retention factor at average concentrations, while C_1, C_2 and C_{12} represent the effect of the concentration of SDS, the proportion of 1-pentanol and their interaction. This equation has been demonstrated to provide adequate values of retention factor for moderately hydrophobic compounds, when analyzed using an SDS-saturated column and a hybrid mobile phase running under isocratic mode [24]. The model was adjusted using the experimental values of k, obtained from the experimental design (5 freedom degrees), by curve-fitting non-linear least-square regression [25]. The values of the regression parameters can be seen in the Table 2. All the constants were incorporated, as their significance was < 4%. The goodness-of-fit of the regression model was demonstrated by the multiple coefficient of determination (> 0.990) and the low error (< 7%) of the predicted values of retention factor for the calibration points. Therefore, these equations provide accurate values of retention factors for the studied analytes at the SDS and 1-pentanol

concentrations in the 0.05–0.15 M and 2–6%, respectively.

Table 2. Regression parameters of the model and calibration curves.

	Axitinib	**Lapatinib**	**Afatinib**
Regression parameters for the model of the retention factor			
$c_0 \pm s_0$ (p_0)	0.456 ± 0.004 (< 0.001)	0.0946 ± 0.0017 (< 0.001)	0.0784 ± 0.0015 (< 0.001)
$c_1 \pm s_1$ (p_1)	0.239 ± 0.005 (< 0.001)	0.042 ± 0.002 (< 0.001)	0.0367 ± 0.0019 (< 0.001)
$c_2 \pm s_2$ (p_2)	0.025 ± 0.005 (< 0.003)	0.0309 ± 0.0019 (< 0.001)	0.0219 ± 0.0019 (< 0.001)
$c_{12} \pm s_{12}$ (p_{12})	0.018 ± 0.006 (< 0.027)	0.015 ± 0.002 (< 0.004)	0.006 ± 0.002 (< 0.040)
R^2	0.998	0.994	0.990
Parameters of the calibration curves (concentrations in mg/L; 6 calibration levels, 3 replicates)			
Slope	8.02 ± 0.09	14.94 ± 0.11	9.54 ± 0.04
y-Intercept	− 0.7 ± 0.4	0.1 ± 0.5	− 0.49 ± 0.19
r^2	0.9995	0.9998	0.99993

The effect of the concentration of SDS, 1-pentanol and their interaction on the retention was evaluated from the values of the constants, and similar results were found for the three drugs. The three factors significantly reduced the retention, according to their positive value. The most important was the concentration of SDS, followed by the proportion of 1-pentanol, and finally their interaction. This increase of the elution strength with the number of micelles points to an attractive interaction between the micelles and the analytes. The effect of SDS is boosted at higher proportions of 1-pentanol, due to the decrease of the CMC and the aggregation number of the SDS-micelles, and then the augmentation of the total number of micelles [26]. Otherwise, we perceived that the retention order was maintained through the studied interval of SDS/1-pentanol concentrations: t_R(AFA) > t_R(LAPA) > t_R(AXI). These deductions coincide with those obtained by the qualitative interpretation of the raw data, performed in Section 3.1.1.

3.1.3. Optimization of the SDS/1-pentanol concentration

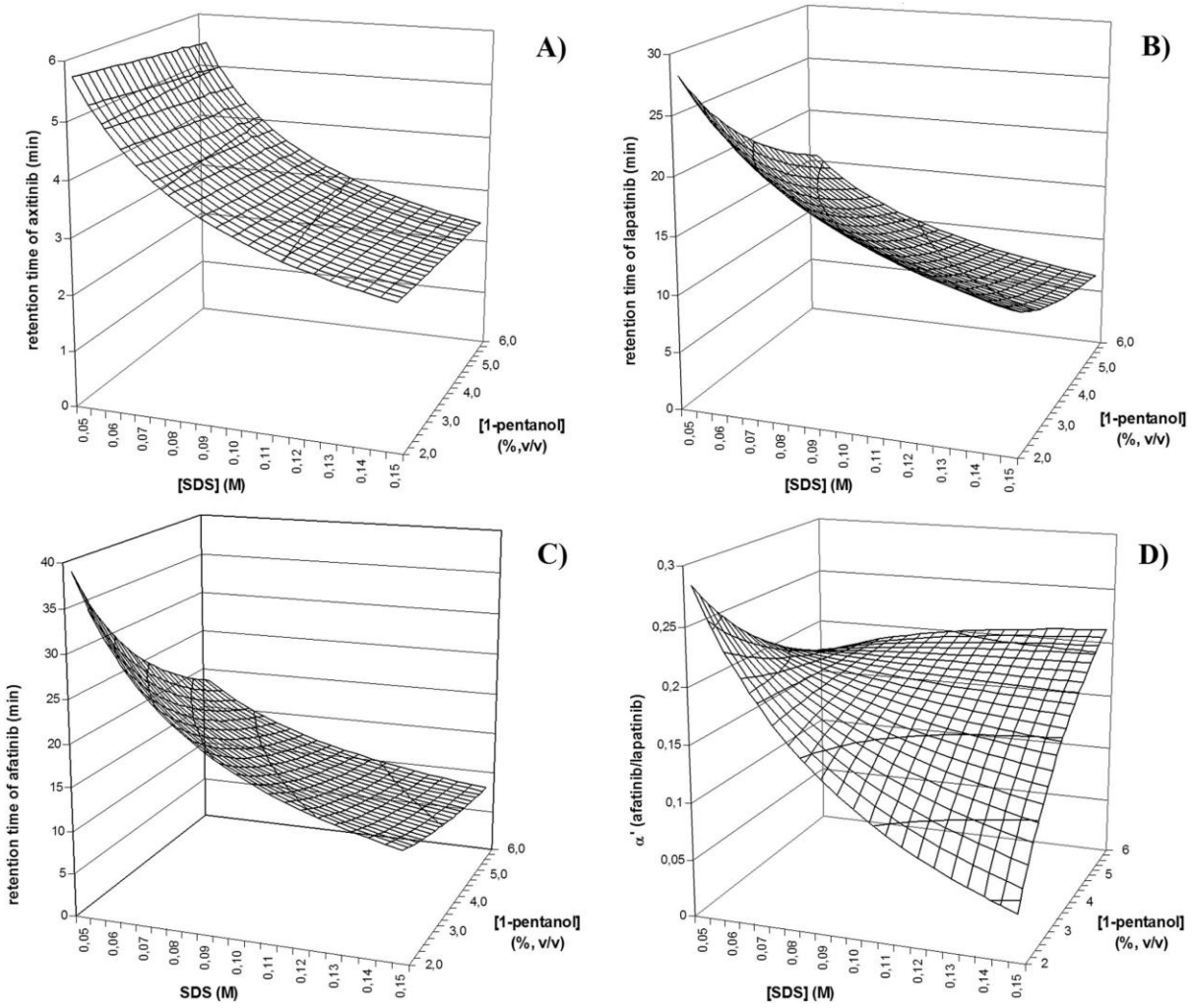
The concentrations of surfactant and organic modifier were selected to reach an adequate analysis time and maximize the resolution between the three TKIs, using an interpretative strategy. These chromatographic parameters were calculated for each concentration of SDS and 1-pentanol from the equations of the retention factors constructed in Section 3.1.2, rather than experimentally determined, in order to save time and effort to achieve the optimization process. As axitinib eluted long before lapatinib, whatever the composition of the mobile phase, only the elementary resolution (paired peaks) between lapatinib and afatinib was considered. It was calculated as the modified selectivity, considering that afatinib is always eluted after lapatinib:

α' (AFA/LAPA) = 1 - k (LAPA) / k (AFA)

α'(AFA/LAPA) = 1 – (0.0784 + 0.0367[SDS] + 0.0219φ + 0.006[SDS]φ / (0.0946 + 0.042[SDS] + 0.0309φ + 0.015[SDS] φ

where α represents the chromatographic selectivity between two peaks and α' the modified selectivity ($\alpha' = 1 - 1 / \alpha$). The concentrations are in normalized unities. The predicted values of the retention times of axitinib, lapatinib and afatinib, and the elementary resolution between AFA and LAPA can be seen in Fig. 2 (Non-normalized concentrations).

Fig. 2. Predicted retention time of A) axitinib, B) lapatinib and C) afatinib, and D) elementary resolution (modified selectivity) between afatinib and lapatinib, depending on the concentration of SDS and 1-pentanol.



In previous studies, we noticed that the protein band is usually eluted at 2.0–4.0 min, when using hybrid mobile phases of SDS/1-pentanol [20], [22], [27], [28]. Therefore, the retention time of axitinib should be > 4.0 min, in order to avoid overlapping with the matrix. Thus, only mobile phases containing > 0.075 M SDS were discarded.

An adequate modified selectivity was reached (0.170–0.285) within the considered interval. Under the maximum resolution-minimum analysis time criteria, the finally selected

mobile phase was 0.07 M SDS - 6.0% 1-pentanol, buffered at pH 7 with 0.01 M phosphate salt, which offers theoretical values of α′ (AFA/LAPA) and t_R (AFA of 0.19 and 14.5 min, respectively. Therefore, a standard solution containing the three drugs was analyzed under the optimized conditions, and the experimental retention times (min) were: AXI, 4.2; LAPA, 12.5 and AFA, 15.2. The peaks showed a nearly normal shape, without noticeable asymmetry. The corresponding chromatogram can be seen in Fig. 3. The error in the prediction of the retention times was < 5.0%.

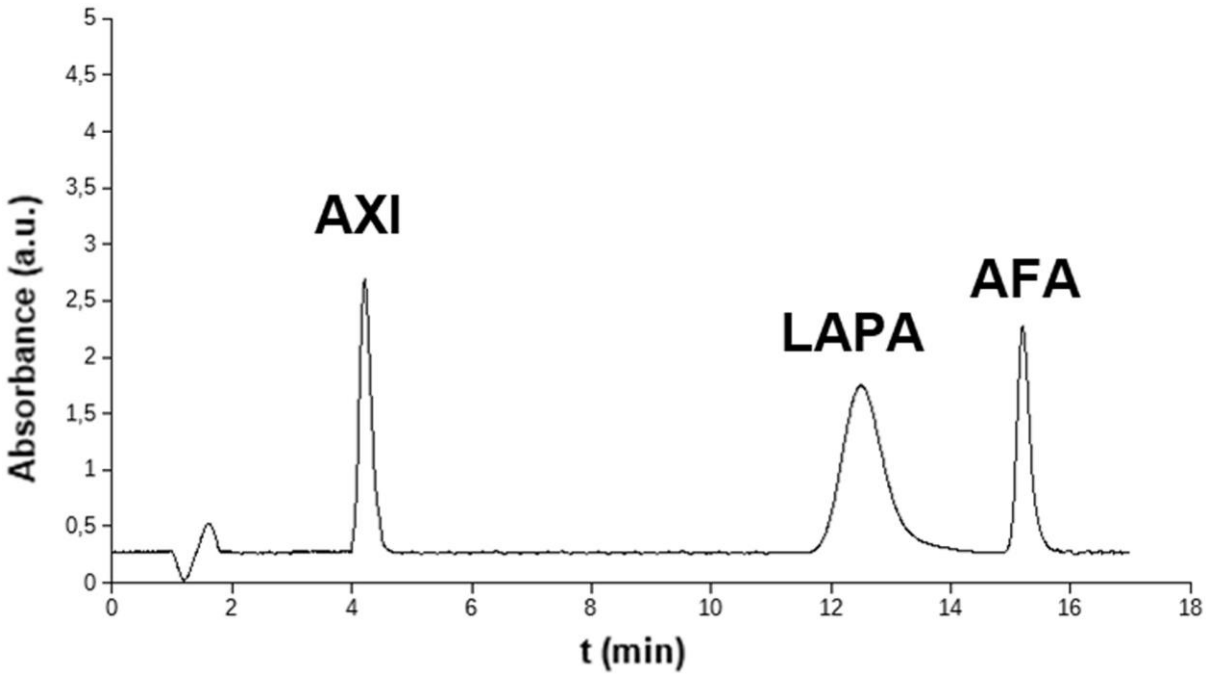


Fig. 3. Chromatogram obtained by the analysis of a standard solution of 1 mg/L axitinib, lapatinib and afatinib (solvent: a micellar solution of 0.05 M SDS buffered at pH 7 with 0.01 M phosphate salt).

The three drugs were resolved in only 17 min using a mobile phase containing a low proportion of hazardous, volatile and flammable organic solvent (< 6.0%). This was possible due to the high solubilization power of micellar solution for small molecules, and the low proportion of organic solvent they tolerate. The interaction of 1-pentanol and SDS-micelles

even presents its evaporation, improving the stability of the mobile phase. In addition, the mobile phase runs under isocratic mode. This improves the reproducibility of the chromatographic response, the baseline stability and reduces the degradation of the stationary phase, if compared to gradient mode. In addition, no equilibration time is required between two injections, saving time and mobile phase volume, thus facilitating the successive analysis of a large set of samples.

3.1.4. Detection conditions

A working standard solution of the three TKIs was analyzed, using the optimal mobile phase. The absorbance spectrum was registered at the retention time of each TKI between 200 and 800 nm. The three analytes have a high absorptivity at 260 nm, and then the detection wavelength was set at this value. No modification of the detection conditions during the chromatographic run was needed.

3.2. Remarks about the practical performances of the procedure

The described method had interesting practical attributes, if compared to RP-HPLC, due to the exclusive use of SDS-micellar solutions as diluting solutions and mobile phases. The main feature of the method is, undoubtedly, the shortening and minimization of the sample treatment. Indeed, the samples can be directly injected after a simple dilution, because of the ability of micellar solutions to solubilize proteins and other macromolecules present in the plasma matrix. This avoids the needing of extraction and purification steps, which are long, tedious, and often offer variable and incomplete recovery, and minimizes the participation of the worker. Besides, the sample is quantitatively introduced in the column, and then the use of an internal standard is not necessary. The sources of variance, and then the uncertainty associated to the result, as well as the analyte loss are diminished. Although the dilution causes a decrease of the sensitivity, this step is compulsory to reduce the signal the front of the chromatogram and enlarge the lifespan of the column.

Another advantage is the low impact of the method for the environment and the health of the laboratory staff. The pure micellar solutions are made of innocuous and biodegradable reagents (SDS and phosphate salt), while the hybrid micellar mobile phase contains, additionally, a low proportion of hazardous chemical (1-pentanol). Consequently, the global volume of harmful chemicals handled and wasted is rather limited, and the cost associated to waste segregation and treatment is negligible. This is in contrast to RP-HPLC, where solutions involved in the sample preparation and mobile phases usually contains large proportions of organic solvents and other toxic chemicals.

The promptness of the procedure can be emphasized, as the entire analysis of a single sample may be performed in only 20 min: 2 min for the sample preparation, 17 for the chromatographic run and 1 for the interpretation of the results. Besides, many samples can be simultaneously processed by a single operator. This makes the method useful to analyze numerous samples per day, thus increasing profitability. Finally, the method is cost-effective, as only common and accessible reagents, as well as laboratory and chromatographic equipment are used. Therefore, the method can be considered useful for routine analysis.

3.3. Method validation

The procedure was in-lab validated to evaluate its analytical performances by the Guideline on bioanalytical method validation, issued by the European Medicines Agency. This guide was specifically developed to assist the researchers in the validation of methods to analyze drugs in biological matrices [23]. The studied parameters were: selectivity, calibration curve, lower limit of quantification, carry-over effect, within-run and between-run accuracy and precision, dilution integrity, matrix effect and stability [23]. The limit of detection [29] and robustness [30] were also evaluated. Unless specified, the validation was performed using spiked matrix-matched blank samples (Section 2.4).

3.3.1. Selectivity

The matrix-matched blank plasma was analyzed before and after fortification at 2 mg/L of axitinib, lapatinib and afatinib. The chromatograms are shown in Fig. 4A and B, respectively.

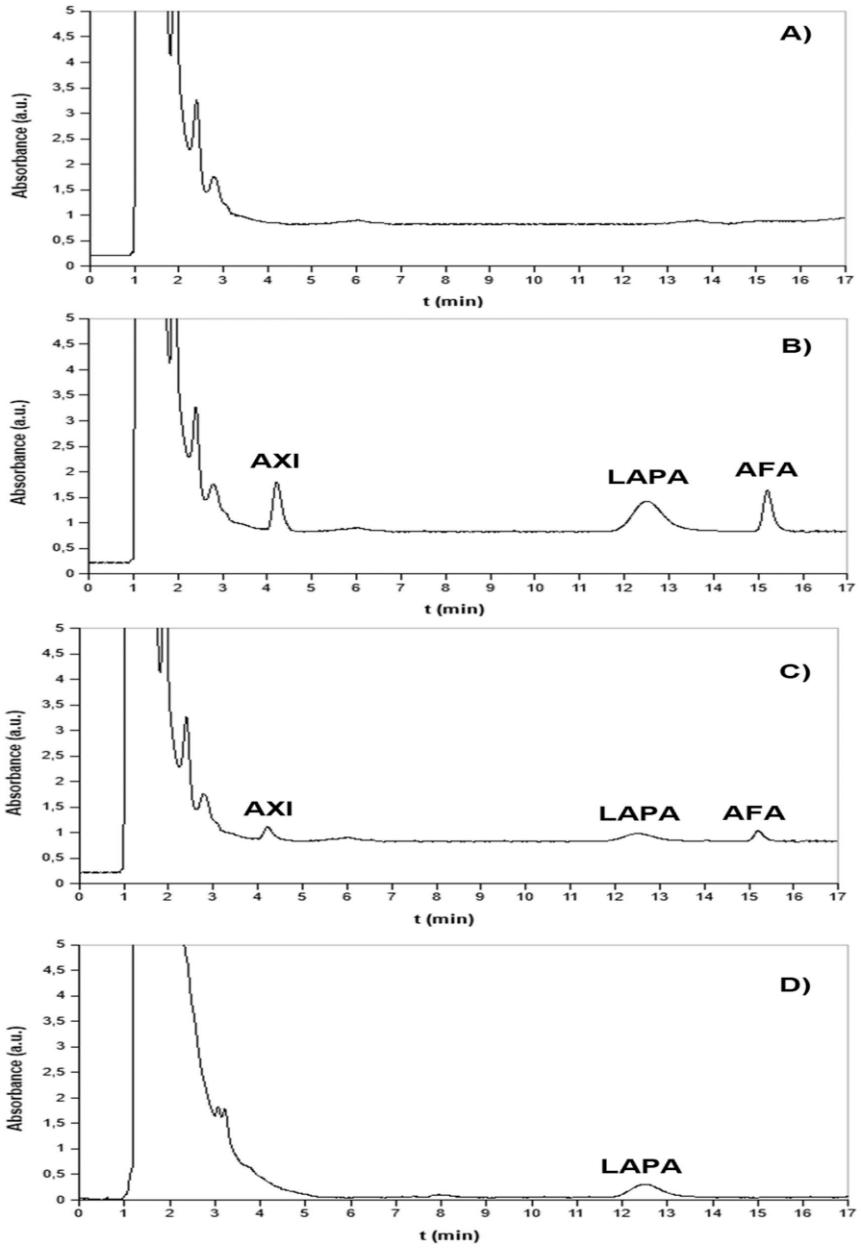


Fig. 4. Chromatograms obtained by the analysis of a plasma sample: matrix-matched A) blank, and fortified at B) 2 mg/L and C) the corresponding LLOQ of each analyte, and D) from patient 3.

In the chromatogram from the blank sample, the protein band elutes from the dead time to nearly 3.7 min, thus before the less retained analyte. The baseline was reasonably steady, and no other peaks were observed. The biomacromolecules of the plasma strongly interact with the micelles, rather than with the stationary phase, and then are quickly eluted.

The chromatograms obtained from the fortified blank samples showed a similar profile, and additionally, the peaks of the three drugs. Retention times, the shape and their respective absorbance spectra were similar to those obtained in Section 3.1.4. Axitinib was eluted far enough from the protein band to be correctly quantified. No peaks appeared at the window time ± 1 min of the analytes, and no overlapping was observed. Therefore, the three drugs can be unambiguously identified in plasma samples, thus assessing the selectivity of the method.

3.3.2. Calibration range and sensitivity

Several portions of the matrix-matched blank were spiked at six increasing concentrations of the three TKIs (up to 10 mg/L), each one analyzed three times. The homoscedasticity of the peak areas through the considered interval was corroborated by the comparison of the higher and lower variance with an F-two-tailed test (confidence level of 5%). The statistical parameters of the calibration curve relating peak area (average) and concentration (slope, y-intercept and determination coefficient) were obtained by least-square regression method [31]. Results can be seen in Table 2.

The lower limit of quantification (LLOQ) was the lowest concentration that can be quantified with a precision and accuracy < 20%, 0.5 mg/L for the three drugs, whereas the upper limit of quantification (ULOQ) was set to 10 mg/L for the three drugs. The linear interval was LLOQ-ULOQ. The limit of detection (LOD) is the smallest concentration that produces a signal reliably over the baseline noise. It was calculated by the 3.3 s criterion: 3.3 times the standard deviation of the blank (taken as the standard deviation of the y-intercept), divided by the slope of the calibration curve. The LOD for the three tyrosine kinase inhibitors was 0.2 mg/L [29].

The goodness of fit was excellent, as the r2 were > 0.9994 and all the calibration points fulfill the accuracy acceptance criteria. Therefore, good linearity was reached within the studied range. A chromatogram obtained by the analysis of a plasma samples spiked at the LLOQ for each drug is shown in Fig. 4C. The signals of the tyrosine kinase inhibitors can be clearly differentiated from the baseline noise.

3.3.3. Carry-over effect

This parameter was investigated to evaluate the cross-contamination of consecutively injected samples within the same analytical run. Two processed blank plasma samples, the first one fortified at 10 mg/L of AXI, LAPA and AFA and the other one non-spiked were processed, and the respective diluted samples were consecutively analyzed. No peak was detected in the second sample at the window time of the analytes. Therefore, the carry-over effect from samples containing the HLOQ was considered negligible.

3.3.4. Accuracy and precision

Blank samples were fortified at four concentrations for the three TKIs: LLOQ, 3 times LLOQ, 3 and 8 mg/L. Those samples were different from that used in the calibration studies. For each level and drug, the within-run measurements were carried out by the successive analysis of 6 samples. These samples were processed in a short-time frame and injected in the same analytical run. The accuracy and the precision were calculated from the respective found concentrations, as the difference between the average and the true value, divided by the true value, and their RSD, respectively. The systematic and random errors from experimental data obtained in different runs performed in different days were investigated. The within-run assay was repeated five different days, over a three-month period, using freshly prepared fortified samples in each occasion. The between-run accuracy and precision were taken from the average found concentration calculated each day, as the difference between their average value minus the true concentration, divided by the true concentration, and their RSD, respectively. Results can be seen in Table 3.

Table 3. Within- and between-run accuracy and precision for the studied drugs.

a=6 ; b=5

Drug	Concentration (mg/L)	Within-run[a]		Between-run[b]	
		Accuracy (ε, %)	Precision (RSD, %)	Accuracy (ε, %)	Precision (RSD, %)
	0.5	+ 1.0	12.1	+ 0.9	13.8
	1.5	+ 5.1	3.4	+ 4.3	4.0
Axitinib	3	− 8.1	3.9	− 6.4	5.2
	8	− 0.1	1.3	+ 0.9	2.5
	30 (1/10)	− 5.3	4.5		
	0.5	+ 6.5	11.6	+ 5.4	12.7
	1.5	+ 3.2	5.2	+ 2.8	7.0
Lapatinib	3	− 1.9	3.6	− 1.0	4.1
	8	+ 0.4	1.1	+ 0.9	2.0
	30 (1/10)	− 1.5	4.5		
	0.5	+ 6.9	11.6	+ 4.7	12.8
	1.5	− 1.5	7.1	+ 0.2	8.4
Afatinib	3	+ 2.6	3.2	+ 1.5	4.3
	8	+ 0.1	1.3	+ 0.4	2.5
	30 (1/10)	+ 2.0	5.8		

The method provides results with a low bias (− 8.1 to 6.9%) and reasonable uncertainty (< 13.8%), partly because of the minimal participation of the operator, the simplification of the sample preparation, elimination of intermediate steps and the quantitative injection of the sample. These values fit the acceptance criteria stated by the guideline (20% for LLOQ and 15% for upper levels, for both parameters), and then the method has been demonstrated to be useful within the examined concentration interval.

3.3.5. Dilution integrity

The effect of the dilution on the reliability of the quantitative data and the suitability of the method to analyze samples containing the TKIs over the HLOQ were examined. The within-run accuracy and precision were determined at 30 mg/L for each drug, using the same protocol as in Section 3.3.4, but introducing an extra- 1/10 dilution in 0.05 M SDS buffered at pH 7. Results can be seen in Table 3.

According to the obtained values, which are similar to those found in Section 3.3.4, this additional dilution does not significantly affect the quality of the quantitative results.

3.3.6. Matrix effect

The potential interference of the matrix compounds on the quantification of the three drugs was investigated. A spiked blank plasma sample and a standard working solution, containing the same concentration were analyzed. The evaluated levels were 2 and 8 mg/L. In all cases, the peak areas were similar, and then the matrix effects can be considered insignificant.

In both cases, the chemical environment can be considered as a micellar solution, because of the dilution ratio of the original solution. The found concentration represents the total concentration of the drug in the diluted solution, and is nearly independent from the composition and origin of plasma. This is because of the preferential interaction between endogenous plasma macromolecules and the TKIs with the micelles. Consequently, the protein-linked drugs are released, and the binding of the matrix compounds and the free TKIs is prevented in the bulk solution [17].

3.3.7. Stability

The degradation over time of the three drugs, in several environments and storage conditions, was explored: working standard solution, in plasma (freeze-and-thaw and long term) and in short term diluted plasma, at 2 and 8 mg/L. The stability was evaluated through the monitoring of the found concentration, and the appearance of unknown peaks in the chromatogram (considered as decay products), for a long period. Degradation was considered significant when the found concentration falls 15% from the initial one. The studies were performed as follows:

a) Working standard solution: the solutions were stored in a fridge (in darkness at + 4 °C), and analyzed each day for two months.

b) Long term stability: 15 samples were fortified at the convenient concentration and stored in a freezer (in darkness at − 20 °C). Each day, one of those plasma samples was analyzed and discarded.

c) Freeze-and-thaw: a fortified blank plasma sample was kept in a freezer. Each day, for 15 days, it was thawed, an aliquot was taken and analyzed, and the original sample was replaced in the freezer.

d) Short-term in processed sample: a fortified plasma sample was diluted in the micellar solution, filtered and injected 80 times (the period between two injections was the duration of the chromatographic run, 17 min). The entire chromatographic sequence took nearly one day.

The results can be seen in Table 4 for lapatinib (the results for the other drugs were similar). In all cases, no significant variation of the found concentration was noticed, and no other peaks were observed in the chromatograms. The three TKIs are quite stable for the above indicated periods and storage/environmental conditions. Therefore, we assume the following statements:

a) The prepared micellar working standard solutions can be used for two months without introducing a systematic error, and should be renewed after this period.

b) The plasma of patients must be analyzed < 15 days being extracted.

c) A spiked or incurred samples can be stored and analyzed several times, for both research and clinical purposes, for 15 days, in spite of the continuous change of temperature and phase.

d) The analytes are stable at room temperature in a processed sample for one day. Therefore, the operator can firstly dilute all the incurred and quality control (QC) samples to-be-analyzed (up to 80 samples times replicate), and thereafter place them in an automated analytical run for injection, instead of having to introduce the processed samples in the column immediately after the dilution. This permits to optimize the laboratory schedule and then the productivity, which is useful for a laboratory with a large workload.

Table 4. Results of stability studies for lapatinib (Ratio peak area at each time/peak area at time 0).

Time (days)	Working standard solution	Long-term stability	Freeze-and-thaw	Time (h)	Short-term in processed sample
1	101.3	100.2	102.1	51	100.4
5	102.0	98.6	97.5	187	99.6
10	98.5	98.4	94.3	357	99.5
15	99.3	96.3	92.8	731	96.8
30	98.1			901	93.6
45	94.8			1088	88.2
60	89.2			1360	85.7

3.3.8. Robustness

The effect of the random errors which can rise up during the preparation of the mobile phase and the possible fluctuations of the instrumental parameters on the retention time and the peak area was investigated. We study the interval within which the parameter can oscillate during the regular work at the laboratory: A) detection wavelength 255–265 nm; B) SDS concentration, 0.065–0.075 M; C) 1-pentanol, 5.8–6.2%; D) pH, 6.8–7.2; E) flow-rate, 0.95–1.05 mL/min; F) injection volume 18–22 μL and G) phosphate salt concentration, 0.008–0.012 M (7 parameters).

The test was performed using a Youden approach. A working standard solution containing 2 mg/L of each TKI was analyzed by eight runs. In each one, the seven parameters were adjusted to their minimal or maximal acceptability limits, the specific combination being selected by a fractional factorial design. Considering the entire assay, each parameter was adjusted to its minimal value in four experiments and at its maximal value in the other four ones. The effect of every factor on the retention time and the peak area was quantified as the difference of the average result obtained at the maximal and the minimal value. This strategy permits the introduction of several variations at once, and it is more rigorous than the study of one alteration at a time, and more cost- and time effective than the testing of all the possible combinations. The effect of a factor was considered relevant if the difference is > 15%. The results are shown in Table 5.

Table 5. Robustness of the method. Difference between the upper and lower level of each factor divided by the optimal value (%).

Factor	Instrumental response	Axitinib	Lapatinib	Afatinib
λ	Retention time	− 0.7%	+ 6.7%	− 0.3%
	Peak area	+ 17.2%	+ 15.8%	− 16.5%
[SDS]	Retention time	− 2.5%	+ 1.0%	+ 2.8%
	Peak area	+ 0.8%	+ 8.6%	+ 1.4%
[1-Pentanol]	Retention time	+ 2.6%	− 11.5%	+ 0.2%
	Peak area	+ 3.7%	− 10.2%	− 4.8%
pH	Retention time	− 7.9%	− 7.0%	+ 11.7%
	Peak area	+ 5.7%	− 2.8%	+ 1.1%
Flow-rate	Retention time	− 16.8%	− 18.2%	− 20.3%
	Peak area	+ 1.0%	+ 4.3%	− 4.7%
Injection volume	Retention time	− 1.2%	+ 4.5%	+ 0.2%
	Peak area	+ 19.7%	+ 19.7%	+ 21.2%
Buffer concentration	Retention time	+ 1.6%	+ 4.3%	− 2.4%
	Peak area	− 0.6%	+ 2.2%	− 11.4%

The elution of the three drugs was significantly influenced by the same factors. Retention times were substantially decreased at higher values of the flow-rate, mainly because of the diminutions of the dead time. The increasing of the injection volume makes an appreciable augmentation of the sensitivity, as a higher amount of analyte is introduced in the column. The absorption wavelength also has a significant effect on the measured peak area, due to the changes in the absorptivity.

Instrumental conditions (wavelength detection, injection volume and the flow-rate) are controlled by the chromatograph and will be unintentionally changed only by instrument damage or unbalance, which are relatively rare and easy to notice. On the other hand, the factors related to the composition of the mobile phase bear a higher uncertainty, as they depend on the manual preparation of a solution by the operator. However, their influence on the

chromatographic response was judged as negligible, thus assessing the robustness of the method.

3.4. Analysis of incurred samples

The developed procedure was applied to quantify lapatinib in plasma samples of cancer patients taking this medication, provided by a local Hospital. Several matrix-matched plasma samples with known concentrations of the three TKIs (blank, and fortified at their LOQ, 2 and 8 mg/L) were also analyzed as quality control (QC), in order to detect any drift in the measurements. All these samples were successively treated and were injected in the same analytical run, in a short period within the same day (Table 6). A chromatogram obtained from patient 3 is shown in Fig. 4D.

Table 6. Concentrations (mg/L) of the studied TKIs in plasma samples from cancer patients and QCs.

Patient	AXI	LAPA	AFA
Medium QC (2 mg/L)	1.95	2.10	2.07
1		1.54	
2		0.94	
3		0.87	
Blank	Under LOD	Under LOD	Under LOD
4		0.2–0.5	
5		0.74	
6		1.25	
Low QC (LOQ)	0.48	0.51	0.54
7		Under LOD	
8		0.64	
9		0.2–0.5	
10		0.58	
High QC (8 mg/L)	7.91	7.84	8.14

In all cases, the TKIs were undoubtedly detected without overlapping. The values obtained for the QC samples were 15% around the true value, and no peaks were observed in the blanks, thus assessing the validity of the overall results obtained for the analytical run. Therefore, the method can be implemented in clinical practice.

4. Conclusions

The analysis of Axitinib, Lapatinib and Afatinib in plasma by direct injection - micellar liquid chromatography in clinical practice was proven as a valuable possibility. The main benefit was the strong shortening and simplification of sample preparation, which was expedited to dilution in a micellar solution and filtration. This enables the analysis of many samples per day with a minimal participation of the operator, without employing hazardous chemicals, and reaching a high sample throughput. The three drugs were resolved in < 17 min using a C18 column and a hybrid micellar mobile phase running under isocratic mode, without interference from the matrix compounds. These achievements were mainly thanks to the solubilization properties of micellar mobile phases, and the interaction of the micelles with the macromolecules and the analytes.

For the three drugs, the retention time and the chromatographic resolution were modeled from sodium dodecyl sulfate and 1-pentanol concentrations in the mobile phase, with the aid of chemometrics. Only nine assays were required to adjust the equations. This permits the determination of the effect of these factors on the elution strength and the optimization of the mobile phase composition, with minimal time and effort.

In order to demonstrate the reliability of the qualitative and quantitative data for clinical purposes of the procedure, a complete and rigorous validation was performed following the guidelines of the European Medicines Agency, using fortified matrix-matched plasma samples. The studied parameters were selectivity, linearity, calibration range, sensitivity, carry-over

effect, accuracy, precision, dilution integrity, matrix effect, stability, robustness, and application to incurred samples (using QC samples in the same analytical run). In all cases, the results comply with the requirements of the guide. The method also holds important practical features, as it is inexpensive, respectful with the workplace safety and the environment, easy-to-conduct, rapid, using, and useful for routine analysis. Besides, it requires available chemicals and instrumentation. These characteristics make the method useful for routine analysis, especially in laboratories receiving many samples per day.

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Chapter 6

Optimization and Validation of a Chromatographic Method for the Quantification of Isoniazid in Urine of Tuberculosis Patients According to the European Medicines Agency Guideline

Chapter 6. Optimization and Validation of a Chromatographic Method for the Quantification of Isoniazid in Urine of Tuberculosis Patients According to the European Medicines Agency Guideline

ABSTRACT

Isoniazid is a drug that is widely used against tuberculosis. However, it shows high interpatient variability in metabolism kinetics and clinical effect, which complicates the prescription of the medication and jeopardizes the success of the therapy. Therefore, in a specific patient, the pharmacokinetics of the drug must be elucidated to decide the proper dosage and intake frequency to make the drug suitable for therapeutic drug monitoring. This can be performed by the quantification of the drug in urine as this process is non-invasive and allows the effects of long-time exposure to be inferred. The paper describes the development of a micellar liquid chromatographic method to quantify isoniazid in urine samples. Extraction steps were avoided, making the procedure easy to handle and reducing the waste of toxic organic solvents. Isoniazid was eluted in less than 5 min without interference from other compounds of the urine using a mobile phase containing 0.15 SDS–12.5% 1-propanol (v/v)–Na2HPO4 0.01 M buffered at pH 7, running at 1 mL/min under isocratic mode through a C18 column with the detection wavelength at 265 nm. The method was validated by following the requirements of the Guidelines on Bioanalytical Method Validation issued by the European Medicines Agency (EMA) in terms of selectivity, calibration curve (r2 = 0.9998 in the calibration range (0.03–10.0 g/mL), limit of detection and quantification (10 and 30 ng/mL respectively), precision (<16.0%), accuracy (-0.9 to +8.5%), carry-over, matrix effect, and robustness. The developed method was applied to quantify isoniazid in urine samples of patients of an Indian hospital with good results. The method was found to be useful for routine analysis to check the amount of isoniazid in these patients and could be used in its therapeutic monitoring.

1. Introduction

Isoniazid (Figure 1) is known for its antibacterial activity, which is caused by the inhibition of the biosynthesis of mycolic acid. It effectively kills bacteria that are actively growing in the body tissues with fluid content just within the first two weeks of treatment [1]. Once administrated, isoniazid is well absorbed and distributed rapidly throughout the fluid part of the body. It is one of the drugs usually used in the treatment of tuberculosis and involves two phases. The initial stage lasts 2 months, and the main objective is to kill as many bacteria as possible. Due to the abovementioned reason, in the first two-month period, rifampicin, pyrazinamide, isoniazid, and ethambutol are given to the patient. After the completion of the first stage, rifampicin and isoniazid are administered until the end of second stage of treatment which lasts another four months. Once ingested by the patient, isoniazid is primarily metabolized in the liver to acetyl isoniazid by acetyl transferase [2,3]. The acetylation is controlled genetically, and it varies from patient to patient. Some of the patients are slow acetylators and some others are fast acetylators. Many researchers have reported that in fast acetylators, 90% of the drug is excreted as acetylisoniazid, resulting in short lasting effects of the drug which may lead to therapeutic failure [4]. In slow acetylators, 67% of the drug is excreted in acetylated form resulting in a higher blood concentration of the parent drug, thus leaving patients prone to more severe side effects, such as peripheral neuropathy, which is the most common Central Nervous System (CNS)-related toxic effect [5]. Due to its complex metabolic process, there is always a need for its monitorization in urine, especially to decide the proper dosage which normally varies from person to person depending on their metabolism, as it depends on highlyvariable factors, like genetics, habits, health, physiological state and the envoirment, among others.

Several methods for the detection and quantification of isoniazid from biological fluids and pharmaceuticals have been developed. These approaches use thin layer chromatography (TLC), high performance liquid chromatography) (HPLC), or liquid and gas chromatography coupled to mass spectrometry detectors (GC/MS, LC/MS) [6–13]. However, these techniques require sophisticated and delicate instrumentation and trained laboratory staff and are relatively expensive in terms of maintenance and purchase. Apart from this, these methods are time-

consuming, and require cumbersome pretreatment and large number of hazardous chemicals. Therefore, they are not suitable for routine clinical use [14,15].

Micellar liquid chromatography (MLC), using a C18 column and the anionic surfactant sodium dodecyl sulfate, is a reverse-phase liquid chromatographic sub-technique that has great potential to overcome the above-mentioned problems [14]. The major advantage of this technique is that it does not require any pretreatment for biological samples, as proteins and other endogenous compounds are denatured and solubilized by the micellar environment and can be harmlessly injected into the column. Besides, they are usually eluted at the front of the chromatogram, thus reducing the risk of overlapping with the analytes, even the barely retained ones [15]. Many drugs, such as antitumorals, antiepileptics, antimalarials, antibiotics, and psychoactive compounds, in biological fluids, such as plasma, serum, and urine have already been successfully analyzed by MLC [16–20]. The experimental protocol is expedited to a simple dilution, usually 1/5 v/v, filtration, and direct injection in the column. The drugs are resolved from the matrix and endogenous compounds using a hybrid micellar mobile phase running under isocratic mode within a reasonable time period.

The aim of the work is to develop a simple, reliable, cost-effective, and rapid method for the determination of isoniazid in urine that can easily be used for the regular monitorization of isoniazid in tuberculosis patients in hospitals. It is validated by the Guidelines of the European Medicines Agency in order to verify its analytical performances, and applied to incurred samples [21,22].

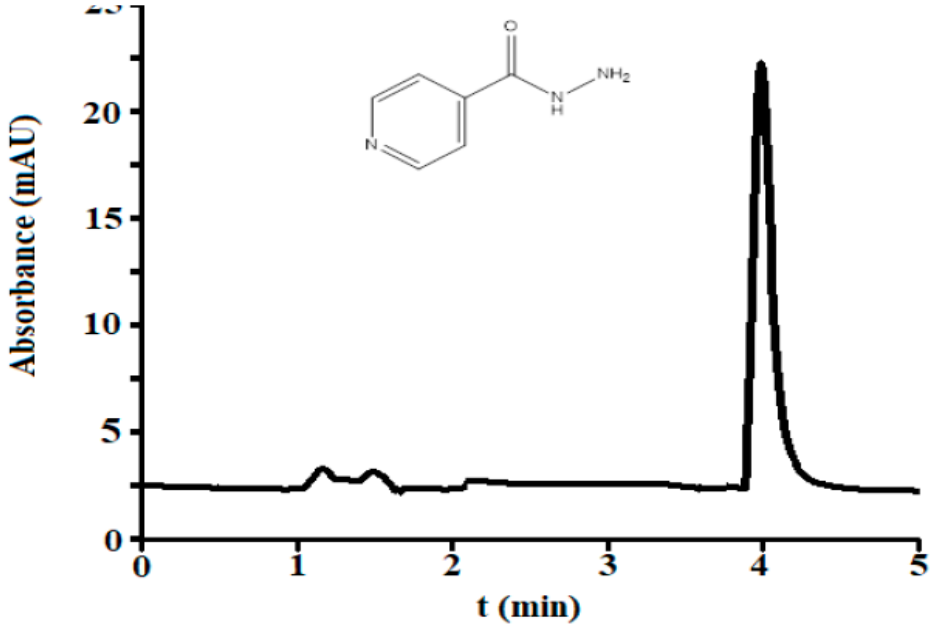

Figure 1. Chromatogram of standard isoniazid (1 g/mL) obtained under the optimal conditions. Structure of isoniazid (MW = 137 g/mol).

2. Experimental

2.1 Chemicals and reagents

Isoniazid (purity >99.0%) was purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS, 99% purity), sodium dihydrogen phosphate, and sodium acetate (analytical grade) were purchased from Himedia Laboratories Private Limited (Mumbai, India). Hydrochloric acid, sodium hydroxide, and HPLC grade 1-propanol were provided by Rankem, RFCL Limited (New Delhi, India). All the solutions were filtered through 0.45 _m nylon membrane filters from Micron Separation (Westboro, MA, USA). An ultrasonic bath (Model Ultrasons-H; Selecta, Barcelona, Spain) was used to achieve the solubilization of the solids. Ultrapure water was in-lab elaborated from deionized water using an Ultrapure water generator device, Simplicity UV (Millipore S.A.S., Molsheim, France). This water was used to prepare aqueous solutions.

2.2 Apparatus and instrumentation

The pH of the mobile phase was measured by using a digital pH meter pH-102/103 Contech, Instruments Limited, (Mumbai, India). The analytical weighing machine used was from Mettler Toledo India Private Limited (Mumbai, India).

Chromatographic analyses were performed on Shimadzu Prominence HPLC System, Shimadzu Corporation, (Kyoto, Japan) equipped with an isocratic pump LC-20 AT, an autosampler SIL-20AC, and a diode array detector SPD-M20 A (190–800 nm). The column used for the analysis was a SPHER-100 C18 100A (250 mm x 4.6 mm x 5 µm particle size) from Princeton Chromatography INC (Cranbury, NJ, USA). The mobile phase was an aqueous solution of 0.15 M SDS–12.5% 1-propanol–0.01 M Na2HPO4 0.01 M buffered at pH 7, running at 1 mL/min under isocratic mode at room temperature. The injection volume and the absorbance detection wavelength were 20 µL and 265 nm, respectively.

All injected solutions, either standard of diluted samples, had been previously filtered through 0.45 µm nylon membrane filters (Micron Separations, Westboro, MA, USA) by pushing with a 3-mL syringe. The processed samples were thrown away after injection. The special care required for the HPLC system when working with micellar mobile phases is detailed in [23].

A personal computer in which Shimadzu LC Solution software version 1.22 SP1 was installed was used to control the instrumentation, register the signal, and determine the main chromatographic parameters: retention time (t_R), retention factor (k), efficiency (number of theoretical plates, N), and the asymmetry (B/A). These parameters were calculated as indicated in [24].

2.3 Preparation of Solutions

Mobile phases were prepared by accurately weighing the proper amount of SDS and sodium dihydrogen phosphate salt, which were then dissolved in HPLC grade water with the aid of a magnetic stirrer. The pH was adjusted by adding drops of NaOH (0.1 M) or HCl (0.1 M). Thereafter, the organic solvent was introduced to reach the desired proportion, and then the volumetric flask was filled up with water, ultrasonicated for 5 min, and filtered through a through 0.45-µm nylon membrane filters (Micron Separations, Westboro, MA, USA) with the help of a vacuum pump.

Standard stock solution of isoniazid 100 $\mu g/mL^{-1}$ was prepared by dissolving it in a small amount of water, following by ultrasonication (5 min). Working solutions were made by successive dilutions in the mobile phase.

2.4 Sample Collection and Processing

Urine samples from tuberculosis patients and healthy volunteers (taking no medications) were collected in glass tubes and stored at -20º C, after consent regulated by the Ethical Committee of the Sagar's Hospital (ethics approval code 2018-ICMR-001027, approved by the Indian Council for Medical Research). The investigations were carried out following the ethical rules of the Declaration of Helsinki 1975, revised in 2013. For confidentiality reasons, no personal or clinical information about the patients or the healthy volunteers (except that indicated below) was provided from the Hospital. The laboratory will not transmit any information to other institutions beyond that included in the publication and will destroy all of the urine samples and chromatogram files one year after publication of the paper.

Urine from healthy volunteers was drug-free. A matrix-matched blank sample was constructed by mixing equivalent volumes from six volunteers—three men and three women, including one individual in their twenties, another in their thirties and the last one in their forties for each gender. This sample was used as "blank sample" throughout the entire research.

Before being injected into the HPLC system, the preserved aliquots were brought to room temperature and 1/5 diluted with the optimized mobile phase without further pretreatment [14]. For spiked samples, the appropriate volume of a working standard solution was added before dilution.

3. Results and discussion

3.1 Optimization of Micellar Chromatographic Conditions

The tandem C18 column/sodium dodecyl sulfate was selected, as it has been largely proven as the best option to resolve drugs in biological fluids [14]. The standard mobile phase flow rate and program were used: 1 mL/min and isocratic. The optimized parameters were the pH, the concentration of SDS, the nature and proportion of organic solvent, and the wavelength absorbance detection. The assays were performed by the analysis of a working solution of 1 µg/mL of isoniazid.

3.1.1 Optimization of the pH

The study was restricted to the working range of the column: 2.5–7.5. Different mobile phases containing 0.15 M SDS–0.01 M buffer salt at pH 3 (phosphate), 5 (acetic), and 7 (phosphate) were assayed. For pH 3, the retention time was too long (15.9 min) and a large tailing appeared and then was discarded. The results were similar for pH 5 and 7; the analyte was eluted at 8.3 min, with an adequate peak shape. The optimal pH was set to 7 as this pH is far enough away from the pKas, has negligible concentrations of the acid/basic forms, and due to being neutral, is less aggressive than acidic conditions for the alkyl C18-silica bonds.

Isoniazid contains three ionizable nitrogen atoms: pyridine N (pKa = 1.8); hydrazine–NH (pKa = 3.6) and hydrazine NH2 (pKa = 10.8) [25]. Therefore, isoniazid has a charge of +2 from pH 2.5 to 3.6; and +1 from 3.6 to 7.5. In both cases, the analyte would be attracted to the sulfate anionic groups located at the outer layer of the modified stationary phase and the pseudo micellar phase by electrostatics. However, for a positively bi-charged molecule, this attraction

would be very strong, slowing down its movement in the bulk mobile phase. This explains the longer retention and peak broadening and distortion.

Under these conditions, the interactions between the analyte and both the stationary phase and the pseudo micellar phase were mainly due to electrostatics, as isoniazid would barely interact with the hydrophobic C18 chains of both environments due to its polarity (log P = −0.7 [26]).

3.1.2 Optimization of SDS and Organic Modifier

In order to accelerate the elution and improve the peak shape, the addition of an organic modifier was considered. The alcohol 1-propanol was selected, as 1-butanol and 1-pentanol are more hydrophobic and would excessively increase the elution strength of the mobile phase. The minimal (2.5%), average (7.5%), and maximal proportions (12.5%) of 1-propanol recommended for MLC were assayed, and the respective experimental values of (t_R, min; N; B/A) were (5.1; 2817; 3.1), (4.3; 3518;1.8), and (4.0; 3912; 1.2). In all cases, the retention times were enough to avoid overlapping with the front of the chromatogram.

According to the criteria of maximal efficiency-minimal analysis time, the optimal mobile phase was 0.15 M SDS–12.5% 1-propanol–0.01 M phosphate salt buffered at pH 7 (Figure 1).

3.1.3 Selection of the Optimal Detection Wavelength

Structurally, isoniazid has a pyridine ring, a carboxylic group, and a hydrazine group (Figure 1), thus, having a high degree of extended conjugation, resulting in its absorption in the UV region. The absorbance spectrum was measured using an UV Visible Spectrophotometer using the mobile phase as a solvent, and the λ_{max} was 265 nm. The UV absorbance spectrum was also taken using the photodiode array detector (PDA) of the chromatogram during analysis at different times of the isoniazid peak: the maximal height, the front/tail half-height, and the front/tail at 0.1-height points. All were alike and had a similar

shape to that obtained under static conditions.

3.2 Method Validation

The method was validated following the Guidelines on Bioanalytical Method Validation issued by the European Medicines Agency (EMA) [21,22]. The evaluated validation parameters were the selectivity, calibration curve, linearity, lower and upper limits of quantification, precision, accuracy, carry-over, matrix effect [21], and limits of detection and robustness [22]. Unless otherwise specified, the validation was performed by using the matrix matched blank, prepared as described in 2.4.

3.2.1 Selectivity

A selectivity study was performed to verify the presence of other endogenous compound co-eluting out near or with the analyte of interest. Ten blank urine samples were injected using the selected optimum conditions, before and after fortification (addition of isoniazid) at 5 µg/mL. The obtained chromatograms for one case are shown in Figure 2A (blank) and Figure 2B (fortified).

Several high and broad peaks corresponding to endogenous compounds of urine were eluted from the dead time to nearly 3.0 min in the blanks. This broadband was also observed in the fortified samples. At higher times, the signal was at a fairly stable baseline. No peaks were observed near or at the window time of the analyte. In order to assess the peak purity, the fortified samples were compared to those obtained in Section 3.1.2 by overlaying, and no distortion was noticed. Besides, the absorbance spectra were taken at the same times as described in Section 3.1.3 and showed a similar shape. Therefore, the analyte can be reliably identified in the urine matrix, and the peak at 4.0 min exclusively corresponds to isoniazid.

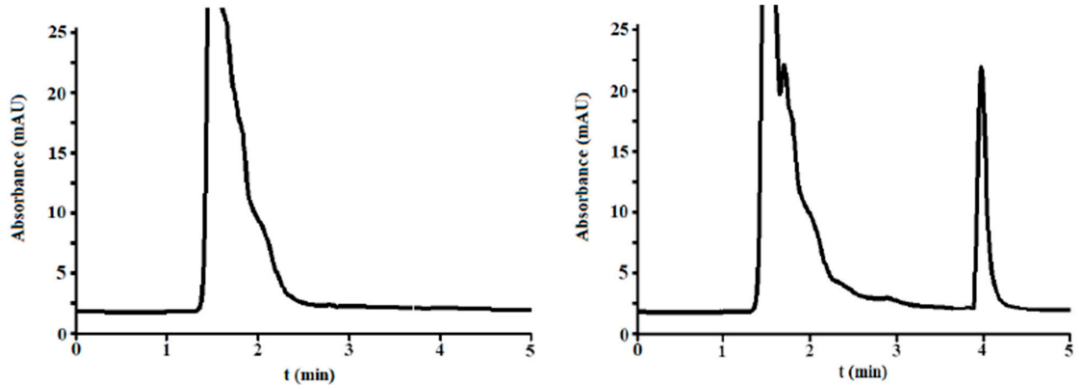

Figure 2. Chromatogram of blank urine from a healthy donor using the optimum chromatographic condition: (A) blank and (B) fortified at 5 µg/mL (by adding isoniazid to the blank sample).

3.2.2. Linearity and Sensitivity

Calibration curves were obtained for nine different concentrations of isoniazid (six replicates), equally distributed in the range of 0.03 (lower limit of quantification, LLOQ) to 10 µg/mL (upper limit of quantification, ULOQ). The calibration range was selected to cover the expected range of isoniazid in the incurred samples [27].

The calibration equation (slope, y-intercept, and determination coefficient) was obtained by plotting the chromatographic peak area of isoniazid versus the concentration using the least-squares linear regression method (Figure 3). To study the variability of the calibration parameters, five curves were obtained by independent measurements on 6 days (one curve each day) over a 3-month period for a different set of standards. A good linear relationship between the independent and observed variables was found. The regression curve, taken as the average of the obtained five calibration curves, was

Peak area = 38,150 ± 21 (isoniazid, µg/mL) + (−4.2 ± 0.3), r^2 = 0.9998

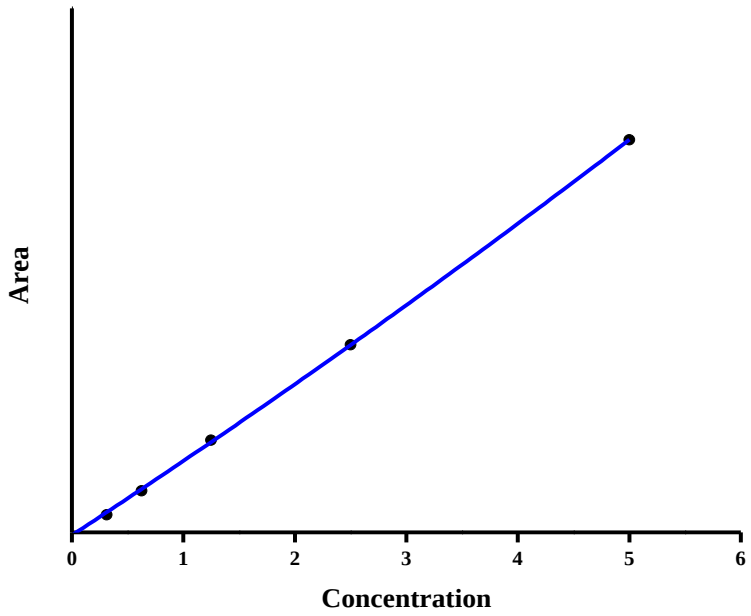

Figure 3. Plot of the peak area vs. the concentration and calibration curve for isoniazid quantification (concentration in µg/mL).

3.2.3. Detection and Quantification Limit

The limits of detection (LOD) and quantification (LOQ) for isoniazid were determined using the 3 s and 10 s criteria—3.3 and 10 times, respectively. The SD of the lowest concentration solution included in the calibration curve was divided by the slope. The LOD and LOQ of isoniazid were 10 and 30 ng/mL, respectively.

3.2.4. Precision and Accuracy

The intraday and interday accuracy and precision of the method were determined by analyzing isoniazid at three different concentrations: 0.03, 0.25, and 2.5 µg/mL. The intraday analyses were performed by analyzing six spiked urine samples on the same day.

The interday analysis was the average of five measurements of the intraday values taken on 6 days over a 3-month period performed by different analysts and equipment at the same concentrations. The results, expressed as the relative standard deviation (RSD) for precision

and the relative error (ε) for accuracy, for both intraday and interday values are shown in Table 1. The values of accuracy (between −0.9 and +8.5%) and precision (RSD less than 16.0%) were below the maximum accepted values according to FDA guidelines (less than 20% for accuracy and precision at the LLOQ, and <15% at higher levels). Thus, the developed procedure can be used in routine analyses of urine samples from tuberculosis patients taking isoniazid medication.

Table 1. Intraday and interday accuracy and precision.

Matrix	Concentration (μg/mL)	Intraday [a]		Interday [b]	
		Accuracy (ε, %)	Precision (RSD, %)	Accuracy (ε, %)	Precision (RSD, %)
Urine	0.03	+8.5	9.3	+6.9	16.0
	0.25	+3.2	6.6	+2.5	7.5
	2.5	−0.9	2.4	−0.4	2.9
Urine (dilution integrity)	15	+1.9	3.2	-	-
Working solution	0.006	+7.3	7.5	-	-
	0.05	+3.0	5.2	-	-
	0.5	−0.2	2.0	-	-

a n = 6; b n = 5. RSD: relative standard deviation.

3.2.5. Carry-Over Effect

A urine sample spiked with isoniazid at 5 μg/mL, and, immediately afterwards, a blank urine sample, were analyzed. In this last one, no peak was observed in the chromatogram at the retention time of the isoniazid. Thus, the carry-over was considered negligible at concentrations within the calibration range.

3.2.6. Matrix Effects

The influence of the endogenous compounds of urine in the quantitative results, either by linking or any interaction interfering with the retention process, was evaluated. Working

standard solutions containing the same concentration of isoniazid as in Section 3.2.4 divided by five were analyzed by following the same protocol to consider the dilution in the experimental procedure. The results for the intraday accuracy and precision were similar to those obtained for urine samples (Table 1). Therefore, no significant matrix effect was detected, regardless of the complexity of the chemical composition of urine. This was probably done by the interaction of the endogenous compounds of the urine and the SDS-micelles.

3.2.7. Dilution Integrity

The effect of the introduction of another dilution step was investigated. Urine samples spiked at 15 µg/mL were 1/10 diluted and then processed as in Section 2.4. The intraday accuracy and precision were determined (Table 1).

The results were inside the acceptance criteria, and then the method allows a sample over ULOQ to be analyzed after the appropriate dilution.

3.2.8. Linearity and Sensitivity

The robustness of the developed method was observed by slightly changing the chromatographic conditions, like the flow rate, percentage of modifier, concentration of surfactant, etc., one by one, keeping the others at their optimal values (Table 2). A standard working solution of 1 µg/mL was used.

Table 2. Robustness evaluation of the developed micellar liquid chromatography (MLC) method (RSD, %).

Chromatographic Condition	Interval	t_R (min)	N	B/A
Flow rate (mL/min)	0.9–1.1	12.1	0.9	1.2
pH	6.8–7.2	0.1	2.6	1.6
1-propanol proportion (%)	12–13	0.1	1.5	2.3
SDS concentration (M)	0.14–0.16	0.7	0.7	2.0

The slight change in the chromatographic parameter did not significantly affect the separation parameter of the selected method; thus, the method can be considered quite robust. As expected, only the variation in the flow rate had an influence on the retention time of the studied compound, whereas the other parameters showed negligible change.

3.3. Analysis of Incurred Urine Samples

The efficacy of any treatment depends on the metabolic activity of the drug, i.e., how fast the drug is eliminated from the body. This fact is very important when discussing drugs that have life-threatening effects or have a short therapeutic window. Tuberculosis (TB) also falls under the above-mentioned category. If the treatment of the diseases is not properly controlled, it may lead to multidrug resistant tuberculosis (MDR-TB). Since the metabolic rate of the medicine differs for every patient, the plasma concentration of the medicine will also be different in different patients.

Therefore, in the treatment of life-threatening diseases, administrating the correct dose to the patient is very important. Therapeutic drug monitoring (TDM) is an area that helps clinicians or pharmacists to calculate the next dose for effective treatment. So, finally, TDM depends on the analytical method which should be very sensitive, selective, fast, reliable, and inexpensive as well as easy to handle.

The developed method was applied to incurred urine samples from 15 TB patients (Table 3) to detect the presence of isoniazid. The chromatogram showed a similar shape to those obtained in Section 3.1.1, and no overlapping peaks were detected. The chromatogram obtained from analysis of urine from patients 1 and 2 is shown in Figure 4A, B, respectively. The results confirm that the excreted concentration of isoniazid differs from patient to patient.

Table 3. Amount of isoniazid found in the urine of 15 tubercular patients.

Patient Number	Amount of Isoniazid μg/mL
Patient 1	2.9
Patient 2	1.34
Patient 3	4.31
Patient 4	8.45
Patient 5	2.98
Patient 6	3.45
Patient 7	5.34
Patient 8	2.84
Patient 9	7.23
Patient 10	6.89
Patient 11	3.14
Patient 12	5.48
Patient 13	5.39
Patient 14	6.78
Patient 15	3.87

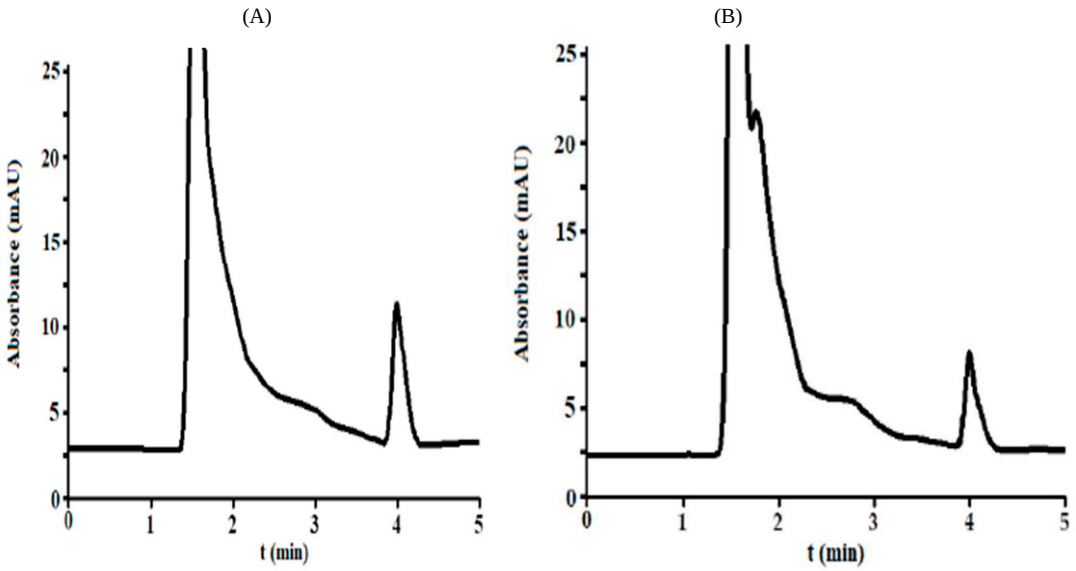

Figure 4. Chromatogram obtained from the analysis of urine from patients 1 (A) and 2 (B)

4. Conclusions

The developed method was applied to real urine samples obtained from patients undergoing treatment for TB to determine the level of unmetabolized isoniazid present in urine. Using the developed method, isoniazid was eluted at 4.0 min. The LOD and LOQ were 0.010 µg/mL and 0.03 µg/mL, respectively. The reported method can easily be used for drug monitorization in clinical laboratories in order to improve drug dosages to help with the optimization of tuberculosis treatment. The present method was shown to effectively detect and quantify isoniazid in urine, which is one of the fluids where the unmetabolized isoniazid can be detected and quantified. Detection of Isoniazid in urine can act like a marker to determine the isoniazid acetylator status and also to check patient adherence to the treatment of TB. It might also be useful for therapeutic monitoring to determine the required dosages of the drugs in the treatment of TB. The developed method could also be used in the pharmaceutical industry for quality control of isoniazid in different pharmaceutical preparations.

5. References

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Chapter 7

Stability studies of Rifampicin in Plasma and Urine of Tuberculosis patients according to the European Medicines Agency Guidelines

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ABSTRACT

- Aim: The macrolide antibiotic Rifampicin is prescribed against several infections, like tuberculosis disease. This drug decays to rifampicin quinone.

- Results/methodology: The biological fluids were diluted in a micellar solution and directly injected. Using a C18 column and a mobile phase of 0.15 M SDS-6% 1-pentanol phosphate-buffered at pH 7, running at 1 ml/min, the analytes were resolved in less than 15 min. The detection was by absorbance at 337 nm. Method was validated by the guidelines of the European Medicines Agency. Decomposition of rifampicin to Rifampicin quinone was also studied.

- Discussion/conclusion: Procedure is rapid, easy-to-handle, economic, eco-friendly and with a high sample throughput. It was successfully used to monitor Rifampicin in the plasma and urine of tubercular patients.

Chapter 7. Stability studies of Rifampicin in Plasma and Urine of Tuberculosis patients according to the European Medicines Agency

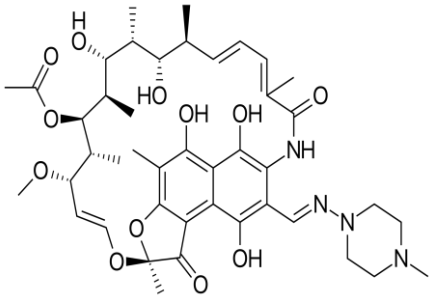
1. Introduction

Rifampicin is a semisynthetic macrocyclic antibiotic (Figure 1A) [1]. It is a lipophilic substance with a log Po/w value of 4.24 and a pKa of 1.7 [2]. The stability of the drug mainly depends on the pH of the medium. It has been reported that in neutral pH the drug is stable; whereas, in acidic and basic pH it is unstable. In acidic media, rifampicin is hydrolyzed to produce 3-formyl rifampicin which is further oxidized to form an inactive quinone derivative, in other words, rifampicin quinone (Figure 1B) [3]. Thus, rifampicin, either pure or in formulations, is an immunosuppressant [4] and is used in tuberculosis (TB), leprosy and legionnaire's disease treatment, but when is stored suffers oxidative decomposition to produce inactive rifampicin quinone.

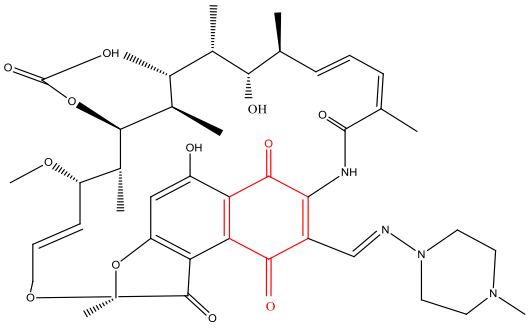
Tuberculosis (TB) is a chronic infectious disease caused by a microorganism of the genus Mycobacterium. It spreads when a healthy person comes in contact with a person who is infected with these bacteria. In this case, if adequate medication is given to the infected TB patients regularly and properly, this disease is completely curable. WHO recommends a four drug combination: rifampicin, isoniazid, ethambutol and pyrazinamide, as the first line anti-TB drugs [5–7]. The entire treatment period is of 6 months which is subdivided into two phases of 4 and 2 months, respectively. In the first phase the four drugs are given to the patient and in the second phase out of the four prescribed drugs only rifampicin and isoniazid are given daily [7]. One 600-mg rifampicin dose is able to kill 99.9% or more of viable organisms. According to the published facts, rifampicin may exert a deferred antibiòtic activity for several days, wherein the organism multiplication is repressed [8]. On the other hand, adverse effects of rifampicin includes nausea, vomiting, stomach pain, heartburn, diarrhea, muscle or joint pain, headache, rash or itching, drowsiness, dizziness, spinning sensation or numbness or tingling in legs [9]. Due to abovementioned side effects patients stop taking their regular dose of medicine. If so, the patients tend to pass to be multidrug resistant patients. Besides, the pharmacokinetics of rifampicin, and then the clinical effects at fixed dosage, exhibits a substantial inter- and intra-patient variability [10,11]. Thus, there is an urgent need for the monitoring of patients who undergo treatment at the Indian Program Directly Observed Short Course (DOTS) center.

Figure 1. Structures of rifampicin (A) and rifampicin quinone (B).

A)



B)



There are several analytical methods reported by researchers for the determination of rifampicin and its oxidative product in different matrices. Spectroscopic methods have been commonly used to determine rifampicin [12,13]. As these methods are known for poor selectivity, there is a need for hyphenated separation technique. To resolve a mixture of isoniazid, ethambutol, rifampicin and pyrazinamide in fixed dose combination, an advanced form of planar chromatography, like thin layer chromatography (TLC) [14] and high performance thin layer chromatography (HPTLC), have been employed [15]. Researchers had also developed a stability indicating [16] and densitometric HPTLC method for the simultaneous analysis of rifampicin and isoniazid in rat plasma [17]. Nevertheless, HPTLC is still considered to be a screening technique and for the purpose of confirmation and quantitative analysis, HPLC remains the standard gold method. As reported by different groups, HPLC is commonly used for the determination of the antitubercular drugs in various matrices, in other words, in formulations, for the purpose of quality control [18] as well as in biological fluids [11,19–27]. Another reported technique for the simultaneous determination of the antitubercular substances in various formulations is capillary zone electrophoresis (CZE) which is also a useful separation technique [28]. Many research groups have also reported the use of LC–MS/MS but the method is quite expensive [29–37] as well as the technique is not readily available in some places of India. The main drawback with all the reported methods is the time of analysis and the fact that sample preparation is tedious and uses large volumes of

hazardous and volatile organic solvents, as well as these techniques required extraction of the drug from blood and other biological matrices.

Micellar liquid chromatography (MLC), using a C18 column and SDS as the surfactant, is as an interesting substitute to hydro-organic reverse phase (RP)–HPLC for the multidrug determination in plasma [38–42]. Micelles strongly bind with proteins of serum and urine, which produces their denaturation, solubilization and the release of bounded drugs. Besides, solubilized matrix biopolymers are barely retained on the modified stationary phase. Therefore, plasma and urine can be directly injected, after a dilution with a micellar solution and filtration, as the matrix is harmless eluted at the front of the chromatogram, thus expediting the experimental protocol [43]. The number of interactions inside the column for the solutes increases by the occurrence of the micelle pseudo phase and the modification of the stationary phase by the adsorption of SDS monomers, as more partition equilibria participate in the retention mechanism. Otherwise, the elution strength and the efficiency can be improved by the addition of a small amount of short-chain alcohol (up to 12.5%) [44]. Therefore, MLC is a highly versatile technique, able to separate drugs with diverse hydrophobicity using an isocratic mode.

The goal of this research is to propose a reliable assay based on MLC to measure the concentration of rifampicin in plasma and urine, and for stability studies. The analytical procedure should be inexpensive, simple, eco-friendly and sensitive enough to detect the usual levels of rifampicin in plasma and urine taken from TB patients following a therapy. It must be validated by the guidelines of the European Medicine Agency (EMA) [45]. The method was applied to study the presence of rifampicin in plasma and urine samples of TB patients and also to decomposition studies to rifampicin to rifampicin quinone.

2. Experimental

2.1. Standard & reagents

Standard of rifampicin and rifampicin quinone (purity>99.0%) was supplied as gift by Wilcure (Wilcure Remedies Private Limited, Indore, India) which is a quality control pharmaceutical company. SDS (purity >99.0%) was bought from Merck (Mumbai, India). Analytical-grade sodium dihydrogen phosphate, hydrochloric acid and sodium hydroxide came from Himedia Laboratories Private Limited (Mumbai, India). HPLC-grade water, 1-propanol, 1- pentanol, 1-butanol and methanol were purchased from Rankem (Rankem RFCL Limited, New Delhi, India). Double distilled water was in-lab produced using a BorosilDistillationUnit (BorosilGlassWorks Limited, Mumbai, India) and all the solution were filtered through 0.45 μm nylon membrane, Micron Separation (MA, USA).

2.2. Apparatus & instrumentation

The analytes were weighed using a Metter–Toledo analytical balance (Mettler Toledo India Private Limited, New Delhi, India). A pH-102/103 meter (Instruments Limited, Mumbai, India) equipped with a combined Ag/AgCl/glass electrode was used to measure the pH. An ultrasonic bath model PCI (Analytics Private Limited, Mumbai, India) was used to dissolve the standards.

A chromatographic system Shimadzu Prominence HPLC (Shimadzu Corporation, Kyoto, Japan), equipped with an isocratic pump LC-20 AT, an auto-sampler SIL-20AC, and a diode array detector SPD-M20 A (190–800 nm) was used. Chromatographic signals were processed on a personal computer which was connected to the instrument using LC-Solution version 1.22 SP1 software (Shimadzu Corporation). Other statistical calculations were performed using Microsoft Office Excel 7 (Microsoft Corporation, WA, USA) and Origin pro 8 (OriginLab Corporation, MA, USA). For the selection of wavelength, a UV-Vis Systronic double beam 2201 spectrophotometer was used (Ahemdabad, India). The incurred samples were centrifuged using Compact cooling centrifuge CM-8 plus, Remi Electrotechnik Limited

(New Delhi, India) and kept in a deep freezer (Remi Electrotechnik Limited, Mumbai, India). The experimental chromatograms were treated by the Michrom Software [46], to measure dead time (t_0) retention time (t_R), efficiency (N), asymmetry factor (B/A) and peak area under the curve (AUC).

2.3. Apparatus & instrumentation

Stock solutions of rifampicin and rifampicin quinone (100 μg/ml) were prepared by weighing the appropriate quantity of powdered standard and dissolving in 5 ml of methanol, completing with mobile phase (see the next subsection). Working solutions were made by sequential dilution of these stock solutions with the mobile phase. These solutions were renewed every 4 days.

To prepare the micellar solutions, the appropriate amount of SDS and sodium dihydrogen phosphate was weighed and then dissolved in double distilled water. The pH was fixed to the desired value by adding drops of 1.0 M hydrochloric acid or 1.0 M sodium hydroxide solutions. The adequate volume of the alcohol was added to reach the chosen proportion and the solution was filled up with double distilled water, ultrasonicated with the aid of a vacuum pump and filtered.

2.4. Chromatographic conditions

The column was a SPHER-100 C18 100A (250 × 4.6 mm, 5 μm particle size) purchased from Princeton Chromatography INC (NJ, USA). The mobile phase was a micellar solution of 0.15MSDS-6% (*v/v*) 1-pentanol-0.01 M phosphate buffer at pH 7, which was running at 1 ml/min isocratic mode. The signal registered by the detector was the absorbance at 337 nm. Injection volume was 20 μl. All the chromatographic system and the injection vials remain at room temperature throughout the analysis.

2.5. Chromatographic care

A special maintenance on the chromatographic system, when using micellar mobile phases, is mandatory to reach a maximal performance and prevent its deterioration. First, a surfactant-free stationary phase has to be saturated with SDS monomers by flushing a micellar solution for at least 2 h at 1 ml/min. When a mobile phase is changed, the new one must be previously pumped for 30 min at 1 ml/min to remove the former one and equilibrate the stationary phase. A micellar mobile phase should never remain static in tubes and in the column, to avoid the precipitation of the salts (surfactant and buffer), which would cause clogging in the components of the chromatographic system, especially those narrow ones (needle, tubes and the column) and seriously harm them. If analyses must be performed for several days under the same conditions, it is advisable to maintain the mobile phase overnight at low flow rate (0.2 ml/min). This contributes to maintain a good equilibrium of the adsorbed SDS-monomers on the stationary phase.

The chromatographic system must be rigorously cleaned before switching off the pump. Pure water must be flushed at 1 ml/min for a minimum of 1 h, to wash away buffer salt and free SDS monomers. Thereafter, the column should be rinsed with 100% methanol at 1 ml/min for more than 1 h, in order to get rid of the water and elute the absorbed SDS-monomers and other strongly retained substances from the stationary phase. At this moment, the chromatographic system power is ready to be turned off. Following these instructions, the column may reach a lifespan of more than 1000 injections.

It should be noted that surfactant monomers are only partially removed from the stationary phase. Therefore, the column remains surfactant-modified its entire lifespan. Consequently, once used with a micellar mobile phase containing a specific surfactant, a column should not be employed with other surfactants or for hydro-organic RP–HPLC. It is not any trouble to separately dispose of columns for hydro-organic RP–HPLC and another ones for MLC, as C18-columns are not so expensive. Therefore, even considering the cleaning procedure, MLC remains more rapid and economic than hydro-organic RP–HPLC.

2.6. Plasma extraction & urine sample preparation

Blood and urine sample of tuberculous patients were supplied by the Clinical Biochemistry Laboratory of the Sagar Central Hospital. The study was approved by the Ethical Committee and conducted by the ethical principle originating in the Declaration of Helsinki. In order to obtain the plasma, blood was centrifuged at 4500 r.p.m. at 4◦C for 5 min. Immediately after, the plasma was stored at -25◦C in amber vials. Drug-free plasma and urine blanks were constructed by mixing the corresponding biological fluids, respectively, obtained from healthy volunteers (three males and three females) who did not use to take any drug.

Before the analysis, the biological fluids were thawed at room temperature. Then, they were 1:5 (v/v) diluted in mobile phase, vigorously shaken, filtered and injected [47,48]. Samples used for the validation and quantification were treated as the same way. Spiked samples were prepared by adding the appropriate volume of working solution before the dilution.

3. Results & discussion

3.1. Optimization of chromatographic conditions: pH, surfactant & modifier

The corresponding assays were carried out using a working solution of rifampicin and rifampicin quinone, both 1 μg/ml. These compounds show acid/base activity, based on this fact and its structure the retention mechanism definitely will depend on the pH of the mobile phase. The normal working range of a C18 column is said to be between pH 2 and 9. However, weak alkaline solutions provoke a slow and continuous hydrolysis of the C18-alkyl-silica bond, resulting in a medium-term loss of performance of the column. Therefore, only acid and neutral pH values were tested. As the pKa of rifampicin is 1.7, the molecule is deprotonated in all the working range of the column and for this reason pH 7 was selected for carrying out the analysis.

SDS is commonly used as an anionic surfactant which is less toxic, economic, eco-friendly and chromatographically compatible. Different concentration of SDS (0.05–0.15 M)

were used for the optimization of desired mobile phase, at the fixed pH 7. When the concentration of SDS was at the minimum, in other words, 0.05 M SDS, both drugs were excessively retained. The elution was enhanced when the concentration of SDS was increased consistently up to its optimum micellar working range, in other words, 0.15 M. At this concentration the chromatographic parameters were: rifampicin 21.3; 2415 and 1.96; rifampicin quinone 27.3; 1124 and 2.87 (retention time, efficiency and asymmetry factor), respectively. As these results were considered not satisfactory, we envisage to incorporate a small proportion of short chain mono-alcohol, to increase the elution strength and ameliorate the peak shape. Variable concentration of organic modifiers was added, in the interval of proportion recommended for MLC [49], in other words, 1-propanol (2.5–12.5%), 1-butanol (1–7%) and 1-pentanol (2–6%) was selected and the effect of these modifiers on the peak parameter was studied. Using 1-propanol and 1-butanol, the chromatographic parameters were not greatly improved but still leaving room for further improvement. This promoted the use of 1-pentanol as organic modifier which showed better results than those using of 1-propanol and 1-butanol.

3.1.1. Optimization of SDS/1-pentanol concentration

Use of an empirical strategy of assaying mobile phases at several values of SDS/1-pentanol to find out the optimum mobile phase was discarded because it would take too long. Therefore, an interpretative strategy based on a chemometric approach was performed, in order to shorten and optimization process [49]. A mechanistic model, able to calculate the chromatographic parameters of moderately hydrophobic solutes from the composition of the mobile phase and four constants with physicochemical meaning, taken from the partition equilibria occurring in the column [49], was used. The Equation 1 is taken to model the retention factor:

$$K'_{AS} / (1+K_{AD\varphi})$$

$$1+ [M] (K_{AM} (1+K_{MD\varphi}) / (1+K_{AD\varphi}))$$

(1)

where [M] and φ are the concentration of SDS (M) and 1-pentanol (%, v/v). The four constants depend on the solute and the surfactant, while KMD and KAD also depend on the organic modifier. The simulation of the peak shape (required to predict the efficiency and asymmetry) was performed using the Equation 2. It calculates the signal h(t) at each time of the chromatographic run caused by the elution of the analyte, which is considered and asymmetrical normal function:

$$h(t) = H \exp\left[-\frac{1}{2}\left(\frac{t - t_R}{s_0 + s_1 (t - t_R)}\right)^2\right] \quad (2)$$

H (maximal height) depends on the concentration and sensitivity, and t_R is the retention time. The constant S_0 represents the central dispersion of the Gaussian curve, whereas S_1 quantify the sleekness. These parameters remain ideally invariant for each solute and mobile phase.

Values of retention factor, N and B/A of rifampicin and rifampicin quinone, measured from the analysis performed using the mobile phases at five combinations of SDS (M)/1-pentanol (%, v/v): 0.05/2; 0.05/12.5; 0.1/7.5; 0.15/2.5 and 0.15/12.5, were treated by the Michrom software [46] to adjust the equations 1 and 2. Thus, the theoretical values of the retention time and peak shape of both analytes can be estimated at any combination of SDS and 1-pentanol inside the range 0.05–0.15 M and 2–6%, respectively, by interpolation. Combining the information by different peaks which are at the same mobile phase, the theoretical values of resolution between both solutes (r_{ij}) were calculated using the valley-peak

criteria. With this information, simulated chromatograms may be drawn, and then the analyst can easily visualize the variation of the chromatogram shape and check the overlapping, when the concentration of SDS or 1-pentanol changes.

According to the chemometric model, the maximum resolution at the minimum analysis time was reached using the mobile phase 0.15 M SDS-6% 1-pentanol-phosphate buffer 0.01 M-pH 7 (r_{ij} = 0.9998). Under these conditions, the experimental values of the chromatographic parameters were rifampicin (t_R: 5.8; N: 6535; B/A: 1.35) and rifampicin quinone (t_R: 11.8; N: 5238; B/A: 1.64). Both compounds were eluted without overlapping with adequate peak shape and the analysis time was less than 15 min.

3.1.2. Advantages of the procedure

The developed procedure shows several benefits over those based on hydro-organic RP–HPLC, because of the use of micellar solutions. Main advantage of the procedure is the simplification of the sample treatment, exploiting the possibility of direct injection, without clean-up, extraction or purification steps, despite the complexity of the matrices. Therefore, the sample is quantitatively transferred to the column, thus reducing the sources of variance.

All these characteristics strongly shorten the global analysis time and facilitate the successive analysis of many samples. Dilution solvent and the mobile phase are mainly made of biodegradable chemicals. They contain only 6% 1-pentanol as toxic and flammable organic solvent which is quite less than that normally used in hydro-organic mobile phases (which is up to 100%). Therefore, the risk for the health of the laboratory staff associated to the handling of toxic reagents and waste disposal are minimized. This fits the current trend in analytical chemistry [50]. Using this analytical method, analyses can be carried out at minor cost, as solely available chromatographic and laboratory instrumentation and low quantity of inexpensive chemicals are needed. Besides, it has a high sample throughput.

3.2. Method validation

The procedure was validated following the Guideline on bioanalytical method validation issued by the EMA. Studied validation parameters were: selectivity, linearity, calibration interval, limit of detection, lower limit of quantification (LLOQ), accuracy, precision, carry-over, matrix effects, robustness and stability [45]. The validation was performed using blank plasma and urine samples spiked with the studied compounds, unless specified.

3.2.1. Selectivity

Plasma and urine blanks were analyzed by the developed procedure. In all cases, a broadband, corresponding to proteins and other endogenous compounds, eluted from the dead time to nearly 4.0 min (Figure 2A for blank plasma and Figure 2B for blank urine), and no other peaks were detected beyond 4.0 min, as obtained in previous work devoted to the analysis of biological fluids by MLC. These substances strongly interact with the micelles of the mobile phase and then are barely retained on the stationary phase [50]. As the analytes show retention times >4.5 min, there are no potentially interfering compounds, thus the method is selective enough to unambiguously detect the analyte, albeit the absence of a purification step. Both blanks were fortified at 1 μg/ml of rifampicin and rifampicin quinone and analyzed. Both compounds were eluted without overlapping at the same retention time as previously found at the optimization step. No other compounds were found close to those corresponding to the analyte (Figure 2C for spiked plasma and Figure 2D for spiked urine).

3.2.2. Calibration range & sensitivity

Nine plasma and urine spiked samples containing concentrations of rifampicin and rifampicin quinone in the range 0.05–50 μg/ml, were analyzed by triplicate. Parameters of the calibration curve and degree of linearity were calculated by least-squares linear regression,

plotting the average AUC versus the corresponding concentration for both compounds. The slope, y-intercept and determination coefficient were: (63024.5; 58402.2); (6.1; -4.2) and (0.9998; 0.9995). The procedure was found linear over the studied range.

Upper limit of quantification was stated at 5 μg/ml, whereas LLOQ was the smallest concentration which can be determined with adequate accuracy and sensitivity (see the next subsection): 0.05 μg/ml, for both drugs. Limit of detection (LOD) was calculated following the 3.3 s criterion (standard deviation of the y-intercept was taken as that of the blank): 0.03 μg/ml, for both compounds. LLOQ of rifampicin was below the lower limit of its therapeutic range.

3.2.3. Accuracy & precision

These parameters were within-run measured at three levels (0.25, 0.50 and 1.5 μg/ml) by analyzing six samples, prepared on purpose from a working solution different to that used in the calibration, in the same day and analysis run. Accuracy was expressed as the bias in percentage calculated from the average of the found concentrations, whereas the precision was taken as the relative standard deviation (RSD) of the measured peak areas.

To determine between-run values, the same assay was performed 3 days over a 1-month period, by renewing the analyzed spiked samples. Accuracy was defined as the bias, in percentage, obtained from the average of the concentration's values found each day. Precision was the RSD of the average values of the peak area taken each day.

Values of accuracy (-3.4 to +4.0%) and precision (RSD < 9.2%) fit the acceptance criteria (less than 20% for LLOQ and less than 15% for higher values). Therefore, the quantitative values for rifampicin and its derivative provided by the method are consistent enough and have a low uncertainty.

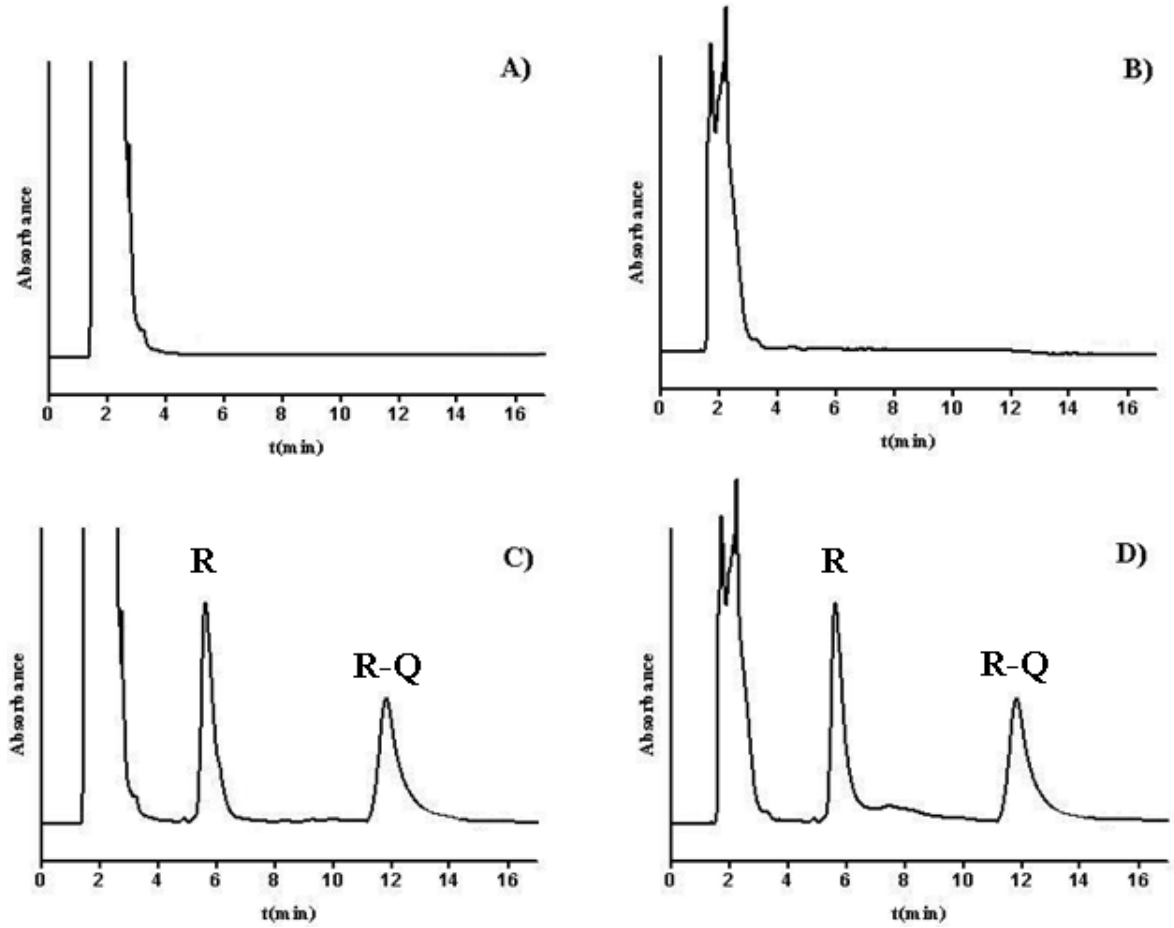


Figure 2. Chromatograms of (A) blank plasma; (B) blank urine; 1-μg/ml rifampicin (R) and rifampicin quinone (R–Q) fortified (C) plasma and (D) urine; using the optimal conditions.

3.2.4. Carryover effect

The carryover effect was investigated by analyzing a plasma and urine sample fortified at 5 μg/ml with rifampicin and rifampicin quinone, and, in the following injection, the corresponding blank. No other peaks, was observed in the chromatogram at the window time of the analytes or nearby for both biological fluids. Thus, the cross-contamination was negligible within the calibration range.

3.2.5. Matrix effects

Possible interference of matrix compounds was examined by analysis of plasma and urine samples fortified at the same levels in accuracy/precision studies, and standard working solutions containing the same concentrations divided by five (to take into account the sample treatment). The peak area values were very similar, irrespective of the starting environment. Therefore, the matrix effect was judged as not significant.

3.2.6. Robustness

In a realistic situation, the experimental parameters may oscillate in a short range, introducing a new source of variance in the chromatographic response. To verify the extent of this fact, the change in the main observed variables for rifampicin and rifampicin quinone (retention time, efficiency, asymmetry and peak area) was studied after small changes, which can occur during the normal laboratory work, of the following experimental conditions: SDS concentration, 0.14–0.16 M; 1-pentanol, 5.5–6.5%; pH, 7.5–6.5; flow-rate, 0.9–1.1 ml/min.

Influence of each factor was separately studied by analyzing the same standard solution at three mobile phases, made by varying the considered factor as follows (keeping constant the other ones): the optimal value, and the lower and upper values of the fixed range. RSD of the three obtained values was calculated for the studied observed response. Results are showed in Table 1. The method was found quite robust, as insignificant variations were observed (less than 5%), within the considered range.

Table 1. Robustness Evaluation of the Developed MLC Method (Variation in RSD, %).

	Chromatographic response	Flow-rate (mL/min)	pH	1-pentanol (%, v/v)	SDS (M)
Rifampicin	t_R (min)	0.06	0.08	4.6	4.6
	N	2.2	3.8	1.9	1.7
	B/A	1.2	1.6	2.3	2.0
	AUC	3.2	2.9	2.8	2.1
Rifampicin quinone	t_R (min)	0.3	0.2	5.2	4.8
	N	3.4	2.9	1.7	2.0
	B/A	1.8	1.5	2.5	2.9
	AUC	4.0	3.4	3.2	3.5

3.2.7. Stability & decomposition studies

Stability means the capacity of the drug to remain unchanged throughout time. It can be studied under different environmental conditions of light, temperature, chemical environment – among others. It is determined by the monitoring of the concentration of analyte in a stored solution or sample. The degradation is noticed by the diminishing of the concentration and the emergence of peaks from decomposition products. As reported in the literature, rifampicin is a highly unstable drug and readily decomposes in rifampicin quinone due to oxidation [3].

The stability was studied from solutions or spiked samples containing 1.0 μg/ml of rifampicin and without rifampicin quinone. Results were found similar for plasma and urine. The investigated conditions were:

- Standard solution in a mobile phase kept in the fridge for one week (Figure 3A). Under these conditions, rifampicin was found stable up to 4 days (90% decay). Therefore, the standard solutions must be renewed after this period;

- Short term stability of the analyte in the processed biological fluid at room temperature for 1 day (Figure 3B). The analyte decays in nearly 1 day, and then a prepared sample must be analyzed a maximum of 1 day after the dilution. This is a quite long period and to not hinder the normal work at the laboratory in routine conditions. The sample throughput is limited to 96 samples per day, a high value;

- In the biological fluid long term freeze at -20◦C for 2 weeks (the usual storage conditions in a hospital). A short decay (90%) was noticed at the end of the experiment. Therefore, the extracted sample can be stored until 15 days before analysis, without affecting the measured concentration of rifampicin.

An additional conclusion of the study was that the decomposition is accelerated as the temperature increased.

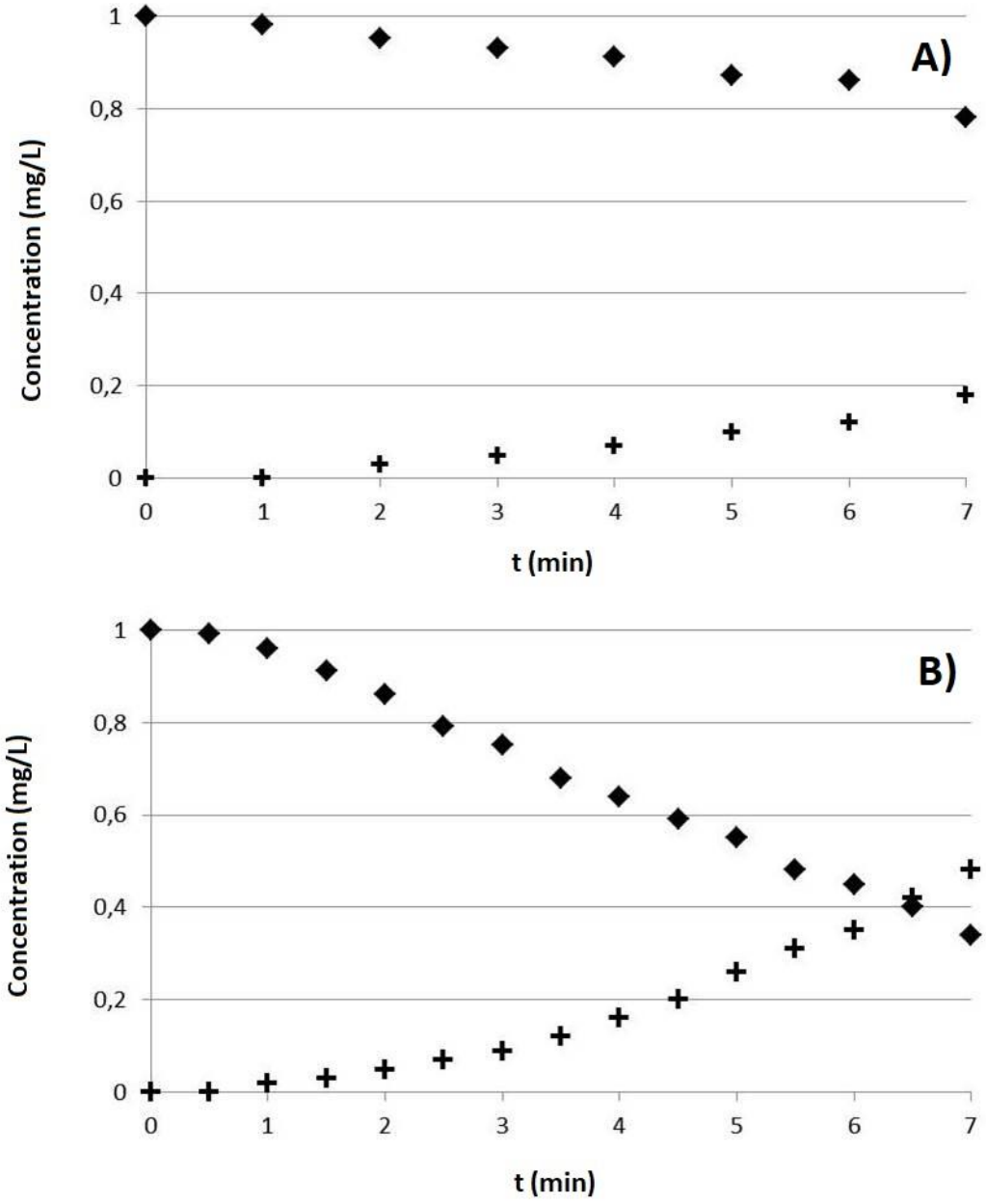


Figure 3. Kinetics of the rifampicin decomposition (•) and rifampicin quinone formation (+) in A) standard solution kept at 4ºC and B) processed biological fluid at room temperature.

3.3. Analysis of incurred samples

The developed method was applied for the determination of rifampicin in incurred samples, in other words, plasma (free plasmatic fraction) and urine of TB patient enrolled in DOTS program (Table 2). The samples were collected from DOTS treatment center generally after 8 h of drug administration.

Chromatograms obtained from the analysis of incurred samples of plasma and urine of TB patients are shown in Figure 4A and B. While observing the chromatogram at the head of chromatogram there is a broad peak which is due to the presence of serum matrix which are normally solubilized by the surfactant and elutes out in the manner as depicted at the beginning of the chromatogram. As observed in the chromatogram, the developed method and obtained results shows that the analysis for the determination of rifampicin present in an incurred sample of TB patients are good in accordance with chromatographic parameters.

Table 2. Amount of rifampicin found in plasma and urine of 8 tubercular patients.

Patient	Rifampicin in plasma (µg/mL)	Rifampicin in urine (µg/mL)
1	2	1.6
2	1.5	0.8
3	1.3	0.93
4	5	3.1
5	0.8	0.56
6	2.2	1.97
7	2.9	0.67
8	4.1	3.12

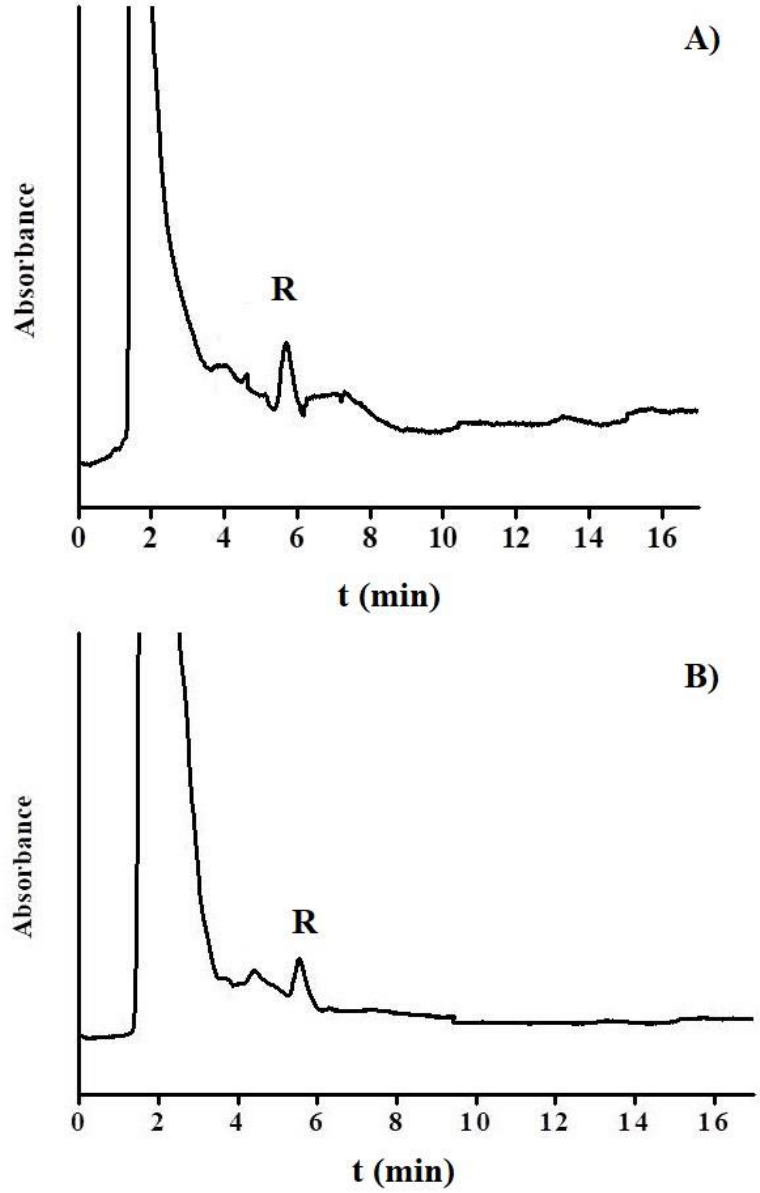


Figure 4. Chromatogram obtained from the analysis of (A) Plasma of tubercular patient (B) Urine of tubercular patient using Optimum Chromatographic Conditions.

4. Conclusion

MLC has been proven as a useful technique for the analysis of rifampicin in plasma and urine. Main advantage of the method is the possibility of direct injection. Plasma and urine samples were analyzed by an easy-to-handle, one-step, rapid and reproducible assay, without long and tedious extractions. Hence, the experimental protocol is simplified, minimizing the probability of handling error and the use of reagents. The analytes were eluted without interference from the matrix in a short time, using an isocratic mobile phase, resulting in a limited global analysis time. These characteristics result in a high sample throughput. Method was fully validated following the directives of an official guideline issued by a renowned institution (EMA), which assesses its reliability. The procedure is environment-friendly and safe for the laboratory operators. Besides, it is quite inexpensive, and hence available to clinical laboratories with limited resources. As there is an urgent need for therapeutic drug monitoring for TB patients to improve the success rate of the treatment and to reduce the side effects. The developed method could be easily used in clinical laboratories as routine analytical tool for monitoring of DOTS patient as well as for adjusting the future dosages. Procedure can also be used for multidrug therapy of leprosy patients.

5. Future perspective

The current tendency in analytical chemistry is the development of short-time, practical, easy-to-conduct, inexpensive, high-sample throughput and eco-friendly procedures, with a minimal operator participation. The implementation of these methods will increase in the future in routine analysis for clinical purposes. Therefore, traditional analytical methods will be substituted by other ones with these characteristics. MLC, which uses direct injection and a little amount of toxic chemicals, can play a major role in this process.

The here-described method may be enlarged to other rifampicin metabolites (like desacetyl rifampicin, rifampicin SV, rifampicin N-oxide and 3-formylrifamycin SV). Besides,

it can also be applied to other biological fluids (cerebrospinal, gastric, saliva, sweat), to solid tissues (feces, organs and muscles), in this last case, with a previous solid-to-liquid extraction. This can be useful for clinical research, point-of-care or punctual determination or therapeutic drug monitoring purposes. It can also be used to analyze pharmaceutical formulations, for quality control or stability studies. The chromatographic conditions will be similar, although the separation and detection conditions may have to be modified, as the micellar environment diminishes the matrix effect. In all cases, the use of statistical tools to model the retention mechanism from the composition of the mobile phase would be highly interesting, in order to get a better knowledge about retention fundamentals and facilitate the optimization of the chromatographic conditions.

A branch of MLC, based on the use of pure mixed micellar mobile phases (using a biodegradable and safe nonionic surfactant), instead of hybrid ones (using toxic, flammable and volatile organic solvent), has been proposed and has raised a huge interest. This new technique can be applied to this method, in order to totally remove the use of hazardous chemicals and then totally fulfill the requirements of 'green' chemistry. Another proposal is the use of microemulsions as mobile phases, which can be useful to determine highly hydrophobic drugs. In both cases, the elucidation of the retention mechanism and the study of the partition equilibria involved in the retention would be useful. The model of the retention parameters from the composition of the mobile phase should be performed to get a better knowledge about retention fundamentals and facilitate the optimization of the chromatographic conditions.

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Chapter 8. A rapid and Reliable assay to determine Flumequine, Marbofloxacin, Difloxacin, and Sarafloxacin in commonly consumed Meat by Micellar Liquid Chromatography

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ABSTRACT

BACKGROUND

Micellar liquid chromatography – fluorescence detection was used to determine the antibiotics flumequine, marbofloxacin, difloxacin, and sarafloxacin in porcine, bovine, poultry, ovine, caprine, rabbit, and equine meat, to verify compliance with EU Regulation 37/2010 with regard to the occurrence of veterinary drugs in food.

RESULTS

The analytes were isolated from the matrix by ultrasonication-assisted leaching in a micellar solution, and the supernatant was filtered and directly injected. The fluoroquinolones were resolved in < 19 min using a C18 column, with an isocratic mobile phase of 0.05 mol L−1 sodium dodecyl sulfate - 8% 1-butanol – 0.5% triethylamine buffered at pH 3. The limits of quantification (0.01–0.05 mg kg−1) were below the maximum residue limits (0.15–0.4 mg kg−1). The method was validated by EU Commission Decision 2002/657/EC guidelines.

CONCLUSION

The method shows practical advantages such as simplicity, low cost, eco-friendliness, safety, and applicability for routine analysis, and is useful for surveillance programs. © 2018 Society of Chemical Industry

1. Introduction

Meat is greatly appreciated as a foodstuff due to its taste and elevated protein, fat, vitamin, mineral, and micronutrient content. These constituents of meat are necessary for a balanced diet and are essential for growth. In recent years, meat consumption has increased worldwide due to increasing population size, urbanization, and increasing income, although it has remained stable at a high level in developed countries.**1**, **2** The production of meat is an important economic activity in the EU because of the high levels of meat consumption and the importance of meat trading. The production of pork (22.6 million tons), beef (7.7 million tons), and poultry (12.6 million tons) meat is both for the home market (110%, slightly above 100% and 104% of the self-sufficiency rate, respectively) and for export, mainly to Russia and East Asia.**3** Although 0.92 tons of sheepmeat and goat meat are produced annually, the EU is a net importer (88% of self-sufficiency rate), mainly from New Zealand and Australia.**4** The production of other kind of meats, such as rabbit (0.6 million tons) and horse (62.8 million tons) has become highly important in economic terms.**5**, **6** Most of these animals are reared in farms at high densities and fed with manufactured feed to reduce the high production costs and maintain an affordable retail price. However, this practice stimulates the incidence and propagation of infectious diseases among the animals, thus increasing their morbidity and mortality and affecting productivity.**7**

Fluoroquinolones are synthetic broad-spectrum antimicrobials and have significant post-antibiotic effects against gram-positive and -negative bacteria. Among them, flumequine (FLU), marbofloxacin (MARBO), difloxacin (DIF), and its main metabolite, sarafloxacin (SAR), are widely prescribed in medical and veterinary practice to combat a wide range of diseases caused by bacterial infections.**8** Their structure and properties can be seen in Fig. **1**(A) and Table **1** respectively.**9**, **10** In farms, antimicrobial drugs are administered orally or injected into the animals as prophylactic and curative agents, to safeguard their welfare, and to promote growth. However, their indiscriminate use has resulted in the occurrence of antibiotic residues in edible tissue. Unnoticed exposure to subtherapeutic amounts has been associated with severe long-term health problems for consumers, such as allergies and the emergence of fluoroquinolone-resistant human pathogens.**11**, **12** This encourages infectious epidemics, which cannot be treated with the current antibacterial arsenal, and may lead to serious consequences for individual patients and increase the costs of medical care.**13**

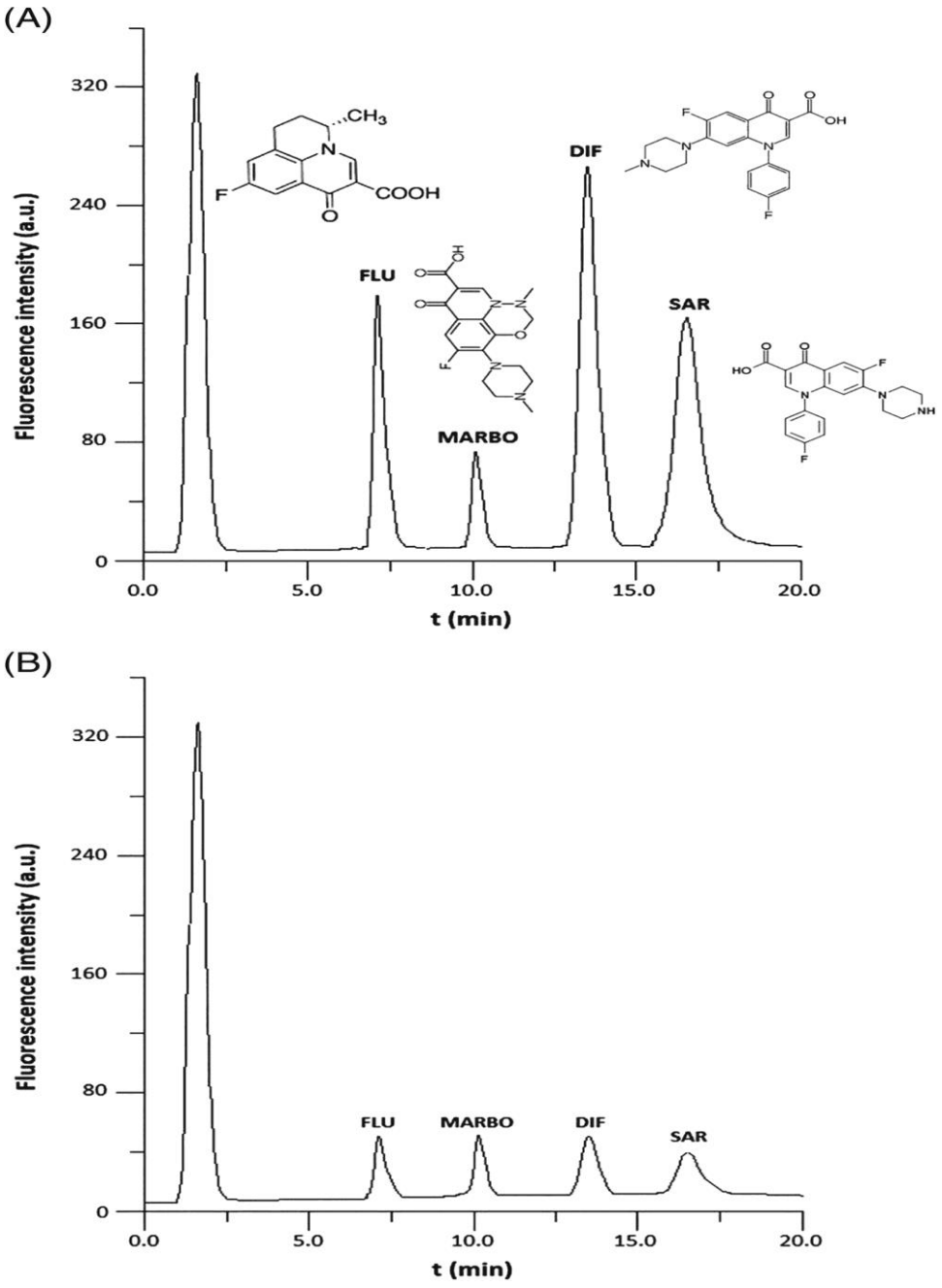

Figure 1. Chromatograms obtained by the analysis of a sample of porcine meat spiked, (A) at 0.2mg kg−1 of each quinolone (the structure of each antimicrobial is also shown), and (B) at their corresponding LOQs.

Chapter 8. A rapid and Reliable assay to determine Flumequine, Marbofloxacin, Difloxacin, and Sarafloxacin in commonly consumed Meat by Micellar Liquid Chromatography

Nowadays, there is worldwide concern among population and international agencies about the potential risks arising from the abusive use of fluoroquinolones.**13** Several governments have therefore established regulations and actions to avoid the misuse of antibiotics in animal farming.**14** Within the frame of its policy of protecting human health and maintaining the image of European meat as a healthy, high-quality product, the EU has set maximum residue limits (MRLs) for FLU, MARBO and DIF in the muscle tissue of several animals (EU Regulation 37/2010).**15** No MRL has been established for SAR but its residue would not be higher than that of DIF (Table **1**). Monitoring is necessary to verify compliance with the regulation and to ensure food safety.

Several multi-residue methods have been developed for the determination of fluoroquinolones in animal muscle tissues using microbiological tests,**16** immunoassay,**17** electrophoresis**18** and reverse-phase high-performance liquid chromatography (RP-HPLC).**19** The latter is the technique of choice due to its higher versatility and selectivity. Several HPLC methods have been developed for the analysis of FLU, MARBO, DIF, and SAR in porcine, bovine, ovine, and poultry meat. In general, they require careful multistep sample preparation.**19** First, the antimicrobials must be extracted by leaching using a solvent (aqueous**20**-**22** or hydroorganic,**11**, **23**-**28** by simple mixing,**20** vortexing,**11**, **21**, **22** shaking,**23**-**26**, **28**, **29** which may be ultrasound assisted**23**, **28** or microwave assisted**24**), eventually followed by centrifugation.**11**, **20**-**26**, **28**, **29** Sometimes, several successive extraction steps may be required. Afterwards, the supernatant is often purified before injection to avoid the introduction of particles, proteins, macromolecules, or other small endogenous compounds, which may be harmful for the column and/or overlap with the analytes, by solid phase extraction using a C18,**20**, **21** hydrophilic-lipophilic **23**, **29** or hydroxylated polystyrene-divinylbenzene,**24**, **25** immunoaffinity**22** or metal chelate affinity **27** coating, liquid/liquid extraction **11**, **28** or Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) procedure **26** extraction. These procedures increase the time, effort, economic, and laboratory resources, and amount of toxic chemicals required for the analysis. They also provide oscillating recoveries and increase the sources of variance in the method. Chromatographic separation has been carried out using polystyrene-divinylbenzene,**28** C8**25** or C18**11**, **20**-**24**, **26**, **29** columns, a mobile phase with a high concentration of organic solvent (up to 100%), usually programmed as a gradient,**11**, **20**-**26**, **28**, **29** and detected by mass spectrometry,**20**-**23**, **25**, **26** and UV-visible absorbance**24**, **25** or fluorescence.**11**, **21**, **22**, **27**-**29** The latter is preferred because of its higher

analytical performance per cost ratio. However, to the best of our knowledge, no HPLC method has been published for the analysis of these antibiotics in goat, rabbit, or horse meat.

Liquid chromatography with acidic hybrid mobile phases, using sodium dodecyl sulfate (SDS) as surfactant and triethylamine (TEA) as sacrificial base, has been proven to be an interesting alternative to the determination of quinolones in food.**30-32** Micellar solutions are able to solubilize compounds within a large range of molecular mass and hydrophobicity. Proteins and other non-water soluble compounds are therefore harmlessly eluted at the front of the chromatogram, and do not interfere with the first eluted analytes. This avoids the injection of aqueous suspensions without cleanup after simple filtration, thus simplifying the sample pretreatment.**33** The negative layer in the stationary phase and the presence of the micellar pseudo phase also increase the versatility and the reproducibility of the retention mechanism, and allow the resolution of a mixture of cationic and neutral drugs with different hydrophobicities in the same run using a mobile phase containing < 12.5% of organic solvents working in isocratic mode. Fluorescence is also enhanced in micellar and microemulsion solutions.**34** Ultrasound-assisted leaching using acidic pure micellar solutions has also been used to extract fluoroquinolones from the meat with a high recovery rate.**30**

The aim of this work was to develop an analytical method for the screening of FLU, MARBO, DIF, and SAR in edible muscle from several animals (pork, beef, chicken, turkey, duck, sheep, goat, rabbit, and horse) using micellar liquid chromatography – fluorescence detection. The method had to be appropriate for quality control, and able to verify the compliance of commercial samples with EU Regulation 37/2010.**15** It should therefore had to be practical, easy to handle, safe, environmentally friendly, cost-effective, with a high sample throughput, and sensitive enough to provide consistent values close to the maximum residue limits for each fluoroquinolone. The analytical performance of the method was verified by validation using the guidelines of EU Commission Decision 2002/657/EC.**35** The suitability of the method for routine analysis in a real situation would be demonstrated by the analysis of incurred samples purchased from retail stores.

Table 1. Characteristics and MRL (mg kg^{-1}) of the fluoroquinolones9, 10, 15

Antibiotic	Flumequine	Marbofloxacin	Difloxacin	Sarafloxacin
pKa COOH group (acidic)	6.4	5.7	5.7	5.6
pKa N-piperazynil moiety (basic)	—	8.0	7.2	8.2
Log Po/w	2.3	−2.9	1.3	1.1
MRL in porcine and bovine meat	0.2	0.15	0.4	0.4[a]
MRL in poultry meat	0.4	0.15[b]	0.3	0.3[a]
MRL in ovine meat	0.2	0.15[b]	0.4	0.4[a]
MRL in caprine meat	0.2	0.15[b]	0.4	0.4[a]
MRL in rabbit and horse meat	0.2	0.15[b]	0.3	0.3[a]

- [a] No regulatory MRL. Practical MRL same as for DIF.
- [b] No regulatory MRL. Practical MRL same as for porcine and bovine meat.

2. Materials & Methods

2.1. Standards & Chemicals

Solid standards of FLU (purity > 98%), MARBO (> 98%), DIF (> 99.8%) and SAR (> 97.2%) were obtained from Sigma (St Louis, MO, USA). Sodium dodecyl sulfate (> 99.0%) was supplied as a powder by Merck (Darmstadt, Germany). Sodium dihydrogen phosphate monohydrate (> 99.0%), 1-propanol, 1-butanol, and 1-pentanol (HPLC grade) were bought from Scharlab (Barcelona, Spain). Hydrochloric acid (37.0%), ethanol (HPLC grade) and trimethylamine (> 99.5%) were purchased from J. T. Baker (Deventer, Netherlands). Ultrapure water was produced in the laboratory from deionized water (supplied by the university as tap water) using an ultrapure generator device, Simplicity UV (Millipore SAS, Molsheim, France).

2.2. Preparation of solutions

Micellar solutions were prepared by weighing the appropriate amount of SDS and $NaH_2PO_4.H_2O$, and dissolving them in ultrapure water using a magnetic stirrer. The appropriate amount of trimethylamine was added, and then the pH was set to 3 by adding drops of HCl solution. The organic solvent was added to reach the selected proportion, and the flask was filled with ultrapure water. Finally, the solution was ultrasonicated for 5 min to achieve solubilization and filtered through a 0.45 μm membrane filter (Micron Separations, Westboro, MA, USA) placed on a Büchner funnel, with the aid of a vacuum pump.

Individual solutions of each fluoroquinolone (100 mg L^{-1}) were prepared by dissolving the appropriate amount of the powdered standard in 5% of ethanol in a volumetric flask, and then a solution of 0.05 mol L^{-1} SDS buffered with phosphate salt 0.01 mol L^{-1} at pH 3 was added up to the mark. These solutions were ultrasonicated for 5 min to ensure that the powder was completely dissolved. Working solutions were prepared by successive dilutions of the stock solutions in the same micellar solution. All the standard solutions were kept at +4 °C for a maximum of 2 months.

2.3. Chromatographic conditions

The chromatograph was an HP1100 (Agilent Technologies, Palo Alto, CA, USA), equipped with an isocratic pump, a degasser, a 20 μL loop, an autosampler, and a fluorescence detector. The instrumentation was controlled and the signal was registered using Chemstation Rev.A.10.01 software (Agilent Technologies). Efficiency (N) was calculated as outlined by Harris,**36** using the half-peak width obtained by the software. The dead time (t_0) and retention time (t_R) were directly taken from the chromatogram. Asymmetry was evaluated visually.

The stationary phase was in a C18 Kromasil column (Scharlab) with the following characteristics: length, 150 mm; internal diameter, 4.6 mm; particle size, 5 μm; pore size, 10 nm. The mobile phase was an aqueous solution of 0.05 mol L^{-1} SDS - 8% 1-butanol - 0.5% triethylamine, buffered at pH 3 with 0.01 mol L^{-1} phosphate salt, running at 1 mL min^{-1} under

isocratic mode. Detection was performed by fluorescence, and the excitation/emission wavelengths (nm) were programmed in time as follows: 0.0–8.5 min, 240/370; 8.5–11.5, 300/488; 11.5–20, 280/455. The solutions were filtered through a 0.45 μm nylon membrane filter before introduction into the vials. The special care required with chromatographic instrumentation when dealing with micellar mobile phases (change of mobile phase, cleaning before switching off, etc.) has been detailed in Rambla-Alegre *et al*.**33**

2.4. Sample processing

Samples of pork, beef, chicken, turkey, duck, sheep, goat, rabbit, and horse meat were bought from a local supermarket, finely minced, and stored at −20 °C in a freezer for a maximum of 2 months. Only muscle was taken for the study, as this is the tissue that is consumed most. Before processing, sample meat was thawed for 30 min at room temperature.

The samples were leached to recover antimicrobial drugs. Thus, 5 g of meat was mixed with 50 mL of a 0.05- mol L^{-1} SDS solution buffered at pH 3. The solutions obtained were placed in an Erlenmeyer flask, shaken using a magnetic stirrer for 1 h, and ultrasonicated for 15 min. Finally, the supernatant was decanted and filtered through a 0.45 μm nylon membrane filter using a Büchner funnel, with the aid of a vacuum pump. This supernatant was immediately injected or kept at +4 °C in the fridge for a maximum of 2 months, until analysis.

For spiked samples, the appropriate volume of the standard solution was injected into the minced meat. The sample was kept overnight at room temperature to cause the slow vaporization of the solvent and the incorporation of the antibiotic into the matrix. These fortified samples therefore adequately imitate biologically contaminated ones.**37** Afterwards, the analytes were extracted as indicated above.

Before the analysis, the stored solutions (standard or supernatant) were warmed at room temperature for 30 min to dissolve the crystals of SDS formed overnight.

3. RESULTS & DISCUSSION

3.1. Optimization of the chromatographic conditions

The main separation conditions were taken from other methods used for the determination of fluoroquinolones in honey**31**, **32** and fish flesh,**30** which have provided adequate results: stationary phase, C18; flow rate, 1 mL min^{-1} under isocratic mode; surfactant, SDS; required organic solvent, 1-propanol or 1-butanol; pH, 3 and 0.5% triethylamine. In this work, we optimized the composition of the hybrid micellar mobile phase (concentration of SDS, and the nature and concentration of the organic solvent) and the detection conditions to resolve a mixture of FLU, MARBO, DIF, and SAR with a good peak shape, using the minimum analysis time. The studies were performed using a standard solution containing 0.02 mg L^{-1} of each fluoroquinolone.

In previous studies, these antimicrobials demonstrated binding behavior with the micelles, and the retention times and the efficiency decreased at higher concentrations of SDS. Indeed, depending on their hydrophobicity and charge, they can interact with the polar, anionic, and hydrophobic sites of the micelles.**34** To maximize efficiency, the concentration was set to the minimal value recommended for MLC: 0.05 mol L^{-1}.

The pure micellar mobile phase provided analysis times that were too long, and broad peaks. To avoid this, the addition of 1-propanol (2.5–12.5%) or 1-butanol (1–10%) was tested.**34** In both cases, lower retention times and higher efficiencies were obtained. This effect was higher using 1-butanol than using 1-propanol, and increased with increasing concentrations of alcohol. Sarafloxacin had a too long retention time using 1-propanol, even at larger proportions, and then it was was not selected. Using 1-butanol, a proportion of 8% provided the maximum resolution with the minimum analysis time. The first eluted compound was FLU ($t_R \approx 7.3$ min), which was far enough from the front of the chromatogram. Adequate efficiency and small tailings were obtained for the four fluoroquinolones.

A standard solution of the four quinolones was analyzed using the optimized mobile phase: 0.05 mol L^{-1} SDS 8% v/v 1-butanol – 0.5% v/v triethylamine, buffered at pH 3 with 0.01 mol L^{-1} phosphate salt. The values obtained for (t_R; N) were: FLU, (7.3 min; 3842);

MARBO, (10.2; 2985), DIF (13.6; 4580) and SAR (16.9; 3214). The analytes were adequately resolved. According to the retention time of the first eluting fluoroquinolone, no overlapping with the front of the chromatogram or the less retained compounds of the matrix was expected.

The antibiotics were resolved using a mobile phase containing a less minor proportion of toxic, volatile and flammable solvent (<8.5%), than usually required in hydro-organic HPLC (up to 100%). The interaction with SDS even reduced its volatility. The mobile phase works under isocratic mode, which improves baseline stability, avoids the emergence of spurious peaks, and enhances the reproducibility of peak shape and retention time. A re-equilibration period is not needed between two successive injections, thus reducing the analysis time per sample.**38**

3.2. Detection conditions

Fluorescence was selected as a detection technique because it has higher selectivity and sensitivity than absorbance and a lower cost than mass spectrometry. Derivatization was not required because the fluoroquinolones that were studied show natural fluorescence. As the spectrophotometric properties of the fluorophore depend on the chemical environment, the excitation/emission wavelengths (nm) of maximum emitted intensity were chosen from several published papers dealing to the determination of these antimicrobials using similar mobile phases: FLU, 240/370; MARBO, 300/488**32**; DIF and SAR, 280/455.**31**

To maximize sensitivity, the detector was programmed to detect each fluoroquinolone at its optimal excitation/emission wavelengths. At the beginning of the chromatography run, the signal was monitored at 240/370. Once FLU had been eluted (8.5 min), the detection wavelengths changed to 300/488, until the complete elution of MARBO (11.5 min). From this point to the end of the chromatograms, the signal was registered at 280/455. The baseline noise was similar for the three sets of wavelengths, and no sudden oscillation of the baseline was observed at the wavelength changes.

3.3. Sample preparation

Sample preparation was based on the leaching described in Rambla-Alegre *et al*.:**30** extraction of the fluoroquinolones from the flesh to a solvent (1/10, w/v) by shaking, followed by filtration of the supernatant and direct injection. Solvents (methanol and 0.05 mol L^{-1} SDS at pH 3) were tested and the duration of the stirring was optimized. The studies were performed using a sample of pig meat spiked at 0.2 mg kg^{-1} of each antibiotic. The recoveries were compared considering the area of the corresponding chromatographic peaks.

It can be easily observed that the micellar solutions contain larger particles, and then they must be ultrasonicated for 15 min to reduce their size to favor the filtration. The chromatographic peaks were sharper using the micellar solution, although recovery was similar with both solvents. The use of methanol was discarded, because the volume of organic solvent handled and wasted would be too high, and it can partially vaporize during the processing, thus providing variable and falsely enhanced recoveries.

Several stirring times, from 10 min to 3 h, were tested. The recovery strongly increased from 0 to 30 min, increased at a low rate to 60 min, and did not show significant variations beyond this value. The stirring time was therefore fixed at 60 min.

Sample preparation was easy-to-handle, as it only included a simple leaching and the direct injection of the supernatant. Time-consuming and cumbersome cleanup steps were not needed, and no reactions were involved. The reagents used are accessible, stable, innocuous, and biodegradable, and no toxic organic solvent was required. The loss of analyte, either by incomplete recuperation or a chemical change, and the risk of contamination of the sample, is therefore reduced, thus enhancing the reliability of the procedure. Several samples can be simultaneously processed by the same operator, which is an interesting practical feature.

3.4. Method validation

3.4.1. Selectivity

Free-fluoroquinolone samples of each studied meat were analyzed using the method developed. The front of the chromatogram covers from the dead time (nearly 1.0 min) to 2.5 min, and other small peaks were observed, but away from the window time ±2.0 min of the studied antibiotics. The chromatograms obtained from all of them were similar.

The same samples were fortified to 0.2 mg kg^{-1} of FLU, MARBO, DIF and SAR, and analyzed. The chromatogram obtained from the spiked pig meat sample can be seen in Fig. **1**(A). In all cases, peaks corresponding to the four antibiotics appeared at similar retention times (< 2%) and peak areas (< 4%) to those obtained by the analysis of a standard solution. The excitation and emission wavelength were taken, and the wavelengths of maximum emitted fluorescence were the same as those indicated in the section on detection conditions above. These results prove the absence of a matrix effect. No overlapping with meat compounds was observed.

The high selectivity of the method occurred because of the low retention of the proteins, fats, and other macromolecules, due to their strong interaction with the micelles, and the specificity of fluorescence, which reduces the number of potential interfering compounds.

3.4.2. Calibration range and linearity

Standard solutions containing increasing concentrations (up to 0.8 mg L^{-1}) of the studied fluoroquinolones were diluted to 1/10, which includes the dilution caused by the transfer of the analytes from the meat to the supernatant, and were analyzed in triplicate. The quantitative values therefore refer to concentrations in meat, not in the injected solution. The average peak area was related to the corresponding concentration by a first-grade equation by least-square linear regression.[39] The slope, *y*-intercept, and determination coefficients can be seen in Table **2**.

Table 2. Calibration curves and sensitivity of the method (concentrations in mg kg^{-1})

Antibiotic	Slope	y-intercept	r^2	LOD	LOQ
FLU	524 ± 3	−2 ± 5	0.9998	0.015	0.05
MARBO	172.9 ± 0.8	3 ± 4	0.9997	0.03	0.1
DIF	2448 ± 5	14 ± 9	0.9996	0.003	0.01
SAR	1055 ± 7	−12 ± 15	0.9994	0.015	0.05

The limits of detection (LOD) and quantification (LOQ) were calculated as 3.3 and 10 times the standard deviation of the blank divided by the sensitivity.**39** The calibration range was from LOQ to 0.8 mg kg^{-1}. The results can be seen in Table **2**. The chromatogram obtained from the analysis of a pig meat sample spiked with the studied antibiotics at their corresponding LOQ can be seen in Fig. **1**(B).

Satisfactory linearity was obtained, with a high goodness of fit of the regression (r^2 > 0.9994). For each fluoroquinolone, the calibration ranges cover the maximum residue limits in porcine and bovine muscle, mainly thanks to the high sensitivity of fluorescence detection.

3.4.3. Recovery and Precision

Intra- and interday recovery and precision (in this last case, also named repeatability and within-laboratory reproducibility) were determined. These parameters were studied for each kind of meat and at three concentrations.

For the intraday measurements, blank samples of porcine and bovine meat were fortified with each fluoroquinolone at 0.5×; 1× and 1.5× the corresponding MRL (the lowest concentration evaluated for MARBO was 0.1 mg kg^{-1}, as the 0.5 × MRL falls under LOQ). For each level, the analysis was performed six times (Six samples fortified at the same concentration were successively analyzed). The recovery was calculated as the average of the concentrations provided by the calibration curve minus the true value, divided by the true value, while the precision was the relative standard deviation of the six peak areas. To determine the interday values for each parameter, the same protocol was performed on five different days over a three-month period, by renewing the fortified samples. The recovery was the average of the five recovery values determined at each day, whereas the precision was the relative standard deviation (RSD) of the five average values of the peak areas obtained each day. The results are shown in Table **3** (for FLU and MARBO) and in Table **4** (for DIF and SAR).

Table 3. Recovery/precision measured in repeatability (R) and within-laboratory reproducibility (WRL) conditions (%/RSD, %) for FLU and MARBO

Meat	Fortified amount	FLU		MARBO[a]	
		R[b]	WLR[c]	R[b]	WLR[c]
Pork	0.5 × MRL	107.8/9.0	106.4/7.5	83.9/8.2	84.8/8.4
	MRL	105.8/5.5	104.4/6.3	90.8/7.3	89.7/7.1
	1.5 × MRL	101.8/4.1	102.4/3.0	97.7/4.2	96.8/4.7
Beef	0.5 × MRL	104.1/6.8	103.8/7.9	85.8/7.5	86.5/9.4
	MRL	102.4/5.1	101.2/4.6	90.5/6.8	91.8/7.7
	1.5 × MRL	97.2/3.5	98.9/2.7	96.1/4.1	97.5/5.5
Chicken	0.5 × MRL	105.2/5.8	104.2/6.1	84.6/8.5	85.2/9.3
	MRL	102.1/3.9	105.5/3.4	91.5/6.9	92.5/7.0
	1.5 × MRL	101.0/1.9	100.9/2.8	96.0/4.2	96.8/5.2
	0.5 × MRL	104.5/4.2	103.9/3.8	87.6/7.5	88.2/7.4
Turkey	MRL	98.0/4.2	98.1/4.1	93.2/5.1	93.0/6.4

Meat	Fortified amount	FLU		MARBO[a]	
		R[b]	WLR[c]	R[b]	WLR[c]
	1.5 × MRL	98.5/3.3	101.0/2.1	96.2/2.9	96.0/3.5
	0.5 × MRL	104.8/5.4	104.0/4.8	86.2/8.0	87.6/7.1
Duck	MRL	102.0/3.1	102.5/3.0	91.6/6.8	91.9/7.0
	1.5 × MRL	101.1/2.8	101.8/2.1	95.9/3.9	95.2/3.4
	0.5 × MRL	104.1/4.1	103.5/3.4	86.7/6.9	85.2/7.4
Sheep	MRL	97.2/3.9	98.3/2.5	90.2/8.1	91.6/7.8
	1.5 × MRL	100.9/2.5	101.0/1.9	94.6/4.5	95.2/4.9
	0.5 × MRL	104.8/5.3	104.0/4.2	87.2/7.9	89.0/8.4
Goat	MRL	103.8/4.1	103.5/2.7	93.1/5.8	93.9/6.7
	1.5 × MRL	102.0/3.4	102.2/1.9	96.4/5.1	95.8/4.7
	0.5 × MRL	106.9/8.7	107.5/7.8	85.5/8.5	86.2/7.4
Rabbit	MRL	105.0/3.9	104.5/4.2	91.6/7.8	91.0/8.1
	1.5 × MRL	103.9/2.7	103.0/3.8	94.8/5.5	93.8/5.7
Horse	0.5 × MRL	107.2/8.1	106.8/7.9	87.2/8.3	88.0/7.9
	MRL	104.9/6.8	104.5/5.5	92.2/7.1	92.0/7.3
	1.5 × MRL	104.0/3.4	103.5/4.2	95.1/6.1	95.6/5.4

- [a] 0.1 mg kg^{-1} instead of 0.5 × MRL.
- [b] n = 6.
- [c] n = 5.

Table 4. Recovery/precision measured in repeatability (R) and within-laboratory reproducibility (WRL) conditions (%/RSD, %) for DIF and SAR

Meat	Fortified amount	DIF		SAR	
		Ra	WLRb	Ra	WLRb
Pork	0.5 × MRL	105.8/7.2	104.5/6.5	93.3/5.8	93.0/4.5
	MRL	101.9/3.9	100.2/3.2	96.4/3.8	97.9/2.8
	1.5 × MRL	103.5/0.8	102.2/1.9	101.2/1.4	99.8/2.1
Beef	0.5 × MRL	105.0/5.7	103.8/6.6	92.8/5.8	92.0/6.8
	MRL	98.4/3.4	99.5/2.5	95.5/3.6	96.6/3.3
	1.5 × MRL	99.7/2.4	98.6/1.7	97.1/2.2	98.2/2.5
Chicken	0.5 × MRL	105.5/6.8	103.4/5.3	94.8/4.6	93.2/7.1
	MRL	102.0/3.8	101.5/2.8	96.3/2.9	96.0/3.8
	1.5 × MRL	100.9/2.4	99.2/1.5	98.3/3.4	98.0/3.0
	0.5 × MRL	104.5/4.0	104.2/4.4	95.9/5.4	95.2/6.0
Turkey	MRL	98.9/2.8	99.1/2.0	97.2/3.8	97.0/4.1
	1.5 × MRL	97.9/3.1	98.5/2.7	99.2/2.0	89.9/2.9
	0.5 × MRL	105.1/6.5	104.9/5.4	97.1/4.5	96.8/4.1
Duck	MRL	100.9/1.8	101.5/2.4	100.8/1.9	100.0/2.8
	1.5 × MRL	101.9/2.5	102.9/3.2	101.2/2.9	101.9/2.4
	0.5 × MRL	103.1/5.9	103.9/4.5	96.1/5.9	96.0/6.8
Sheep	MRL	103.5/4.2	104.0/3.8	97.3/3.5	97.4/4.5
	1.5 × MRL	102.9/3.4	102.5/3.0	98.3/2.5	98.2/3.5
	0.5 × MRL	104.0/3.9	103.8/4.9	94.2/4.6	94.5/4.0
Goat	MRL	99.1/4.1	98.9/3.8	97.0/3.9	96.5/4.0

Meat	Fortified amount	DIF		SAR	
		Ra	WLRb	Ra	WLRb
Rabbit	1.5 × MRL	96.5/2.9	97.1/2.4	98.8/2.4	98.0/3.1
	0.5 × MRL	103.9/4.2	104.1/5.0	95.2/5.9	96.1/6.0
	MRL	100.8/1.9	99.7/3.9	98.1/3.4	97.1/4.0
Horse	1.5 × MRL	102.1/3.5	100.9/2.9	100.5/2.8	99.5/2.1
	0.5 × MRL	105.1/3.5	104.5/2.9	95.0/3.5	95.8/4.2
	MRL	97.5/5.4	98.5/3.2	97.1/4.6	96.5/3.8
	1.5 × MRL	100.4/3.9	100.5/2.8	99.6/3.3	99.0/2.5

- [a] n = 6.
- [b] n = 5.

The values of the extraction throughput (83.9 to +107.8%) and variability (RSD < 9.4%) provided by the procedure were adequate for the levels, analytes, and matrices studied, and fulfill the requirements of the EU guidelines. This demonstrated a high and stable extraction rate of the leaching step, and the advantages of the direct injection of the supernatant.

3.4.4. Decision limit and detection capability

Parameters have been proposed by the EU Commission Decision 2002/657/EC, to address the difficulty in distinguishing between compliant and non-compliant samples because of the uncertainty of the quantitative measurements.**35** In brief, the decision limit is a regulatory concentration, higher than the MRL, and refers to the concentration found in a sample. If a sample containing between the MRL and the CCα is rejected, the probability of having made a wrong error is 5–50%. If a sample is rejected at concentrations over the CCα, this probabilty is less than 5%. Therefore, the rejection limit is usually fixed at the CCα instead of at the MRL. However, this increases the probability of accepting a contaminated sample. The CCα is the minimal concentration in a sample that the method is able to classify as non-compliant with a certainty of >95%.

CCα and CCβ were measured separately for each kind of meat and fluoroquinolone. The decision limit was the MRL plus 1.64 times the standard deviation obtained by the analysis of a muscle piece spiked at the MRL (n = 20). The detection capability was the CCα plus 1.64 times the standard deviation obtained by the analysis of a sample fortified at the CCα.**35** The results can be seen in Table **5**.

Table 5. Decision limit/detection capacity for each quinolone in the studied meats (concentrations in mg kg^{-1})

Meat	FLU	MARBO	DIF	SAR
Pork	0.22/0.24	0.17/0.18	0.43/0.45	0.42/0.44
Beef	0.22/0.23	0.17/0.18	0.42/0.44	0.42/0.44
Chicken	0.43/0.45	0.17/0.18	0.32/0.33	0.31/0.33
Turkey	0.43/0.46	0.16/0.18	0.31/0.32	0.32/0.34
Duck	0.42/0.44	0.17/0.18	0.31/0.32	0.32/0.33
Sheep	0.21/0.22	0.17/0.19	0.43/0.46	0.32/0.34
Goat	0.21/0.22	0.16/0.18	0.43/0.45	0.42/0.45
Rabbit	0.21/0.23	0.17/0.19	0.31/0.33	0.32/0.34
Horse	0.22/0.25	0.17/0.19	0.33/0.35	0.32/0.33

For the meats and antimicrobials studied, the decision limits (< 13% over MRL) and the detection capabilities (< 27% over MRL) were close to the MRL. The probability of obtaining a result leading to the acceptance of a potentially non-compliant sample is therefore relatively low. The concentration range within which the method is unable to classify a contaminated meat sample correctly is quite narrow. Random errors would therefore only lead to an incorrect decision in a few situations.

3.5. Ruggedness

The changes in retention and sensitivity caused by small variations in experimental conditions were examined in the range that can occur in the normal laboratory practice, using a Youden approach.**35** Ruggedness was studied separately for each fluoroquinolone and instrumental response (retention time and peak area), using a standard solution of 0.02 mg L^{-1} of FLU, MARBO, DIF and SAR.

The factors that were considered, and their intervals, were: SDS, 0.045–0.055 mol L^{-1} (A); 1-butanol proportion, 7.8–8.2% (B); pH, 2.8–3.2 (C); TEA, 0.45–0.55% (D); flow-rate, 0.98–1.02 mL min^{-1} (E); excitation wavelength; optimal value ±5 nm (F) and emission wavelength: optimal value ±5 nm (G). The standard deviation of the method was determined as the within-laboratory reproducibility, using the optimal instrumental conditions, as indicated in the section on recovery and precision above, but using the standard solution.

For both peak area and retention time, the differences obtained for each factor were similar. These differences, and the standard deviations of the differences, were slightly above the standard deviations obtained under optimal conditions. The method is therefore robust enough to be unaffected by modifications in the instrumental conditions in the ranges considered, mainly because of the reproducibility of MLC.

3.6. Stability

The degradation of the fluoroquinolones in the standard solutions, bench-top in the extract obtained in the leaching step, and in the muscle tissues studied, was investigated under their common storage conditions (as indicated in the materials and methods section above), to corroborate the adequacy of the selected storage time.

A standard solution of MRL/10 mg L^{-1} of each fluoroquinolone was stored in a fridge and analyzed each day. The peak areas remained nearly constant for 2 months, and no other peaks appeared in the chromatogram.

To assess bench-top stability, a processed meat sample (fortified at the MRL for each veterinary drug) was placed in the autosampler at room temperature and repeatedly injected for one day. The peak area of each analyte remained roughly constant, and no other peaks appeared. A large number of samples can be processed and further analyzed on the same day. Samples of each meat were fortified at the MRL for the antimicrobials that were studied and kept in a freezer. On the day 0 and each week, a sample was analyzed. The concentration of the antibiotics does not decline significantly after 2 months, and no degradation products were observed.

The fluoroquinolones remained stable both in standard micellar solution at +4 °C and in meat at −20 °C, in the darkness, for at least 2 months. The standard solutions were discarded after 2 months, and the meat samples could be stored during this period until analysis.

3.7. Analysis of samples

The method developed here was used to determine the quantity of FLU, MARBO, DIF and SAR in samples from pig, beef, chicken, turkey, duck, sheep, goat, rabbit, and horse meat (five samples of each) purchased from a local supermarket, to evaluate the applicability of the technique for routine analysis. Fluoroquinolone residues were not detected in any sample, meaning that the meat could be sold without risk to the general population.

A single operator was able to analyze the whole set of samples in one day. Indeed, the meat pieces were simultaneously processed in < 2 h, and the total chromatographic sequence takes nearly 14.5 h. The participation of the operator was restricted to the preparation of the solutions, mixtures, filtration, control of the instrumentation and apparatus, and the supervision of the whole process, as the other tasks (stirring, ultrasonication, injection, and chromatographic separation) were fully automated.

The procedure offers a high sample throughput, using basic laboratory instrumentation and material, and requires a small amount of chemicals. It does not pose a risk to the health of the operator or to the environment because of the limited toxicity of the solutions that are

prepared. It also allows the costs of waste segregation and treatment to be reduced and the analyses were performed at a reasonable price. These practical features make this method useful for routine analysis.

4. CONCLUSIONS

Residues of FLU, MARBO, DIF and SAR in the meats that are most commonly consumed can be reliably determined by micellar liquid chromatography – fluorescence detection. The procedure designed here allowed a high sample throughput with easy-to-handle pretreatment and minimal participation from the operator, despite the complexity of the matrix. It was eco-friendly, safe for the laboratory staff, relatively inexpensive, and useful for routine analysis. These can be considered the main advantages of the procedure. The analytical quality (selectivity, calibration range, linearity, recovery, precision, decision limit, detection capability, robustness and stability) was thoroughly evaluated following the guidelines of EU Commission Decision 2002/657/EC, with satisfactory results. It was observed that the method provides consistent quantitative values around the maximum residue limits (0.15–0.4 mg kg^{-1}). This was mainly accomplished as a result of the specific properties of micellar solutions. This analytical method is a suitable alternative allowing quality-control laboratories to evaluate the compliance of commercial edible animal muscle samples with EU regulation 37/2010 with regard to the occurrence of the antimicrobials FLU, MARBO, DIF, and SAR.

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Chapter 8. A rapid and Reliable assay to determine Flumequine, Marbofloxacin, Difloxacin, and Sarafloxacin in commonly consumed Meat by Micellar Liquid Chromatography

Chapter 9

Micellar Liquid Chromatography Determination of Rivaroxaban in Plasma and Urine. Validation and Theoretical Aspects

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ABSTRACT

A micellar chromatographic method to determine rivaroxaban in plasma, urine and pharmaceutical formulations has been developed. The samples were dissolved in the mobile phase (SDS 0.05 M – 1-propanol 12.5 %, phosphate-buffered at pH 7) and 20 µL directly injected, avoiding the extraction and purification steps. Using a C18 column and running under isocratic mode at 1 mL/min, analyte was eluted without interference from the matrix in less than 6.0 min. The detection absorbance wavelength was set to 250 nm. The procedure was validated by Food and Drug Administration guidelines in terms of: system suitability, calibration range (0.005 – 1 mg/L; limit in injected solution), linearity, sensitivity, robustness, carry-over effect, specificity, accuracy (-11.1 to 4.2%), precision (<19.9%), stability and analysis of incurred samples. The method was found reliable, practical, easy-to-conduct, rapid, relatively eco-friendly, safe, inexpensive, widely available and with a high sample throughput. The method was applied to the analysis of incurred samples, including incurred sample reanalysis and quality control samples to verify that the instrumentation works correctly. In addition, the constants of the different partition equilibria occurring in the column were elucidated, in order to have a better comprehension of the theoretical aspects of the retention mechanism. A moderately strong association between rivaroxaban and the stationary phase and the micelles was found, weakened by the alcohol.

Chapter 9. Micellar Liquid Chromatography Determination of Rivaroxaban in Plasma and Urine. Validation and Theoretical Aspects

1. Introduction

Rivaroxaban (RIV, Fig. 1) is an oxazolidinone belonging to the group of the direct or novel oral anticoagulants (DOAC or NOAC, respectively) commonly used to prevent blood clots. Its activity is attributed to a potent, irreversible and selective inhibition of free and clot bound Factor Xa [[1], [2], [3], [4], [5], [6], [7], [8], [9], [10]], thus blocking thrombin generation [[11], [12], [13], [14], [15]]. RIV was the first DOAC and was approved by the FDA in 2011. It is currently one of the most prescribed anticoagulants [11]. RIV is indicated to treat and/or prevent thromboembolic disorders, like venous thromboembolism (VTE), deep vein thrombosis (DVT), pulmonary embolism, etc. It is used for the prophylaxis of VTE after total orthopedic hip or knee replacement therapy and stroke or systemic embolism in patients with atrial fibrillation [[1], [2], [3], [4], [5], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15]]. It is not recommended for prescription in patients under 18 years old, pregnancy and those with severe renal or liver failure [2,10,11]. RIV may cause excessive bleeding at overdose [11]. Its therapeutic and adverse effects are closely related to the plasmatic concentration [2,4,10,12,15].

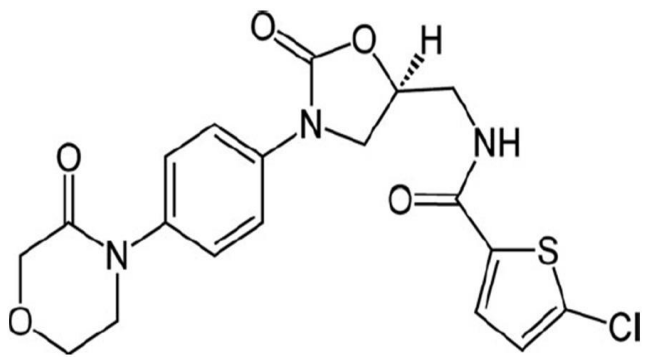

Fig. 1. Structure of Rivaroxaban or (S)-5-chloro-N-{[2-oxo-3-[4-(3-oxomorpholin-4-yl) phenyl] oxazolidin-5-yl] methyl} thiophene-2-carboxamide.

Chapter 9. Micellar Liquid Chromatography Determination of Rivaroxaban in Plasma and Urine. Validation and Theoretical Aspects

RIV displays a more favorable benefits-to-risk profile than conventional anticoagulants. It is administered orally and rapidly absorbed by the gastrointestinal system. It has high efficacy and tolerability, a rapid onset of action, predictable pharmacokinetics (Table SM1) and pharmacodynamics, large therapeutic window (nearly 6 to 491 μg/L [2,12]), a low risk of interaction with food, absence of accumulation in patients without co-morbidities, low likelihood of adverse effects and rapid elimination. Clinical effects are dose-dependent and no patient-adapted dosage is usually required. In fact, RIV can be prescribed at a fixed daily dose for the most patients [[1], [2], [3], [4], [5], [6], [7], [8], [9], [10], [11], [12],16,17].

Therapeutic Drug Monitoring (TDM) of RIV in a given individual may be relevant in certain circumstances in order to guide the decision making about the treatment ensuring the optimum efficacy. Plasma levels might be influenced by several factors altering the drug metabolism exposing the patients to a significant risk of under- or over anticoagulation. The direct quantification of RIV in plasma is preferred for drug testing [[1], [2], [3], [4], [5],[7], [8], [9],[13], [14], [15], [16], [17]], although in urine is also feasible [3,9,14,15] and has the advantage of being non-invasive. The level of RIV in plasma reflects the presence of the drug at a specific point in time, since in urine infers the exposure for an extended period [9]. Therefore, clinical laboratories require rapid and sensitive analytical assays able to identify and quantify RIV in both biological fluids to implement an effective TDM approach.

Micellar liquid chromatography (MLC) using a C18 column, and anionic sodium dodecyl sulfate as surfactant, and short-chained monoalcohol as organic modifier has been proven a reliable tool for the determination of drugs in plasma and urine [18], avoiding the major drawbacks of hydro-organic HPLC: use of sophisticated/expensive detectors and devices, need of large, cumbersome and variable-recovery extraction or purification steps, use of a large quantity of hazardous chemicals, extensive manipulation, etc. Indeed, micellar solutions are able to solubilize small drugs [18]. Besides, micelles and monomers strongly interact with proteins, lipids, glycosides and other macromolecules, usually present in the biological fluids, provoking its denaturing, solubilization, releasing bounded drugs and preventing further interaction with the analytes [19]. In the column, these biopolymers remain

solubilized in the micellar mobile phase without risk of precipitation, and are barely retained, being harmless eluted at the front of the chromatogram. Therefore, after a sample dilution, the biological fluids, can be directly injected in the chromatographic system [18]. Hybrid micellar solutions contain innocuous and biodegradable salts, and only up to 12.5% of organic solvent [20]. The retention mechanism in MLC depends on two partition equilibria for moderately hydrophobic solutes (bulk hybrid micellar mobile phase – micellar pseudo phase; bulk hybrid micellar mobile phase - monomer-modified stationary phase). Besides, the chromatographic behavior of a solute can easily be predicted from the composition of the mobile phase, using equations constructed from the three-phase model [21].

The aim of the work is developing a reliable, rapid, practical, easy-to-handle and inexpensive procedure to determine RIV in plasma and urine by MLC. The method will be fully validated to evaluate its analytical performance and used in incurred plasma samples and commercial pharmaceutical formulations to ensure its suitability for routine analysis. Another goal was to investigate the interaction of RIV with the different chemical environments occurring in the column, for a better comprehension about the theoretical aspects of the retention mechanism and the role of the main components of the mobile phase on the retention factor, by calculating the physicochemical constants of the corresponding partition equilibria.

2. Experimental

2.1 Standard and chemicals

Powdered standard of Rivaroxaban (purity>99.4%) was purchased from InterQuim (Barcelona, Spain). Sodium dodecyl sulfate (>99%), dimethyl sulfoxide (DMSO, >99.6%) and sodium dihydrogen phosphate dihydrate (>99%) were bought from Scharlab (Barcelona, Spain). The solvent 1-propanol (HPLC grade) came from Merck (Darmstadt, Germany). Hydrochloric acid 37%) was from J.T. Baker (Phillipsburg, NJ, USA). Sodium hydroxide (>98%) was purchased from Riedel-deHaën (Hannover, Germany). Ultrapure water was in-lab

produced using an ultrapure water generator device Simplicity UV (Millipore S.A.S., Molsheim, France), from deionized water, provided as tap water by the university. All aqueous solutions were prepared using this water.

2.2 Preparation of solutions and mobile phases

To prepare the micellar solutions, the adequate amount of SDS and NaH2PO4·H2O (according to the desired concentration) was solved in ultrapure water and the pH was fixed to the selected value by adding drops of HCl or NaOH solutions. Then, the appropriate volume of the organic solvent (depending on the chosen proportion) was introduced, and the volumetric flask was filled up with ultrapure water. Finally, this solution was ultrasonicated for 5 min to attain a complete solubilization and filtered through a 0.45 μm-Nylon membrane filters (Micron Separations, Westboro, MA, USA), with the aid of a vacuum pump, to remove solid particles. These solutions were kept in amber bottles (to avoid bacterial growth) at room temperature.

A stock solution of rivaroxaban of 100 mg/L was prepared by weighing the proper weight and dissolving it in DMSO. The working solutions were made by dilution of the stock solution in mobile phase. These solutions were stored in a fridge at 4 °C.

2.3 Chromatographic conditions

The chromatographic system was a HP1100 (Agilent Technologies, Palo Alto, CA, USA) equipped with an isocratic pump, a degasser, an autosampler and an UV–Visible absorbance diode array detector (DAD), connected to a PC. The software Agilent Chemstation (Rev. B.04.03(16) 2010) was used to control the instrument, visualize and register the signal, and the measurement of the chromatographic parameters (dead time, t_0, retention time, t_R and

peak area, PA). The experimental dead time was 1.0 min. Retention factor (k), efficiency (number of theoretical plates, N) and tailing factor (T) were calculated as in [22].

The stationary phase was in a C18 Kromasil C18 column (AkzoNobel, Amsterdam, The Netherlands) with the following parameters: 150 × 4.6 mm; 5 μm particle size; 10 nm pore size. Injection volume and absorbance wavelength were 20 μL and 250 nm, respectively. The mobile phase was an aqueous solution of 0.05 M SDS - 12.5% 1-propanol buffered at pH 7 with a phosphate salt. It runs under isocratic mode at 1 mL/min. All the analyzed solutions were filtered through a 0.45-μm Nylon membrane filter (Micron Separations) with the aid of a 3 mL syringe, and then introduced in the chromatographic vial. The special care of the chromatographic instrumentation required when working with micellar mobile phases can be seen in [23]. Under these conditions, the column has a lifespan of at least 1000 injections [23].

2.4 Sample collection and processing

The study was approved by two local Ethics Committees, Hospital and the University Ethics Committee for Analysis of Research Projects. Written informed consent was obtained from all participants and all research was performed in accordance with the 2013 Helsinki Declaration principles. Plasma and urine samples from patients and healthy volunteers were provided by a local Hospital, after consent of doctors and patients. The "healthy volunteers" do not take any medication and their blood is free of drugs. They were six: three men and three women, and, for each gender, one in his/her twenties, another in his/her thirties and the last one in his/her forties. For confidentiality reasons, the samples from the patients were sent unlabeled. No personal and clinical information about the patients or the healthy volunteers (except that indicated above) was provided from the Hospital. The laboratory undertakes to do not transmit any information to other institutions (except that here indicated).

The blood was collected using a BD SST tube (SBD Vacutainer Systems, Plymouth, UK), and centrifuged at +4 °C at 3000 rpm for 10 min (3000 x g), to get the non-cellular

fraction, which was immediately frozen and stored at −20 °C. The reference validated and accredited standard method used by the Hospital includes this kind of tubes, then no loss of RIV at this step is expected. Urine samples were collected in glass tubes and kept at the same temperature. Afterwards, these samples were sent to the laboratory, where they were kept under the same conditions. A matrix-matched blank was made by mixing equivalent volumes of the plasma or urine, respectively, of the six healthy volunteers.

Plasma and urine, either blank or patient samples, were treated in the same way. The samples were thawed until the onset of melting, the same day of the analysis. Afterwards, an aliquot was 1/5-dissolved in mobile phase, filtered and directly injected [21]. The fortification was performed by adding the appropriate volume of a standard solution of Rivaroxaban, before the dilution. All the validation studies "in matrix", for these biological fluids, were performed using spiked blank samples.

3. Results and discussion

3.1 Optimization of the chromatographic conditions

The main chromatographic conditions to optimize in MLC method [18,21] are: surfactant, modifier concentrations and pH. Using C18 and SDS as column and surfactant, respectively, the stationary phase is saturated with SDS monomers adsorbed on its surface, with the sulfate group oriented to the mobile phase, at concentrations >10 mM, slightly over the critical micellar concentration (CMC) of 8 mM. Working above this concentration, SDS monomers in the mobile phase are organized as micelles, which number per volume unit is proportional to the initial concentration of SDS. The amount of free monomers remains nearly constant and equal to the CMC. Therefore, we can work a relatively low concentration of surfactant (recommended working concentrations 0.05–0.15 M) in which the mobile phase has a low viscosity and low background signal.

According to the moderate hydrophobicity (log Po/w = 1.7) of RIV [11], a pure micellar mobile phase would provide too long capacity factors. Therefore, 1-propanol was added to reduce the retention time and, additionally, enhance the efficiency. The studied interval of concentrations for 1-propanol was that recommended in MLC, 2.5 to 12.5% [18].

The effect of the pH was studied at 3, 5 and 7, all of them inside the working range of the C18 column (2.5–7.5), by the analysis of a working solution of 0.5 mg/L of RIV. At pH 3, no peak was observed, and at pH 7 the best peak appeared with a nearly Gaussian shape at reasonable retention time. Therefore, these values were taken as optimal. According to the acid/basic activity of RIV (pKa 1.0 and 13.4), the molecule is neutral under the working conditions [11].

The qualitative influence of the SDS and 1-propanol on the elution parameters was examined. A working solution of RIV (0.2 mg/L) was analyzed using nine mobile phases selected following an experimental design based on a face-centered composite design plus the central point. We take the combination of the minimum (−1) and maximum (+1) levels for both factors ($2^2 = 4$ assays), the combination of the average value (0) of one of them and the minimal and maximal of the other one ($2 \times 2 = 4$ assays), and the combination of the average values (1 assay). The −1/0/+1 values were the minimal, average and maximal concentration of each component recommended in MLC, respectively [24]. The composition of the assayed mobile phases and the results are shown in Table 1. From these results, we deduce that RIV has a binding behavior face to the micelles in the mobile phase, as the retention factor and the efficiency decrease at increasing values of SDS-micelles. By the way, the elution strength of the mobile phase and the efficiency grow up at higher values of 1-propanol, as usual in reverse phase MLC. The optimal mobile phase composition was set to 0.05 M SDS – 12.5% 1-propanol – pH 7. The analyte elutes at a short time, but far from the expected front of the chromatogram for biological fluids [18], and the efficiency is maximal. Therefore, the chromatographic analysis of a diluted sample can be carried out in only 6.0 min, using a low amount of organic solvent and a simple isocratic program.

Detection conditions were optimized by Agilent ChemStation Rev. B.04.03(16) 2010 software. Absorbance spectrum of rivaroxaban (200–500 nm) was on-line measured at the maximum height during the chromatographic run, in order to obtain its spectrophotometric properties in the same micellar environment as used for the analysis. The maximum of absorbance was found at 250 nm, and then this value was selected to maximize the signal-to-noise ratio. For peak purity studies, the absorbance spectra were taken at different points of the peak (at half-height and 0.1-height, both fronting and tailing), and they show the same shape as above.

Table 1. Chromatographic parameters of rivaroxaban depending on the composition of the mobile phase (Experimental dead time was 1.0 min).

[SDS] (M)	1-propanol (%)	t_R (min)	Efficiency (number of theoretical plates)
0.05	2.5	10.03	1362
0.05	7.5	6.71	1418
0.05	12.5	4.64	2029
0.10	2.5	5.92	1104
0.10	7.5	4.34	1275
0.10	12.5	3.28	1912
0.15	2.5	5.08	913
0.15	7.5	3.53	1189
0.15	12.5	2.78	1763

3.2 Determination of the partition physico-chemical constants

According to the three-phase model, in MLC using pure micellar solutions, the solutes are partitioned between three phases: surfactant-modified stationary phase, bulk aqueous solvent, and micellar pseudo-phase. The equilibria are described by two partition constants: K_{AS} (between the bulk mobile phase and stationary phase; thus increasing the retention) and K_{AM} (between the mobile phase and the micelles, thus decreasing the retention). Therefore, K_{AS} and K_{AM} quantify the degree of interaction of the solute with the modified stationary phase and the micelles, respectively. In hybrid micellar mobile phases, we assume that this model is also applicable, but the organic solvent displaces the equilibria. This is only applicable at low concentration of organic solvents (<22 % for 1-propanol), as higher values cause the disaggregation of the micelles. We introduce two constants, K_{AD} and K_{MD}, which evaluates the shift in the equilibrium to the solvent and the micelle, respectively, with respect to the pure micellar solution (thus decreasing the retention in both cases). According to this three-phase theory, in MLC the retention factor of a moderately hydrophobic solute can be modeled from the concentration of surfactant and alcohol by the following mechanistic equation [20,22]:

$$k = \frac{\Phi K_{AS} \frac{1}{1 + K_{AD}\varphi}}{1 + K_{AM} \frac{1 + K_{MD}\varphi}{1 + K_{AD\varphi}} [SDS]} \qquad \text{Eq. 1}$$

where Φ is the phase ratio (volume of stationary phase divided by the volume of mobile phase in the column), φ is the proportion of 1-propanol in %, v/v and [SDS] in (M). If φ = 0, the constants K_{AD} and K_{MS} are removed from the equation, and if [SDS], the retention is ΦK_{AS} (as in hydro-organic HPLC). The equation may be reorganized to:

$$\frac{1}{k} = \frac{1}{\Phi K_{AS}} + \frac{K_{AD}}{\Phi K_{As}}\varphi + \frac{K_{AM}}{\Phi K_{AS}}[SDS] + \frac{K_{AM}K_{MD}}{\Phi K_{AS}}[SDS]\varphi \quad \text{Eq. 2}$$

Eq. 2 was fitted using the experimental values of *k* obtained from the experimental design (Table 2), by curve-fitting non-linear least-square regression (5 freedom degrees) [28], in order to calculate values of the physico-chemical constants. An adequate goodness-of-fit was reached (R^2>0.991). The values of the constants were: ΦK_{AS} = 45.3; K_{AM} = 44.9; K_{AD} = 3,9 and K_{MD} = 0.15.

A moderate/strong interaction was found between both the stationary phase and the micelles with RIV, probably by hydrophobic interactions. The presence of 1-propanol significantly modifies the two equilibria towards the bulk mobile phase, by the resulting increase of the hydrophobic interactions in it. However, the occurrence of 1-propanol barely affects the interaction of the analyte with the micelles.

3.3 Method validation

The method was fully validated under guidelines of the FDA guidance for Industry, Bioanalytical Method Validation 2018 (developed for the analysis of biological fluids) [26] and several guides about validation of chromatographic methods [18,27]. The general parameters were determined in plasma samples: system suitability, linearity, calibration range, sensitivity, robustness (as the results are similar for both biological fluids [28]), while specificity, carry-over effect, accuracy and precision were separately determined for plasma and urine. Stability was determined an in-stock solution, diluted plasma and urine, and plasma and urine.

3.3.1 Specificity

The ability of the procedure to distinguish the analyte from endogenous compounds of the biological fluids was evaluated. The following analyses were performed: plasma (Fig. 2A and Fig. 2C, respectively) and urine (Fig. 2B and D) were analyzed before and after fortification at 1 mg/L. Additionally, the absorbance spectrum was measured at five points as in 3.1.

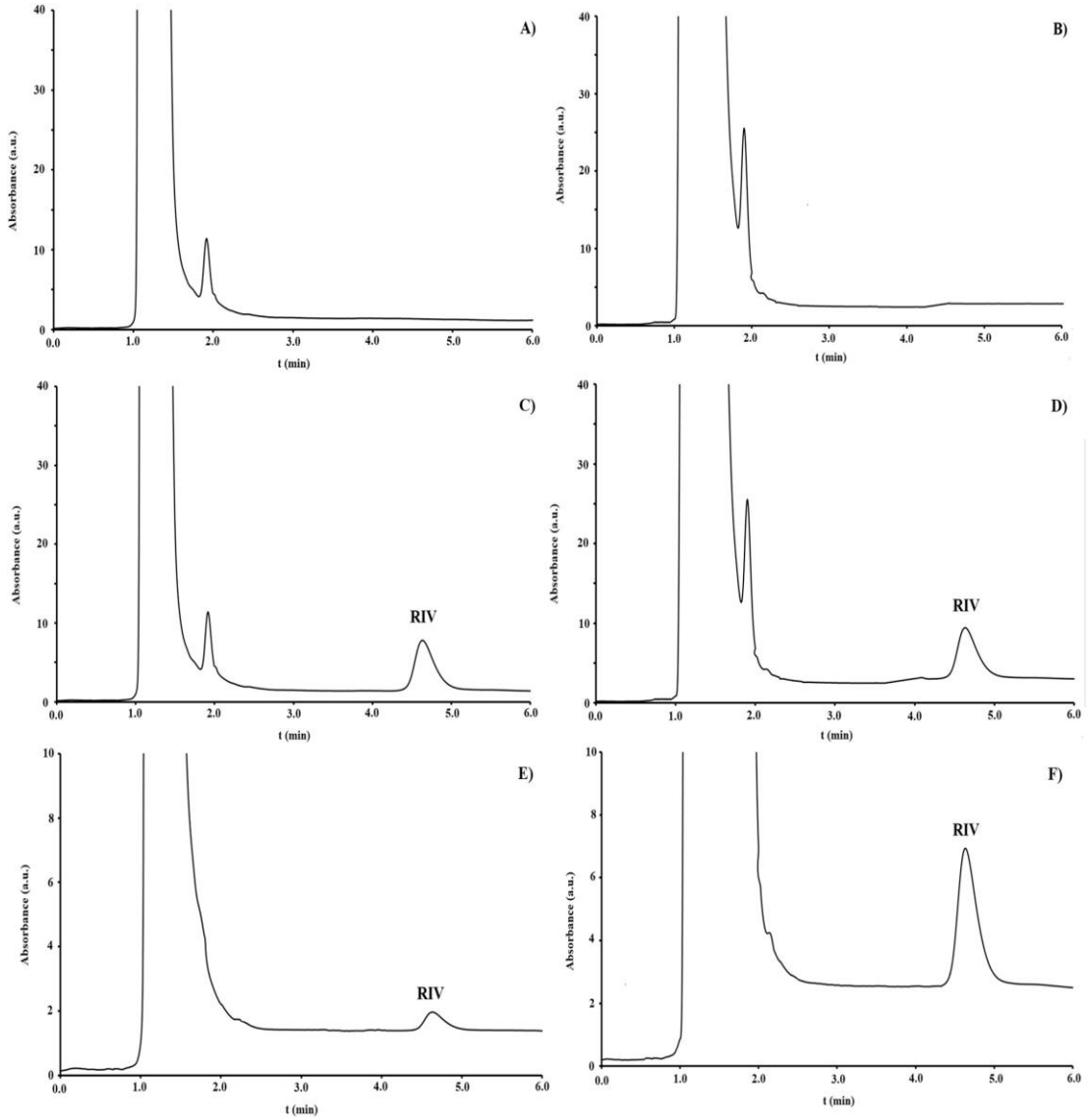

Fig. 2. Chromatograms obtained by the analysis of: plasma A) blank, C) fortified at 1 mg/L with RIV and E) from patient 4; and urine B) blank, D) spiked at 1 mg/L of RIV and F) from patient 4.

In blank samples, a broadband (<3.5 min) was observed at the beginning of the chromatogram, corresponding to proteins and other macromolecules, strongly associated to the micelles, thus far enough from the analyte [18]. The baseline was quite stable, and no peaks were visualized close or at the window time of the analyte. In the fortified samples, no peaks were observed near or partially overlapping the analyte.

The following experiments were performed to assess the peak purity:

- For plasma and urine, the spiked samples were compared to those non-fortified. The chromatograms show similar shape, except the peak corresponding to RIV.

- The chromatograms from processed samples were overlaid to that obtained in 3.1, and the peaks assigned to the analyte were alike.

- The absorbance spectra were compared and were found rather similar, regardless of the time taken, and were comparable to that obtained in 3.1.

These results point to the absence of compounds co-eluting with RIV, in the different matrices. Therefore, the method provides reliable qualitative and quantitative signals about the analyte.

3.3.2 System suitability testing (SST)

The main chromatographic parameters and their variability were determined, in order to dispose of reference values to further check the normal running of the instrumentation and evaluate the reproducibility of the chromatographic response. A blank plasma sample fortified at 2.5 mg/L of drug was injected by n = 6. The evaluated parameters and acceptance criteria were: tailing factor, 1.41, <0.8–1.6; retention factor, 3.64, >2.0; number of theoretical plates, 2325 > 2000; RSD of the retention time 1.3%, <1.5% and RSD of the peak area 1.4%, <1.5%.

According to the results, the chromatographic response is enough consistent and useful for a reliable qualitative/quantitative analysis.

3.3.3 Calibration range & Linearity

Several blank plasma spiked samples containing increasing concentrations of RIV were prepared in the range 0.025–5 mg/L, and analyzed by triplicate. The average peak area (PA) values (response variable) and the corresponding concentration (independent variable) were processed by least-square linear regression [29]. The slope was 119.8 ± 0.6 and the y-intercept was 0.7 ± 0.6. The reliability of the model was verified by the following tests:

- The residuals were homoscedastic, as the variances of the observed variable at the different concentration levels (calculated from the PA replicates) were found equal ($p = 4.1\% < 5\%$).

- No trend was detected for the residuals, by visual examination of a residual plot vs. concentration.

- No outliers were found in the regression model: the relative residuals were $< 1.66\%$ (acceptance criteria <3) and the Cook's squared Distances were < 0.5 (acceptance criteria <1) for all points.

- The y-intercept confidence (0.7 ± 0.7) interval includes zero, then no systematic error was noticed.

- A significant correlation was found by a t-test on r ($p = 0.000\%$, acceptance criteria <5%) and comparing the model and residual variance by a F test ($p = 0.000\%$, acceptance criteria <5%).

- The determination coefficient ($r^2 = 0.9997$) and the relative residual standard deviation (<1.3%) meet the acceptance criteria (> 0.990 and 5%, respectively) and then an adequate

linearity was found between the two variables in the studied interval. The lower limit of quantification (LLOQ) was the lowest concentration fitting the acceptance criteria for accuracy and precision, 0.025 mg/L, and the upper limit of detection (ULOQ) was 5 mg/L.

3.3.4 Sensitivity

The limit of detection is the smallest concentration of RIV injected that provides a peak clearly differentiable from the baseline noise, whereas the limit of quantification was the minimal concentration of analyte that can be reliably determined. These parameters were calculated as 3.3 and 10 the standard deviation of the y-intercept; 0.008 and 0.025 mg/L. The calibration range covers the expected range of concentrations in patient's incurred sample (Table SM1), except for the lower range, between 8 and 25 μg/L [3,8,13]. However, the no-detection of RIV points to the absence of therapeutic concentrations of the drug (under 6–8 g/L).

3.3.5 Carry over effect

The possible increase of the signal due to the presence of analyte remaining from previous injections was investigated. For each biological fluid, two blank samples, fortified at the ULOQ (5 mg/L) and unfortified, were consecutively analyzed. No peak was observed at the window time of RIV in the chromatogram obtained from the blank. Therefore, the occurrence of cross-contamination was considered negligible.

3.3.6 Accuracy & Precision

These parameters were determined in matrix at four levels: LLOQ, 0.1; 0.25 and 2 mg/L. Six samples were successively prepared and analyzed in the same run. The accuracy

was the closeness of agreement (in %) between the average value of the found concentrations and the true value, while the within-run precision (repeatability) was the dispersion (RSD) of the obtained concentrations. The effect of time in the variability was studied. The same experimental protocol was conducted five different days (using renewed solutions) over a three-month period. The between-run precision (intermediate precision) was the relative standard deviation of the five average values of found concentration. The results are shown in Table 2.

Table 2. Accuracy and precision studies (%).

an = 6; bn = 5.

Matrix	Level	Accuracy[a]	Within-run Precision[a] (repeatability)	Between-run (intermediate) Precision, %
Plasma	0.025 mg/L	−11.1	19.9	18.5
	0.1 mg/L	+5.6	7.4	6.9
	0.25 mg/L	+3.8	5.5	6.1
	2 mg/L	+0.0	1.2	1.4
Urine	0.025 mg/L	−9.5	17.4	16.2
	0.1 mg/L	+5.0	9.1	8.4
	0.25 mg/L	+4.2	7.0	5.8
	2 mg/L	+0.2	1.5	2.3

All the values were under the acceptance criteria (for LLOQ <20% and for higher levels, <15% [26]), the method provides adequate quantitative values for bioanalysis. These

performances were reached by the use of an easy-to-handle dilution as sample preparation, where the sample is quantitatively introduced in the column with the absence of extraction/purification steps, decreasing the subsequent sources of variance and the risk of analyte loss.

3.3.7 Robustness

The variation of the main chromatographic responses (retention factor, peak area and efficiency) was examined at small, but deliberate, changes in the main operational parameters. These were changed from their optimal value, and the tested oscillations were that we considered that may occur during the laboratory work and chromatographic run, in a usual situation. The studied factors and the studied interval were: A) detection wavelength ± 5 nm; B) SDS in mobile phase ±0.05 M; C) 1-propanol in mobile phase ±0.2%; D) pH in mobile phase ±0.2; E) flow-rate ± 0.05 mL/min and F) injection volume ± 2 μL.

A blank plasma sample spiked with 2.5 mg/L was analyzed by testing eight different set of experimental conditions, which value was fixed by an experimental design following a Youden approach [30]. Differences >8.0% were judged significant, meaning that they may be especially controlled during the analysis. The retention factor was significantly affected by the concentration of SDS in mobile phase and the flow-rate, the peak area by the concentration of SDS and the injection volume, and the efficiency by the flow and the injection volume. Therefore, these factors operational parameters must be especially controlled during analysis to achieve adequate analytical results.

3.3.8 Stability

The degradation of RIV was studied in the below mentioned chemical environments and external conditions. In each case, the tested conditions were those likely to be encountered

during the actual storage, handling and analysis, either in a Hospital or a clinical laboratory. The stability was examined by the monitoring of the concentration of RIV/nominal value (0.2 mg/L in the injected solution, in all cases) and the emergence of peaks from the decomposition products. The degradation was considered significant if >5% for standard solutions and 15% for processed and unprocessed biological fluids.

The experiments were conducted as follows. For all the cases, no new peaks were detected and the concentration of RIV remains quite invariant, and then the drug was found stable during the studied periods.

a) Stock solutions: A standard solution of 0.2 mg/L was prepared and stored in amber vials in a fridge at +4 °C. Each day, for two months, the solution was thawed, analyzed and re-introduced in the fridge. Therefore, the analyte is stable during this period, and the working standard solutions can be safely used without introducing a systematic error in the results.

b) Bench top Processed Sample Stability: Samples from both biological fluids were diluted and placed in the autosampler. For one day, the samples were injected each hour (six replicate injections). Standard Operating Procedure indicate that, each day, all the samples received by the laboratory can be diluted and afterward injected in the chromatograph successively in the same sequence run, up to 240 analyses. Could be used in QC (quality control) and system suitability solutions to-be-analyze. This optimization of time management permits to increase the number of samples that can be analyzed in a day and the productivity of the laboratory.

c) Long term storage of the biological fluids: a set of 15 spiked samples of plasma and urine were stored at −20 °C in the darkness. Each day, a sample was analyzed (six replicate injections). Therefore, the biological fluids should be analyzed up to 15 days after sampling.

d) Long term freeze-and-thaw for the biological fluids: a fortified sample of plasma and another of urine were kept at −20 °C in the darkness. Each day (15 days), they were thawed, an aliquot was analyzed (six replicate injections), and reintroduced in the freezer. Therefore, an

individual sample can be stored and analyzed several times at least during this time, albeit undergoing abrupt temperature changes, without affecting the reliability of the results.

3.4 Analysis of incurred samples

The suitability of the assay was evaluated by the analysis of biological samples provided by a local Hospital. QCs samples were incorporated, using spiked samples of plasma and urine. System Suitability Test, blank samples, quality control (QCs) and Incurred Sample Reanalysis were also performed. All the samples were analyzed in the same run. The results can be seen in Table 3. Samples of plasma and urine with the same number correspond to the same patient. The chromatogram obtained from the analysis of plasma and urine samples from patient 4 is shown in Fig. 2E and F, respectively.

Table 3. Analysis of incurred samples (Px indicated the patient's number).

Sample	[Rivaroxaban]
Blank plasma sample (1 mg/L) ×6	Fit the acceptance criteria (see section 3.3.2)
Plasma QC (0.075 mg/L)	0.069 mg/L
Plasma P1	0.125 mg/L
Plasma P2	Not detected
Plasma QC (0.2 mg/L)	0.190 mg/L
Plasma P3	0.420 mg/L
Plasma P4	0.090 mg/L
Blank plasma QC	Not detected
Plasma QC (0.5 mg/L)	0.470 mg/L
Plasma P5	0.321 mg/L
Plasma P6	0.259 mg/L
Plasma QC (0.075 mg/L)	0.070 mg/L

Sample	[Rivaroxaban]
Plasma P7	0.128 mg/L
Plasma P8	0.032 mg/L
Plasma QC (0.2 mg/L)	0.185 mg/L
Plasma P9	0.059 mg/L
Plasma P10	0.226 mg/L
Plasma QC (0.5 mg/L)	0.523 mg/L
Plasma P3 reanalyzed	0.392 mg/L
Plasma 6 reanalyzed	0.228 mg/L
Urine QC (0.075 mg/L)	0.072 mg/L
Urine P1	0.821 mg/L
Urine P2	0.059 mg/L
Urine QC (1 mg/L)	1.123 mg/L
Urine P3	4.826 mg/L
Urine P4	0.685 mg/L
Blank urine	Not detected
Urine P5	3.325 mg/L
Urine P6	2.394 mg/L
Urine QC (2.5 mg/L)	2.387 mg/L
Urine P7	0.995 mg/L
Urine P8	0.325 mg/L
Urine QC (0.075 mg/L)	0.083 mg/L
Urine P9	0.587 mg/L
Urine P10	1.715 mg/L
Urine QC (1 mg/L)	0.926 mg/L
Urine 2 reanalyzed	0.318 mg/L
Urine 5 reanalyzed	3.654 mg/L
Urine QC (2.5 mg/L)	2.323 mg/L

The results of the SST fall within the acceptance criteria, indicating that the instrument components are roadworthy and ready to perform the analyses. In all the incurred samples, RIV peak was observed without overlapping from endogenous compounds. No unknown peaks or cross-contamination were detected in the blank QCs. The quantitative results of the other QCs (same acceptance criteria as accuracy in 3.3.5) and the reanalyzed incurred samples (difference < 20%) were close to the nominal and the previous found concentration, respectively, thus pointing to the absence of drift in the measurements. Despite the high number of analysis (44 injections), the entire batch was examined in a short period, with a minimal participation of an operator, and using available instrumentation, laboratory material and reagents. Besides, the volume of hazardous chemicals manipulated and wasted, as well as the charge associated to waste treatment, is rather limited. Therefore, the practical performances of the method allow its implementation in a clinical laboratory.

3.5. Application to drug monitoring

The procedure was applied to determine the pharmacokinetics of RIV in a healthy subject, in order to prove its suitability to this sort of clinical studies. Following oral administration of a single 20-mg tablet to a healthy volunteer, the plasma was extracted each hour over 12 h and analyzed. The chromatograms were similar to those obtained in 3.4. The peak corresponding to the analyte was observed and none metabolite or interfering compounds were detected. Fig. 3 depicts the curve RIV concentration vs. time after ingestion. Peak-plasma was reached at nearly 2 h, and the C_{max} was 0.2 mg/L. The elimination half-life was 4.5 h.

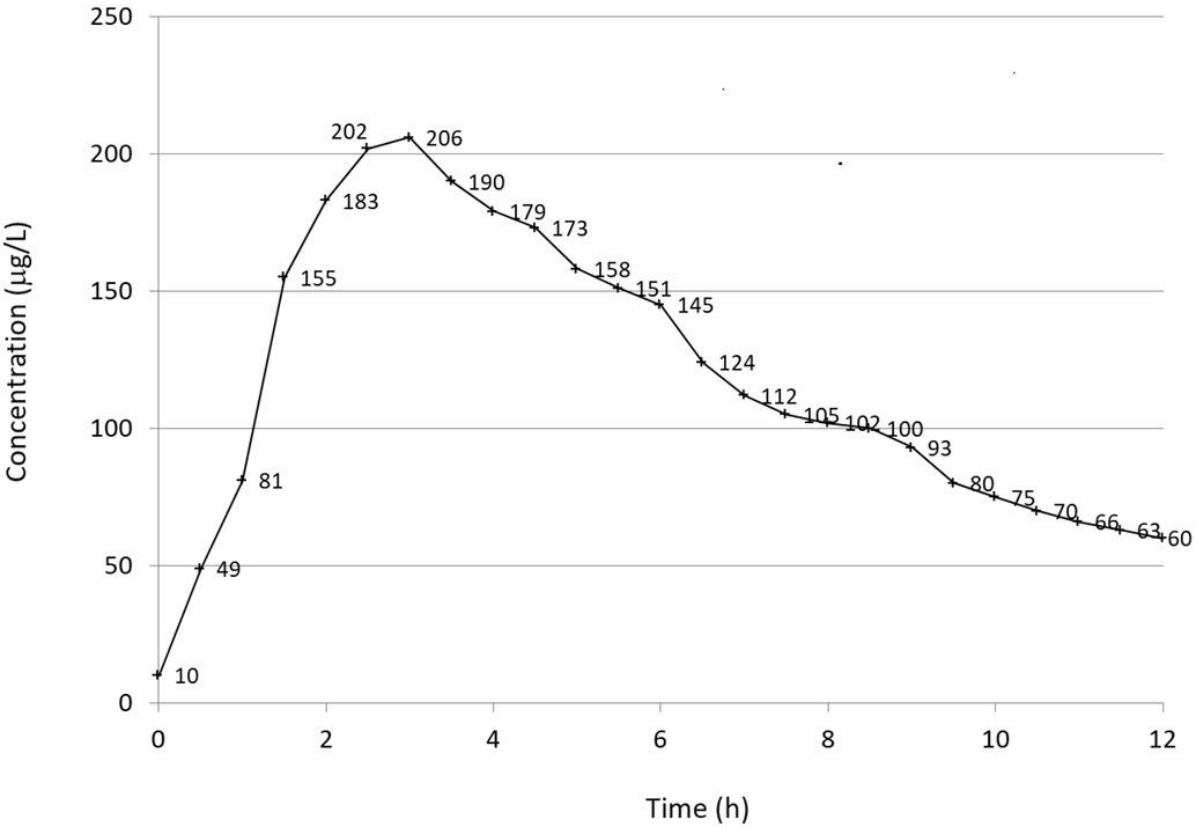

Fig. 3. Plasmatic concentration-time profile of RIV after a single ingestion of a 20-mg tablet by a healthy volunteer (the measured concentrations are shown with the same unity as the y-axis).

4. Conclusions

The use of micellar liquid chromatography with direct injection of the samples has been demonstrated to be a valuable alternative for the determination of RIV in plasma, urine and pharmaceutical formulations. The main advantage is, without doubt, the shortening and simplification of the sample preparation. In addition, the analyte was eluted in less than 6.0 min, without interferences and risk for the column, and clearly identifiable and quantifiable, in spite of the occurrence of macromolecules in the matrices. These advantages were achieved because of the remarkable properties of micellar solutions to bind and solubilize biopolymers and drugs. The method was rapid, easy-to-handle, semi-automated, economic, eco-friendly, safe, with a high sample throughput, and useful for a laboratory with a large workload, like a clinical/pharmaceutical one. The procedure was rigorously and fully validated in terms of system suitability, specificity, linearity, calibration range, sensitivity, carry-over effect, accuracy, precision, robustness, stability and analysis of incurred samples (including blanks, QCs and ISR in the same batch); and the results fit the acceptance criteria. The procedure was found suitable for the determination of RIV in the three studied matrices for pharmaceutical and clinical purposes. Finally, the retention equilibria in the column were investigated, from the data obtained using an experimental design of only 9 assays, by chemometrics. The binding between RIV and both micelle and stationary phase were moderately strong, and essentially by hydrophobic interaction. The binding weakened by the occurrence of 1-propanol in the bulk mobile phase.

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Chapter 10
An assay to determine Rivaroxaban in Pharmaceuticals formulations by Micellar Liquid Chromatography.

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ABSTRACT

Rivaroxaban is one of the most prescribed anticoagulants. The ingestion of the correct dosage is a key for the success of the therapy. A procedure to evaluate the content of active principle ingredient in rivaroxaban pharmaceutical formulations, based on micellar liquid chromatography has been developed. Tablets were solved in mobile phase, and directly injected; thus avoiding the use of large volumes of organic solvents. Analysis were performed using a C18 column and a mobile phase 0.05 M sodium dodecyl sulfate – 12.5% 1-propanol, buffered at pH 7 with phosphate salt; running under isocratic mode at 1 mL/min. The analyte was resolved in less than 6.0. Detection was by UV absorption at 250 nm. The method was successfully validated by the guidelines of the International Conference of Harmonization in terms of: specificity, calibration range (0.01 – 1 mg/L), linearity, limit of detection (0.002 mg/L), limit of quantification (0.006 mg/L), trueness (-0.4 to -0.3%), precision (<2%), robustness, system suitability, carry-over effect and stability; thus proven the adequate quality of the analytical results. A set of incurred samples and quality control was analyzed in the same run; and adequate results were obtained. The procedure was found practical, easy-to-conduct, rapid, safe, eco-friendly, inexpensive, and with a high sample throughput; and then useful for routine analysis in pharmaceutical quality control.

Chapter 10. An assay to determine Rivaroxaban in Pharmaceuticals formulations by Micellar Liquid Chromatography

1. Introduction

Rivaroxaban (RIV) is an anticoagulant drug belonging to the group of the direct, non-vitamin K or target-specific antagonist oral anticoagulants (DOAC, NOAC, or TSOAC, respectively) [1,2]. In fact, it was the first developed DOAC. The drug was approved for marketing by Health Canada and the European Commission in 2008 and by FDA in 2011 [2,3]. Since then, it has been increasingly prescribed as an alternative to heparin or vitamin K antagonist, as it exhibits a more favorable benefits-to-risk profile, being nowadays one of the most administered anticoagulant [1,4]. In clinical studies, rivaroxaban has demonstrated a consistent and strong capacity to prevent blood clotting and the development of thrombi [1,2,5]. However, it may cause bleeding complications and no antidote has been developed to reverse its effects [1,2].

The medication is distributed as tablets with the brand-name of Xarelto® (Janssen Pharmaceuticals, Titusville, NT, USA or Bayer A.G., Leverkusen, Germany) [2]. Currently, it is indicated to treat, prevent and/or avoid the recurrence of thromboembolic disorders, like venous thromboembolism (VTE), deep vein thrombosis (DVT), pulmonary embolism, *etc.* [1,2,6]. It is especially prescribed for the prophylaxis of VTE after total orthopedic hip or knee replacement therapy [1-3,5,7,8,9] and of stroke and systemic embolism in patients with atrial fibrillation [1,2,9]. In combination with antiplatelet agents, it is approved to avoid atherothrombotic events in adults who have acute coronary syndrome and elevated cardiac biomarkers [10]. It has also been prescribed against venous thrombosis induced by homozygous protein S deficiency [11].

Chapter 10. An assay to determine Rivaroxaban in Pharmaceuticals formulations by Micellar Liquid Chromatography

RIV acts by a selective, potent and irreversible inhibition of free and clot bound Factor Xa [1-3,5,7-9,11], thus terminating the amplification of thrombin generation and interrupting the pathway of the blood coagulation cascade [2-5,7,10]. The drug does not decrease protein C/S activity [11], does not inhibit thrombin, has no effect on platelets [3], and do not require the coadministration of cofactors [12]. Chemically, it is an oxazolidine-derivate small molecule (MW = 436 g/mol) almost insoluble in water [3], with a strong absorption in the UV [6].

For the most of the patients, clinical effects of RIV are dose-dependent [4] and then it can be prescribed at a fixed daily dose without regular measurement of RIV exposure for dose adjustment, which is one of the major advantages of RIV-based treatments [1,2,9]. The usual dosing regimen of RIV-therapies is 2.5 mg to 20 mg taken once or twice daily, depending on the treated disorder [2,7]. The success of the therapy requires the patient takes the proper dose, and then the assessment of the medication quality is of the utmost significance. Therefore, the pharmaceutical industry must check the chemical composition of the tablets in all stages of the manufacture and in the finished products, as emphasized by renowned drug regulatory agencies (International Conference on Harmonization (ICH), US Food and Drug Administration, Canadian Drug and Health, Agency, European Medicines Agency, World Health Organization, *etc*…) [3, 6-8,13]. On the other hand, due to the high price of the formulations, a black market (essentially on-line) has emerged around the world, where pharmaceutical formulations are sold at minor cost by non-certified producers, most of them counterfeit or with dubious quality, which must be controlled by national health regulatory agencies [14,15]. Quality control laboratories require rapid, practical, reliable and highly sample-throughput analytical assays

able to identify and quantify RIV in pharmaceutical formulations. According to its analytical characteristics, separative techniques are the most adequate for this sort of analyses. However, as far as we know, few methods have been reported to determine RIV in tablet dosage forms [3,6-8].

Methods based on RP-HPLC using surfactant-mediated solutions in the sample preparation (as solubilizing solutions) and chromatographic analysis (as mobile phases) steps, have been successfully developed to determine the active principle ingredient in pharmaceutical formulations. The most approaches combine the use of a C18-column and micellar solutions containing the anionic surfactant sodium dodecyl sulfate (SDS), an organic modifier (0 to 22% v/v) and a phosphate-buffer [14,15]. The main features of these solutions are their safety and eco-friendliness (as they use innocuous and biodegradable salts and only a minor proportion of toxic, volatile and flammable chemical, which evaporation is hindered by the interaction with the micelles) and their strong solubilization power (they can solubilize small drugs within a large interval of hydrophobicity, even those non-water soluble ones). Therefore, the sample treatment is expedited to a simple one-step solubilization, filtration and direct injections. The use of large volumes of hazardous solvents, and intermediate steps, like drying and resolubilization, are avoided in the sample preparation. MLC also exhibits interesting advantages in the chromatographic separation. Indeed, the retention mechanism is highly reproducible, and then provides stable chromatographic responses. The toxicity of the hybrid micellar mobile phases is lower than hydro-organic ones, as these last ones can contain

up to 100% of organic solvents. In addition, the spectrophotometric properties are enhanced in an organized media [16].

The aim of the work is the development of a reliable, rapid, practical, eco-friendly, easy-to-handle and inexpensive procedure to determine Rivaroxaban in tablets by micellar liquid chromatography. The method will be fully validated by the guidelines of the International Conference Harmonization and relevant documents about this topic [13,17,18], in order to evaluate its analytical performances. It is supposed to be used to analyze a set of commercial pharmaceutical formulations to evaluate its suitability for routine analysis.

2. Materials and Methods

2.1 Standard and chemicals

Powdered standard of Rivaroxaban (purity>98%) were bought from InterQuim (Barcelona, Spain). Sodium dodecyl sulfate (>99%), dimethyl sulfoxide (DMSO, >99.6%) and sodium dihydrogen phosphate dihydrate (>99%) came from Scharlab (Barcelona, Spain). The solvent 1-propanol (HPLC grade) was from Merck (Darmstadt, Germany). Hydrochloric acid 97-38%) was purchased from J.T. Baker (Phillipsburg, NJ, USA), while sodium hydroxide (>98%) was from Riedel-deHaën (Hannover, Germany). Ultrapure water was *in-lab* produced from deionized water (provided as tap water by the university) using an ultrapure water generator device Simplicity UV (Millipore S.A.S., Molsheim, France). All aqueous solutions were prepared using this water.

2.2 Preparation of solutions and mobile phases

The micellar solutions were prepared as follows: the proper mass of SDS and $NaH_2PO_4.H_2O$ was solved in ultrapure water, and the pH was adjusted by adding drops of HCl or NaOH solutions. Then, the adequate volume of the organic solvent was introduced, and the volumetric flask was filled up with ultrapure water. Finally, this solution was ultrasonicated for 5 min to achieve a complete solubilization and filtered through a 0.45 µm-Nylon membrane filters (Micron Separations, Westboro, MA, USA), with the aid of a vacuum pump, to remove solid particles. These solutions were stored in amber bottles at room temperature.

A stock solution of rivaroxaban of 100 mg/L was prepared by weighing the appropriate weight and solving it in DMSO. The working solutions were made by successive dilution of the stock solution in mobile phase. These solutions were kept in a fridge at 4ºC.

2.3 Chromatographic conditions

The chromatographic separation was performed in an HP1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with an isocratic pump, a degasser, an autosampler with a 20-µL loop, and an UV-Visible absorbance diode array detector (DAD), and connected to a PC. The software Agilent Chemstation (Rev. B.04.03(16) 2010) was used for the visualization and registration of the signal, and the measurement of the chromatographic parameters (dead time, t_0, retention time, t_R and peak area, PA). The experimental dead time

was 1.0 min. Retention factor (*k*), efficiency (number of theoretical plates, N) and tailing factor (T) were calculated as in [13]. The special care of the chromatographic instrumentation required when working with micellar mobile phases can be seen in [19]. Under these conditions, the column has a lifespan of at least 1000 injections.

The chromatographic separation was carried out using a Kromasil C18 column (150 x 4.6 mm; 5 µm particle size; 10 nm pore size) from AkzoNobel (Amsterdam, The Netherlands). The mobile phase was an hybrid micellar solution made of 0.05 M SDS – 12.5 % 1-propanol buffered at pH 7 with a 0.01 M-phosphate salt, and run under isocratic mode at 1 mL/min. RIV was eluted at 4.61 min, and the chromatographic run lasted 6 min, and does not require a re-equilibration time *prior* to the next injection.

The absorbance detection wavelength was set at 250 nm. All the analyzed solutions were filtered through a 0.45-µm Nylon membrane filter (Micron Separations) with a 3-mL syringe, and then introduced in the chromatographic vial.

2.4 Sample collection and processing

Several tablets from Xarelto® were purchased in local pharmacies, and stored in the fridge at -4ºC until analysis. They were powdered using an Agatha mortar, and an aliquot was solved in mobile phase to a target concentration of 0.2 mg/L, otherwise specified, and the solution was ultrasonicated to achieve solubilization [15]. In all cases, once injected, the processed samples were discarded.

3. Results and discussion

The chromatographic conditions were taken from [4]. The present work focuses on the optimization of the sample treatment, and the method validation.

3.1 Optimization sample solubilization

In hydro-organic HPLC methods, sample preparation is a large and complex process, which involves the solubilization of tablets using an organic solvent, the evaporation of the solvent of an aliquot by gentle heating in a nitrogen atmosphere, following by redissolution in mobile phase and injection. In MLC, the pharmaceutical formulations are directly injected, after a simple ultrasonication-assisted solubilization in a micellar solution.

In this case, the solvent was optimized. Several solvents were studied: a micellar solution of 0.05 M SDS at pH 3, and the mobile phase. The recovery was evaluated by the peak area, compared to that obtained by the analysis of a tablet solved in methanol. Similar recovery was obtained using methanol and mobile phase, while it was lower for the pure micellar solution. Therefore, mobile phase was chosen.

The association of RIV with the micelles is only based on hydrophobic and dipole-dipole interactions, and do not involve electrostatic ones [4], and then is too weak to achieve a complete solubilization in a pure micellar solution. This was attained by the addition of the organic solvent, which increases the hydrophobicity of the solution. The combined interaction

of the aqueous micellar pseudo phase and a small proportion of organic solvent reach similar solubilization power than a pure methanol solution, despite water remains the main component and the solution is essentially hydrophilic.

The main advantage is the low amount of toxic and volatile solvent used and wasted, thus minimizing the occupational hazard and the impact on the environment. Besides, intermediate solvent-change step, involving evaporation and heating and requiring specific devices, are avoided, and the solubilized sample is quantitatively introduced in the column, thus reducing the loss of the analyte and the sources of variance. The sample preparation is short-time, easy-to-conduct, one-step and employs limited resources (considering chemicals, material and apparatus) and manpower. Many samples can be simultaneously processed by the same operator, thus increasing the sample throughput.

3.2 Method validation

The method was fully validated by the guidelines of the International Conference of Harmonization [17], the Method Validation in Pharmaceutical Analysis book (specifically developed for the analysis of pharmaceutical formulations) [13], and a guide about validation of chromatographic methods [18]. The evaluated parameters were: specificity, linearity, calibration range, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, robustness and system suitability testing (SST).

3.2.1 Specificity

The ability of the procedure to discriminate the analyte from other compounds of the matrix (excipients, impurities or degradation products) was investigated. We analyze a solubilized tablet to a target concentration of 0.2 mg/L. The chromatogram shows a baseline oscillation from the dead time (1.0 min) to ≈3.0 min, and a quite stable and straight baseline. No peak was detected close to the window time of RIV (retention time of 4.61 min). The analyte was observed without overlapping.

A peak-purity test was performed to found co-eluting compounds. The UV-absorbance spectra (190 to 400 nm) were taken at five points of the peak: maximum, and 50% and 5% peak height at the leading and tailing edge. The spectra were normalized at the maximum height and overlaid. No significant differences were found by visual observation.

These results point to the absence of interfering compounds in the tablets. Therefore, the method is effectively able to unequivocally identify the analyte in the presence of other components of the tablets. Besides, the entire value of the peak area can be assigned to RIV.

3.2.2 Calibration range and linearity

Several solutions containing increasing concentrations of rivaroxaban were prepared in the range 0.01 – 1 mg/L, and analyzed by triplicate. The average peak area values (response variable) and the corresponding concentration (independent variable) were processed by least-

square linear regression [20]. The slope was 596.7±1.7 and the y-intercept was 0.7±0.4. The reliability of the model was verified by the following tests:

- The residuals were homoscedastic, as the variances of the observed variable at the different concentration levels (calculated from the peak area replicates) were found equal (α = 5%).

- No trend was detected for the residuals, by visual examination of a residual plot *vs.* concentration.

- No outliers were found in the regression model: the relative residuals were <3 and the Cook's squared Distances were <1 for all points.

- The y-intercept confidence interval includes zero, and the signal of the y-intercept was <1.5% the target concentration. Therefore, no systematic error was noticed.

- A significant correlation was found by a t-test on *r* and comparing the model and residual variance by a F test.

- The determination coefficient (r^2 = 0.99996) and the relative residual standard deviation (<0.94%) meet the acceptance criteria (> 0.990 and 1.5%, respectively) and then an adequate linearity was found between the two variables in the studied interval. The lower limit of quantification (LLOQ) was 0.01 mg/L and the upper limit of detection (ULOQ) was 5 mg/L. The calibration range effectively covers 50-150% from the target concentration

3.2.3 Sensitivity

The limit of detection is the smallest concentration of rivaroxaban injected that provides a peak clearly differentiable from the baseline noise, whereas the limit of quantification was the minimal concentration of analyte that can be reliably determined. These parameters were calculated as 3.3 and 10 the standard deviation of the y-intercept; 0.002 and 0.006 mg/L. These were <0.03% and <0.05%, respectively times the target concentration [13].

3.2.4 Trueness and precision

These parameters were determined in matrix at three levels: 0.8; 1 and 1.2 times the target concentration.

Six samples were successively prepared and analyzed in the same run. The accuracy was the closeness of agreement (in %) between the average value of the found concentrations and the true value, while the repeatability was the dispersion (RSD) of the obtained concentrations. The effect of time in the variability was studied. The same experimental protocol was conducted five different days (using new samples in each occasion) over a three-month period. The intermediate precision was the relative standard deviation of the five average values of found concentration. The results can be seen in Table 1.

All the values were under the acceptance criteria (accuracy, <2.0%; repeatability, <2.0%; and intermediate precision, 3% [13]) Method provides adequate quantitative values for

pharmaceutical control purposes. These performances were reached mainly by the use of an easy-to-handle dilution as sample preparation, where the sample is quantitatively introduced in the column, and the absence of intermediate steps, thus minimizing diminishing of the sources of variance and the risk of loss of the analyte.

Table 1. Trueness and precision studies (%)

Concentration	Trueness[a]	Repeatability[a]	Intermediate Precision[b], %
0.16 mg/L	-0.4	1.5	2.0
0.2 mg/L	-0.3	1.6	1.8
0.24 mg/L	-0.3	1.1	1.4

an=6; bn=5

3.2.5 Robustness

The effect of small, but deliberate, changes in the main instrumental/operational factors on the chromatographic responses (retention time and peak area) was investigated by a one-factor-a-time approach, by analyzing a standard working solution of 0.2 mg/L. The studied factors and alterations were: A) SDS in mobile phase ±0.05 M; B) 1-propanol in mobile phase

± 0.2 %; C) pH in mobile phase ±0.2; D) Injection volume ±2µL; E) Flow-rate ±0.05 mL/min; and F) Detection wavelength ± 5 nm. In each case, the RSD of the instrumental responses found at the low, optimal, and high levels was calculated. Results can be seen in Table 2. Values >4% were judged significant, meaning that they ought to be especially controlled during the analysis

The concentration of SDS in the mobile phase significantly affects the retention time and the peak area. As expected, the retention time changes with the flow rate, while the peak area with the injection volume. Consequently, the steadiness of these operational parameters must be especially surveyed during analysis to attain a reasonable analytical quality.

Table 2. Robustness of the method (%)

Parameter	Retention time	Peak area
SDS in mobile phase	5.1	4.7
1-propanol in mobile phase	2.4	1.8
pH in mobile phase	0.9	0.7
Injection volume	2.7	6.3
Flow-rate	5.8	1.6
Detection wavelength	0.5	2.4

3.2.6 System suitability testing

The correct performance of the equipment and mobile phases was evaluated by the determination of the main chromatographic parameters and their variability under the minimal precision level (replicate injections).

A working standard solution of 0.2 mg/L of drug was injected by n = 6. The evaluated parameters and acceptance criteria were: tailing factor, 1.37, <0.8-1.6; retention factor, 3.61, >2.0; number of theoretical plates, 2368 >2000; RSD of the retention time 0.9%, <1.0% and RSD of the peak area 0.9%, <1.0%. According to the results, the chromatographic response is enough consistent and useful for a reliable qualitative/quantitative analysis. The obtained values can be used as a reference to assess the suitability of the instrumentation when the method will be implemented for routine use in a clinical laboratory.

3.2.7 Carryover effect

A standard solution of RIV at the ULLOQ 1 mg/L and a blank (only mobile phase) were successively injected. In the second chromatogram, neither peak nor baseline excessive noise appeared at the window time of RIV. Therefore, the carry-over effect was considered as insignificant at the studied concentrations.

3.2.8 Stability

The degradation of rivaroxaban was studied in solved tablets under bench-top conditions, at room temperature and indoor daylight. Different aliquots of a single diluted sample (0.2 mg/L) were located in the autosampler tray and injected six consecutive times each hour for one day. The found concentration was determined for each aliquot.

No significant decrease (<2%) of the found concentration of RIV was noticed and no peaks from degradation products were observed in the chromatograms; then the analyte was found stable.

Therefore, all the samples and standards (either for the SST or the calibration curve) had better be analyzed the day prepared. They can be first processed then analyzed in the same sequence run, until 240 injections. These operating instructions represent an optimization of the work-time and permits to increase the number of samples that can be analyzed in a single day; and have to be included in the Standard Operating Procedure.

3.3. Analysis of incurred samples

The suitability of the method for routine analysis was verified by the determination of the label claim of tablets from two manufacturers and different amount of active principle ingredient, purchased from local pharmacies (10 in total). A System Suitability Test was performed before the analysis of the samples. To follow the reliability of the quantitative results

throughout the analysis, several quality control (QCs) were analyzed among the samples, and some samples were analyzed twice, in non-consecutive injections. All the samples/standard were first prepared and then placed in the autosampler tray, and finally analyzed in the same sequence run. The results can be seen in Table 3. The chromatogram obtained from the analysis of a Xarelto® tablet 2.5 mg is shown in the Fig. 1.

Table 3. Analysis of incurred samples

Injection nº	Sample	[Rivaroxaban]
1-6	Standard solution 0.2 mg/L x6	Fit the acceptance criteria (see 3.3.6)
7	Blank QC (mobile phase)	Not detected
8	Xarelto® tablet 2.5 mg (Janssen Pharmaceuticals)	96%
9	Xarelto® tablet 5 mg (Janssen Pharmaceuticals)	103%
10	QC 0.160 mg/L	0.158 mg/L
11	Xarelto® tablet 10 mg (Janssen Pharmaceuticals)	104.2%
12	Xarelto® tablet 15 mg (Janssen Pharmaceuticals)	102.2%
13	QC 0.200 mg/L	0.202 mg/L
14	Xarelto® tablet 20 mg (Janssen Pharmaceuticals)	98.8%
15	Xarelto® tablet 2.5 mg (Bayer A.G.)	100.0%
16	Blank QC (mobile phase)	Not detected
17	QC 0.240 mg/L	0.238 mg/L
18	Xarelto® tablet 5 mg (Bayer A.G.)	95.6%
19	Xarelto® tablet 10 mg (Bayer A.G.)	98.8%
20	QC 0.16 mg/L	0.157 mg/L
21	Xarelto® tablet 15 mg (Bayer A.G.)	97.5%
22	Xarelto® tablet 20 mg (Bayer A.G.)	100.2%
23	QC 0.200 mg/L	0.198 mg/L
24	Xarelto® tablet 10 mg (Janssen Pharmaceuticals) reanalyzed	101.6%
25	Xarelto® tablet 10 mg (Bayer A.G.) reanalyzed	96.1%
26	QC 0.240 mg/L	0.245 mg/L

Chapter 10. An assay to determine Rivaroxaban in Pharmaceuticals formulations by Micellar Liquid Chromatography

The analytical operations and equipment were serviceable and ready to be used; as the results of the SST fit the acceptance criteria. In all the chromatograms, rivaroxaban peak was observed without overlapping with impurities or excipients, and the label claim was 95-105%. No unidentified peaks or cross-contamination were noticed in the blank QCs. No drift in the quantitative values was detected, as the QCs provide results inside the acceptance criteria for accuracy; and the reanalyzed samples showed differences <5%. The whole batch was analyzed in a short time, although 26 injections were made. Participation of the operator was minimal and only one solution (mobile phase) had to be prepared, and only common available instrumentation, laboratory material and reagents were required. In addition, the volume of toxic chemicals manipulated and wasted, and the charge associated to waste managing and processing treatment, was relatively low.

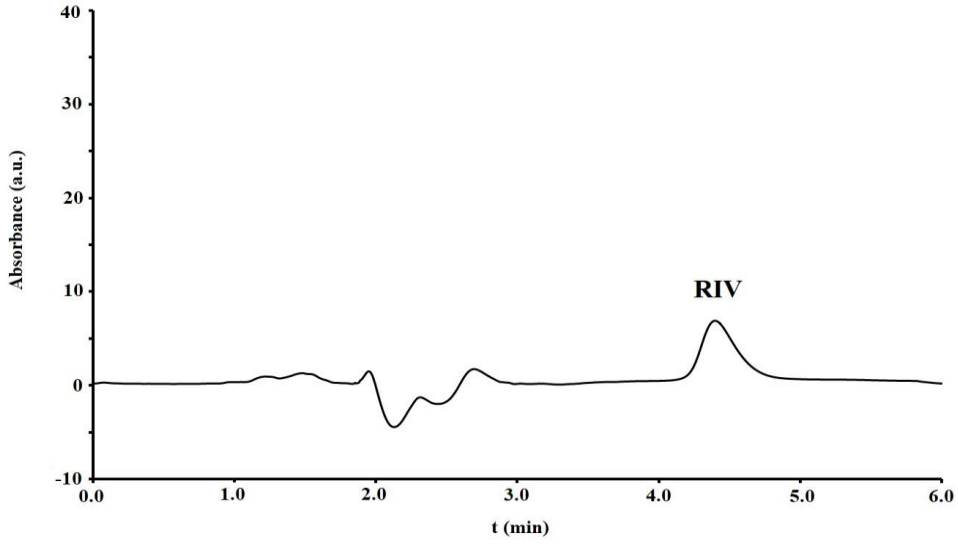

Fig. 1. Chromatograms obtained by the analysis of a tablet solved to 0.2 mg/L of rivaroxaban

4. Conclusions

Direct injection - micellar liquid chromatography has been demonstrated to be a valuable alternative for the determination of rivaroxaban in pharmaceutical formulations. Method fits the requirements of “green chemistry” (rapid, semi-automated, economic, eco-friendly and safe), involves an easy-to-handle/short sample preparation and is globally available, which can be considered its main advantages. Besides, the procedure exhibits high sample throughput, and useful for a laboratory with a large workload. These features were achieved due to the remarkable properties of micellar solutions to solubilize drugs. Procedure was rigorously and fully validated in terms of system suitability, specificity, linearity, calibration range, sensitivity, carry-over effect, accuracy, precision, robustness, stability and analysis of incurred samples (including blanks, QCs and ISR in the same batch); with adequate results, thus assessing the reliability of the analytical information. Therefore, the procedure was found suitable to the determination of rivaroxaban for pharmaceutical quality control purposes.

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Chapter 11

Procedure for the Screening of Eggs and Egg products to detect Oxolonic acid, Ciprofloxacin, Enrofloxacin and Sarafloxacin using Micellar Liquid Chromatography

Chapter 11. Procedure for the Screening of Eggs and Egg products to detect Oxolonic acid, Ciprofloxacin, Enrofloxacin and Sarafloxacin using Micellar Liquid Chromatography

ABSTRACT

A method based on micellar liquid chromatography has been developed to determine oxolinic acid, ciprofloxacin, enrofloxacin and sarafloxacin in eggs and egg products. The antimicrobial drugs were obtained in a micellar solution, which was directly injected. The analytes were resolved using a C18-column and a mobile phase of 0.05 M sodium dodecyl sulfate – 7.5% 1-propanol – 0.5% triethylamine, buffered at pH 3 with phosphate salt, running under isocratic mode. The signal was monitored by fluorescence. Validation was successfully performed by the EU Commission Decision 2002/657/EC in terms of specificity, calibration range (LOQ to 1 mg/kg), linearity ($r^2>0.9991$), limit of detection and decision limit (0.01-0.05 mg/kg), limit of quantification (0.025-0.150 mg/kg), detection capability (<0.4 times decision limit), trueness (-14.2 to +9.8%), precision (<14.0%), robustness and stability. Procedure was environmentally-friendly, safe, easy-to-conduct, inexpensive, and highly sample-throughput, thus useful for routine analysis as screening method in a laboratory of food residue control.

Chapter 11. Procedure for the Screening of Eggs and Egg products to detect Oxolonic acid, Ciprofloxacin, Enrofloxacin and Sarafloxacin using Micellar Liquid Chromatography

1. Introduction

Antibiotic refers to a large category of drugs used to kill or avoid the growth of bacterial microorganism. In human medicine, they are prescribed to cure microbial infection diseases. In husbandry, they are administered for the same reason, but also unethically for prophylaxis purposes and to promote growth increasing the economic profit of the farm [1,2]. Quinolones are among the most important antibacterial agents used in human medicine, and are active against both Gram-positive and Gram-negative bacteria through the inhibition of their DNA gyrase. They are well absorbed after oral administration and extensively distributed in tissues, and they display a stable and favorable pharmacokinetics and dose-dependent activity. Such characteristics make these drugs suitable to act as the therapy for a large number of infections on animal farms [3]. However, inappropriate or abusive use of antibiotics in poultry farms (or poultry producers) might provoke the transfer and accumulation of their residues in food products. This causes the exposure of the consumer to low concentrations of these compounds, which may produce toxic reactions, and stimulates the emergence of quinolone-resistant pathogens [4,5]. It has been largely proven eggs from poultry treated with pharmaceutical products contain drug residues, even laid days to weeks after treatment cessation [6].

Nowadays, there is a worldwide concern among the society and public agencies about the dramatic consequences of quinolone misuse in population health [7]. In the frame of food safety, the European Union (EU) has regulated the use of quinolones as veterinary drugs in food-producing animals and has established maximum residue limits (MRLs) of antibiotics in food. According to EU regulation, the quinolones, like ciprofloxacin (CIPRO), enrofloxacin

(ENRO), sarafloxacin (SARA), and oxolinic acid (OXO), are “not for use in animals from which eggs are produced for human consumption”, which means they are strictly forbidden in laying hens rearing. No MRL have been stated for them, and eggs and industrially-elaborated egg products containing quinolone residues at any level must be rejected [8,9]. Accordingly, their residues need to be controlled to verify the compliance of producers and importers with the regulation.

The determination of quinolones in complex food samples requires a previous extraction step of the analyte from the matrix, followed by different clean-up steps that involve liquid–liquid extraction (LLE) [10,11], solid-phase extraction (SPE) [12,13], dialysis [14] or supercritical fluid extraction (SFE) [15]. A comprehensive review on current trends in sample preparation to isolate veterinary drugs and growth promoters from foods has been presented [16]. Turbulent-flow chromatography coupled with tandem mass spectrometry (TFC-LC-MS/MS) is a technique that eliminates time-consuming simple clean-up steps, increases productivity and reduces solvent consumption without harming sensitivity or productivity.

The most recent studies of antibiotics in eggs have been published using high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) [17,18,19,20,21,22,23,24]. However, this equipment is very expensive and only a few laboratories can afford it. Other methods involve HPLC with fluorescence detection [25,26,27]. As previously commented, one of the main problems in multi-residue antibiotic analyses in incurred samples is the extraction; cleanup and preconcentration of the matrix analytes before the instrumental analysis are excessively tedious and complex.

Chapter 11. Procedure for the Screening of Eggs and Egg products to detect Oxolonic acid, Ciprofloxacin, Enrofloxacin and Sarafloxacin using Micellar Liquid Chromatography

Micellar liquid chromatography (MLC) [28] is an attractive alternative to conventional HPLC methods that uses a surfactant solution above the critical micellar concentration instead of aqueous-organic solvents as mobile phases. It has been previously used for the determination of quinolones in a large variety of food matrices with direct injection from liquid samples and, by a simple solid-to-liquid extraction followed by direct injection of the supernatant, from solid samples. MLC allows the analysis of physico-chemically complex food matrices, usually without the aid of a purification step, thus considerably reducing the cost, manipulation, use of hazardous and volatile organic solvents and analysis time. Indeed, SDS-micelles and SDS-monomers tend to bind proteins and other biomacromolecules competitively, provoking its denaturing (in the case of proteins) and solubilization. Therefore, they are washed harmlessly away to elute with the solvent front rather than precipitating into the column, reducing the probabilities of overlapping with the analytes. Besides, protein-bound drugs are released and can be determined [7,29,30,31,32].

The aim of this work was to develop a multi-residue HPLC procedure with micellar mobile phases to simultaneously determine several quinolones (ciprofloxacin, enrofloxacin, sarafloxacin, and oxolinic acid) in fresh eggs and industrially-made egg products: boiled egg; liquid pasteurized egg white and yolk; powdered whole egg; powdered egg white and yolk; and omelette. The method was validated in line with Commission Decision 2002/657/EC [33] in terms of selectivity, linearity, decision limit, detection capability, precision, and robustness. The proposed method was applied to determine these compounds in a large number of incurred samples of egg and egg-product samples, distributed in retail shops to consumers.

2. Materials and Methods

2.1 Standards and reagents

Powdered standards of oxolinic acid (purity > 97.0%), enrofloxacin (>98.0%), ciprofloxacin (>98%) and sarafloxacin (>97.2%) were bought from Sigma-Aldrich (St-Louis, MO, USA). Sodium dodecyl sulphate (>98.0%) and sodium dihydrogen phosphate monohydrate (>99.0%) were from Scharlab (Barcelona, Spain). HPLC grade 1-propanol was purchased from Merck KGaA (Darmstadt, Germany). Hydrochloric acid (37.0 %), ethanol (HPLC grade), and triethylamine (>99.5 %) were supplied by J.T. Baker (Deventer, The Netherlands). Ultrapure water was in-lab produced from deionized water (provided by the University as tap water) using an ultrapure generator device Simplicity UV (Millipore S.A.S., Molsheim, France). All the aqueous solutions were prepared using freshly made ultrapure water. The ultrapure water was not stored.

2.2 Preparation of solutions

The micellar solutions were made as follows: the proper quantity of SDS and $NaH_2PO_4.H_2O$ were weighed and solved in ultrapure water using a magnetic stirrer. Later, the adequate volume of triethylamine (TEA) was added, and the pH was adjusted by adding drops of an HCl solution. Afterwards, the appropriate volume of 1-propanol was introduced to obtain the desired proportion, and then the flask was filled up with ultrapure water. Finally, the solution was ultrasonicated to ensure solubilization and filtered, with the aid of a vacuum

pump, through a 0.45 µm-membrane filter (Micron Separations, Westboro, MA, USA) placed on a Büchner funnel.

Stock solutions of the quinolones (100 mg/L) were made by solving the appropriate amount of the powdered standard in 5% of ethanol in a volumetric flask, which was then filled up with a solution of 0.05 M SDS buffered at pH 3. These solutions were ultrasonicated to achieve solubilization. Working solutions were made by consecutive dilution of the stock solutions in the same micellar solution. All the standard and stock solutions were stored at +4ºC in amber vials a maximum of two months. Before their utilization, these stored solutions were warmed at room temperature until the completion of dissolution of the crystals of SDS that were formed overnight.

2.3 Chromatographic conditions

An HP1100 (Agilent Technologies, Palo Alto, CA, USA), equipped with an isocratic pump, a degasser, an autosampler, a 20-µL loop and a fluorescence detector, was used for the chromatographic analyses. No module worked at a regulated temperature. The software Chemstation Rev.A.10.01 (Agilent Technologies) was used to control the instrumentation, as well as to register and process the signal. The dead time ($t_0 \approx 1.0$ min) and the retention time (t_R) were directly taken from the chromatogram. We calculated the retention factor (*k*) [34], efficiency (*N*, number of theoretical plates), asymmetry (B/A), and resolution (*Rs*) [35].

The column was a C18 Kromasil (Scharlab) with the following features: length, 150 mm; internal diameter, 4.6 mm; particle size, 5 µm; pore size 10 nm. The mobile phase was an aqueous solution of 0.05 M SDS – 7.5% 1-propanol – 0.5% triethylamine, buffered at pH 3 with 0.01 M phosphate salt, running at 1 mL/min under isocratic mode. The detection was performed by fluorescence, applying for the following excitation/emission wavelength program: 0 – 10, 260/366 nm, and 10.01 to 30: 280 – 455 nm. The working and cleaning specifications regarding the utilization of the chromatographic instrumentation when using micellar mobile phases are described in [36].

All the samples to-be-injected were filtered through a 0.45-µm-Nylon membrane filter, with the aid of a 3-mL syringe, before introduction into the vials.

2.4 Sample processing

Fresh eggs and industrially-made egg products (boiled egg, pasteurized egg white and yolk, powdered whole egg, powdered egg white and yolk, and omelette) were purchased in local supermarkets. White and yolk were separated for fresh and boiled eggs.

The liquid samples (fresh and pasteurized egg yolk and white) were kept in the freezer at -20ºC. The solid samples (boiled egg yolk and white; powdered whole egg; powdered egg white and yolk; and omelette) were finely ground using a mincer (Model MZ10, Petra Electric, Burgau, Germany) at 5000 rpm for 5 min and kept in the freezer at -20ºC. All the samples were

stored a maximum of 2 months, and thawed the day of the analysis until reaching room temperature

Liquid samples were homogenized for 10 min through shaking using a magnetic stirrer. An aliquot was 1/5 w/v, diluted in mobile phase, filtered and directly injected. For spiked samples, the appropriate volume of quinolone standard was added before the dilution.

A Stirring Batch Solid-to-Liquid-Extraction [37] (SBSLE) procedure was used to extract the quinolones from the solid samples [7,32]. Five grams were introduced in an Erlenmeyer flask containing 50 mL of mobile phase; and the solution was shaken in a magnetic stirrer for 1 h and ultrasonicated for 15 min. The supernatant was decanted, filtered through a 0.45-μm-Nylon membrane filter using a Büchner funnel, with the help of a vacuum pump, and injected. For fortified samples, the proper amount of quinolone standard solution was injected in the crushed sample, and the mixture was left 24 h at room temperature to stimulate a slow elimination of the solvent and maximize the integration of the matrix and the analytes [32]. Therefore, these artificially contaminated samples were similar to the incurred ones.

3. Results and Discussion

3.1 Optimization of the chromatographic conditions

General chromatographic conditions were taken from previously published methods about the determination of quinolone residues in food matrices, like fish flesh [29], honey [30,31] and meat [7,32]: flow rate, 1 mL/min; isocratic mode; not controlled temperature;

stationary phase, C18; surfactant, SDS; buffer, 0.01 M phosphate salt; pH, 3; sacrificial base, TEA at 0.5% and the optimal values of fluorescence excitation/emission wavelength. The wavelength program used to register the signal was designed to determine each quinolone at their optimal detection conditions. Under these conditions, OXO is neutral and ENRO, CIPRO and SARA, positively monocharged. Oxolinic acid exhibits a low retention and requires a hybrid mobile phase with limited elution strength. Therefore, 1-propanol was selected as organic solvent. In the context of this work, we optimized the concentration of SDS and 1-propanol in the hybrid mobile phase to determine OXO, ENRO, CIPRO and SARA with a maximum resolution, peak shape, and the minimum analysis time.

We have previously demonstrated the retention factor and efficiency of the quinolones using hybrid mobile phases decreases at increasing concentrations of SDS, as the quinolones bind the micelles [7,32]. Indeed, the number of micelles is directly related to the total concentration of SDS. In order to maximize the peak shape, the concentration of SDS was fixed to the minimal value recommended in MLC, 0.05 M.

A standard solution of 0.1 mg/L of the four quinolones was analyzed using several mobile phases, by varying the proportion of 1-propanol in the interval 2.5-12.5%, v/v. The elution strength of the mobile phase increased at higher proportions of 1-propanol. In all the assays, the order of retention times was the same OXO < CIPRO < ENRO < SARA, and OXO was eluted far from the other ones. Low proportions of 1-propanol resulted in broad peaks and an excessive duration of the run. Otherwise, at higher proportions, OXO was eluted too close to the dead time and the resolution CIPRO/ENRO; and ENRO/SARA diminished. The optimal value was set to 7.5%.

Finally, the detection conditions were set. A wavelength program was introduced, by changing the excitation/emission wavelengths during the run, in order to determine each quinolone at its maximal signal-to-noise ratio of emitted intensity. Therefore, the initial conditions were 260/366 nm (optimal values for OXO) and were maintained until 10.0 min (far from both the endpoint of OXO peak and the starting point of CIPRO peak. Then, the wavelengths were shifted to 280 – 455 nm, the optimal conditions of the other quinolones, which were kept until the end of the chromatogram. Neither baseline drift nor baseline noise were affected by the wavelength change.

A system suitability test (SST) was performed, by determining the experimental values of the main chromatographic responses/parameters, under the optimized operating conditions. Results can be seen in Table 1. The values complied with the acceptance criteria [35]. Additionally, the excitation and emission spectra were registered at five points of the peak signal: maximum height, and at front and tail, 0.5- and 0.1-maximal-height. For each quinolone, the spectra showed alike, thus proven the stability of the signal. Therefore, the chromatographic separation was successfully achieved.

The four antibiotics did not overlap and were reliably distinguished. Besides, OXO was eluted distantly enough to the dead time to prevent its overlapping with the front of the chromatogram. Besides, the analysis time was not excessively long.

The antimicrobial drugs were resolved using a mobile phase essentially made of innocuous and biodegradable reagents. Only a low proportion of hazardous and volatile organic solvent (7.5%) was employed, less than usually required in hydro-organic HPLC (up to 100%).

SDS-micelles interact with 1-propanol molecules and reduce its volatility, making the mobile phase more stable and less toxic. The four compounds were resolved using isocratic mode, despite the different hydrophobicity and charge. This avoids the problems of baseline shift, spurious peaks from solvent impurities or gases, solvent de-mixing, and limited reproducibility of the chromatographic response (due to the difficulties in maintaining constant the separation conditions through several injections, as the proportion of solvent pumped to the system changes, and the homogenization of the mixture may not be complete), inherent to gradient approaches. Besides, there is no need of re-equilibration time for the column, thus shortening the effective analysis time per injection.

Table 1. System suitability testing. Main chromatographic parameters obtained under the optimal conditions (n=6).

Parameter	OXO	CIPRO	ENRO	SARA	Acceptance criteria
t_R (min) (RSD, %)	5.48±0.05 (0.9)	16.08±0.14 (0.9)	18.79±0.17 (0.9)	22.3±0.2 (0.9)	(<1.0)
RSD of peak area, %	0.6	0.9	0.5	0.7	<1.0
RSD of peak width at half-maximum height, %	0.5	0.8	0.7	0.9	<1.0
Retention factor	4.48	15.08	17.79	21.3	>2.0
Efficiency	2172	2842	2584	2158	>2000
Asymmetry	1.4	1.2	1.2	0.9	0.8-1.6
Resolution with the next eluted quinolone	High	1.9	2.4	-------	>1.5

3.2 Sample preparation

Sample processing and optimization of the experimental conditions were separately carried out for liquid and solid egg-derived foodstuff. In both cases, a sample spiked at 0.5 mg/kg with the four studied antimicrobial drugs was used.

3.2.1 Liquid samples

Liquid egg-derived samples contain large amounts of non-water-soluble macromolecules, like proteins, and in the case of those containing yolk, also fats; blended to other nutrients and the quinolones. The sample pretreatment was based on those detailed in [30,31], but using mobile phase as a dilution solvent, instead of a pure micellar solution. When the sample is mixed with the hybrid micellar solution, the quinolones and some of the biological macromolecules were solubilized by interaction with the SDS micelles. The remaining macromolecules form insoluble aggregates were dispersed through the solution, but they were easily removed by at the filtration step.

Several dilution ratios (1:1; 1:2; 1:5; 1:10; 1:25 and 1:50, v/v) were assayed. The values 1:1 and 1:2 were discarded as the filter was early blocked and the obtained volume was very low. In the other cases, more than 2 mL were collected. The matrix was eluted as a broadband, which tallness decreased at higher dilution ratios, at the front of the chromatogram, but the signal fell to the baseline before the elution of OXO. Therefore, 1:5 was chosen, in order to get a reasonable sensitivity.

3.2.2 Solid samples

The sample pretreatment was a SBSLE, similar to those described in [7,32], but using mobile phase as extraction solvent, for the same reasons as in 3.2. The quinolones, some of the macromolecules and micro-particles moved from the solid to the liquid phase during the stirring phase. The ultrasonication step was introduced to achieve this process and reduce the size of the particles, which were then removed by filtration. Two factors, the stirring time and the sample-to-extractant solution (S/E, w/v) ratio were optimized. The absolute recovery was determined by the peak area, corrected by the S/E ratio.

The effect of the stirring time was investigated over the range 5 min to 2 h. The absolute recovery rose from up to 30 min, slowly increased to 50 min, and remained nearly invariant to 2 h. Consequently, the stirring time was set to 60 min.

Several S/E ratios were tested: 1:1; 1:2; 1:5; 1:10; 1:25 and 1:50. For 1:1 and 1:2, the mixture was a viscous paste, which cannot be stirred, and then were directly rejected. The absolute recovery was nearly similar for the other values. In all cases, the matrix compounds were eluted at a broadband at the front of the chromatogram, but without overlapping with OXO. The optimal value was set to 1:5, in order to maximize the sensitivity.

3.2.3 General comments on the procedure

In both cases, the sample pretreatment included the minimum number of easy-to-conduct steps, some of them semi-automated. The diluting/extracting solutions were directly

injected in the column, hence avoiding additional tedious and time- and resources-consuming extraction or purification steps and chemical reactions. Participation of the operator was minimal. A derivatization was not needed, as the quinolones exhibited natural fluorescence. Consequently, the probability of loss of the analyte and contamination of the sample during the preparation was strongly diminished. This contributed to ameliorate the reliability and consistency of the results.

The most used reagents were widely obtainable, stable, biodegradable and innocuous. Only a minimal volume of toxic and volatile organic solvent was used. All the laboratory material and apparatus required are general and accessible, as no specific devices, complex assembly or hyphenated extraction techniques are used.

3.3 Method Validation

The method was validated following the guidelines of the European Commission Decision 2002/657/EC [33], specifically developed to determine organic residues in foodstuff, and other documents about validation [35,38]. Because several and physico-chemically different kind of samples are studied, the general parameters (instrumental calibration range and sensitivity, and ruggedness) were determined in standard solution, while the most specific ones (specificity, method calibration range and sensitivity, trueness, precision, decision limit, detection capability and stability) were determined in each matrix.

3.3.1 Instrumental calibration range and linearity

Standard solutions (n=7) containing increasing concentrations of OXO, CIPRO, ENRO and SARA (up to 0.2 mg/L) were analyzed by triplicate. The variances of the response (peak area) at the different levels were found significantly equivalent by an F-test, and then the residuals of the calibration curve can be considered homoscedastic.

The average peak area was plotted *vs.* the corresponding concentration. Both variables were related by a first-grade equation using least square regression method [38,39]. Table 2 shows the constants (slope and y-intercept, and their respective standard deviations, SD), the goodness-of-fit parameters (determination coefficient, r^2, and relative residual standard deviation, RRSD), outliers study (standardized residuals, $\varepsilon_R/s_{y/x}$ and Cook's squared distance, CD^2), and the sensitivity parameters (limit of detection, LOD, and limit of quantification, LOQ: as 3.3 and 10 times the deviation standard of the blank, taken as the standard deviation of the y-intercept, divided by the slope). No constant error was found, as the y-intercept was significantly equal to 0. Normality of the residuals was checked by the visualization of the residual plot *vs.* concentration. According to the results, a proper linear relationship was demonstrated between the observed and independent variable, in the range LOQ to 0.2 mg/L.

Table 2. Calibration and sensitivity parameters.

Parameter	OXO	CIPRO	ENRO	SARA	Acceptance criteria
Slope ± SD	1353 ± 5	16970 ± 30	8240 ± 20	10760 ± 80	
y-intercept ± SD	-7 ± 4	18 ± 8	6 ± 4	-12± 16	$<t_{(5;0.05;2\ tails)}$ x SD
r^2	0.9991	0.9994	0.9997	0.9992	>0.990
RRSD	1.3	0.8	0.6	0.9	<1.5
Max. $\varepsilon_R/s_{y/x}$	1.2	0.8	0.5	1.1	<3
Max. CD^2	0.8	0.4	0.3	0.8	<1
LOD (μg/L)	10	2	2	5	
LOQ (μg/L)	30	5	5	15	
MLOD (μg/kg)	50	10	10	25	
MLOQ (μg/kg)	150	25	25	75	
Method calibration range (mg/kg)	0.15-1.0	0.025-1.0	0.025-1.0	0.075-1.0	

3.3.2 Ruggedness

The effect of small changes in the operating conditions on the main chromatographic responses (retention time and peak area) was investigated for each quinolone, using a Youden approach [33]. Minimal (x) and maximal (X) values were set at the same distance from the nominal value (the optimal one) to those can be reached in the usual laboratory work (preparation of the mobile phase and random instrumental fluctuation). Studied factors and x - X values were: A) SDS concentration, 0.045-0.055 M; B) 1-propanol, 7.3 – 7.7 %; C) TEA, 0.4-0.6%; D) pH, 2.8-3.2; E) flow rate, 0.95 – 1.05 mL/min; F) injection volume, 18 – 22 μL and G) emission wavelength ±5 nm. The assays were carried out using a standard solution of

0.1 mg/L of each antibiotic. The difference in response between the extreme values was judged significant if >5%.

Modification of the flow rate (>7.3%) and TEA (>6.8%) caused a change on the retention time, while that of the injection volume on the peak area (>8.6%), for the four studied quinolones. The concentration of SDS influenced: the retention time, for OXO, ENRO and SARA (>6.5%) and the peak area for ENRO (5.4%). The proportion of 1-propanol affected the peak area for ENRO (5.3%). In the other cases, the response parameters remained nearly unchanged. Therefore, the method can be considered robust enough to provide consistent values of chromatographic responses, although the main instrumental parameters undergo slight oscillations from their optimal value.

3.3.3 Specificity

The ability of the method to discriminate OXO, ENRO, CIPRO and SARA from endogenous compounds in the studied egg-derived samples was evaluated by analyzing blank samples, and blank samples fortified with the four analytes at 0.5 mg/kg. The concentration of the quinolones in the injected solutions was 0.1 mg/L, as the standard solution analyzed for the optimization and the SST (described in 3.1). In the fortified samples, the excitation and emission spectra were taken for the four analytes as described in 3.1.

In all cases, a wide band (probably containing most of the macromolecules from the matrix) was eluted at the front of the chromatogram, from the dead time to nearly 3.0 min. No

peaks from endogenous compounds were observed close (± 2.0 min) at the window time of the quinolones, and the baseline was quite stable.

In the fortified samples, the retention time and the peak width at a half-maximum height of the quinolones were similar (<3% and 96-103%, respectively) to that obtained from the analysis of the standard solution (section 3.1). In order to evaluate peak purity, the chromatograms and the excitation and emission spectra, respectively, obtained from fortified samples and from the standard solution, were overlaid and compared by visual observation. No significant differences were perceived among them, thus assessing the absence of coeluting substances and matrix effects. The chromatogram obtained by the analysis of the fresh-egg yolk (blank and spiked) is shown in Figure 1.

Using the here-described experimental conditions, each analyte gave the same response regardless of the original chemical environment, and no overlapping or coeluting compounds were noticed in the egg-derived foodstuff matrices. Therefore, the method is specific enough to confirm the absence or presence of the analytes reliably, and to enable their quantification (as the entire peak area can be assigned to the corresponding quinolone) in an incurred egg-derived sample. This was mainly because of the strong association of the macromolecules (fats and proteins) with the micelles, which prevent their interaction with the quinolones or the modified stationary phase; and the use of natural fluorescence, which limits the number of potential interfering substances.

3.3.4 Method calibration range and sensitivity

These parameters refer to the amount of quinolone that can be found in the unprocessed egg-derived sample. The method limit of detection (MLOD), the method limit of quantification (MLOQ) and the method upper limit of quantification (MULOQ) were calculated from the instrumental ones, considering the sample treatment. The values were the same for all the studied samples. The results can be seen in **Table 2**. We can see that even extremely low amount of quinolone residues can be detected in incurred samples.

As no permitted limit has been stated by the EU Regulation 37/2010 [8], a Minimum Required Performance Limit (MRPL) has been fixed for each quinolone at their corresponding MLOQ.

3.3.5 Trueness and precision

These parameters were separately determined for each quinolone and kind of egg-derived foodstuff, at three concentrations: 1x; 1.5x and 2x MRPL.

The trueness was determined by the consecutive analysis of six blank egg-derived sample spiked at the corresponding concentration. It was calculated as the difference between the average found concentration and the fortified concentration, divided by the fortified concentration, times 100. The repeatability was calculated from the same experiment, as the relative standard deviation (RSD) of the six values of found concentration. The same approach was made five days over a three-month period (using freshly prepared samples in each case),

in order to evaluate the consistency of the precision through time. The within-laboratory reproducibility was the RSD of the five average found concentrations. The results can be seen in **Table 3**.

The values (-14.2 to +9.8% for trueness and (<14.0% for precision) comply with the acceptance criteria stated by the guideline. Therefore, the method provides reliable quantitative data at concentrations close to the MRPL and inside the calibration curve. This performance was reached because of the simplicity of sample preparation and the stability of MLC responses.

Table 3. Trueness (%), repeatability[a] and within-laboratory reproducibility[b] (RSD, %) values for the quantification of the studied quinolones in fresh eggs and several egg-derived foodstuffs (Acceptance criteria: trueness, -20 to +10%; precision, <15.1%). For each matrix, the first second and third lines refers to the fortified amount MRPL; 1.5xMRPL and 2x MRPL)

Sample	OXO	ENRO	CIPRO	SARA
Fresh-egg yolk	+7.5/6.2/7.0	-6.9/7.1/6.8	-10.7/9.3/9.2	-9.3/8.9/10.6
	+6.5/6.1/7.2	-4.0/5.2/4.7	-7.8/8.2/7.9	-8.5/10.5/9.5
	+4.2/4.8/5.3	-2.9/4.8/6.0	-6.5/7.3/6.5	-4.2/6.2/6.5
Fresh-egg white	+7.3/6.9/6.7	-7.0/6.5/8.0	-9.9/9.6/9.0	-8.9/8.5/8.7
	+5.2/6.2/7.6	-4.9/6.0/7.2	-7.5/6.8/7.2	-7.8/7.0/6.8
	+5.0/5.9/7.1	-2.6/3.9/4.4	-5.9/6.8/6.4	-5.5/6.0/6.9
Boiled-egg yolk	+9.5/8.0/9.8	-9.8/8.5/8.9	-14.2/13.3/12.5	-13.8/14.0/12.9
	+7.9/7.1/8.0	-7.2/6.8/7.0	-9.5/8.3/7.7	-10.0/12.2/11.8
	+6.8/4.9/6.4	-5.6/7.2/6.8	-6.9/7.1/5.8	-7.5/8.5/7.2
Boiled-egg white	+9.2/7.4/8.7	-9.4/9.0/10.2	-13.8/13.4/14.0	13.2/11.4/13.9
	+7.0/6.8/8.1	-6.9/5.9/6.8	-10.5/9.2/8.9	-9.7/10.2/11.0
	+4.9/5.0/5.8	-4.8/4.3/5.2	-7.5/8.0/8.6	-7.0/6.5/7.3
Pasteurized egg yolk	+6.9/7.2/7.5	-6.5/7.2/7.5	-9.7/8.5/7.9	-8.9/9.3/9.8
	+5.0/4.8/5.9	-4.3/6.8/7.0	-7.9/7.5/8.0	-7.5/6.7/7.0

	+3.9/4.0/4.9	-3.0/4.9/5.0	-6.2/6.0/6.5	-5.2/6.5/5.8
Pasteurized egg white	+6.8/6.8/7.2	-7.0/6.9/7.2	-9.2/7.8/8.2	-8.6/9.0/9.4
	+5.8/6.1/6.0	-5.0/6.2/6.3	-8.2/7.4/7.8	-7.2/7.3/6.9
	+4.0/3.8/3.6	-3.4/4.2/4.7	-6.1/6.4/6.9	-4.9/5.2/5.7
Powdered egg yolk	+9.5/8.2/8.4	-10.9/9.9/10.6	-13.5/12.8/11.9	-12.9/10.0/10.9
	+7.5/7.0/6.5	-8.0/8.2/7.9	-11.0/9.9/10.5	-9.4/8.5/8.1
	+5.2/5.0/5.8	-5.9/5.8/6.0	-7.3/7.5/7.2	-7.3/7.0/7.4
Powdered egg white	+9.8/8.5/8.9	-8.2/7.9/8.5	-13.6/12.1/12.8	-12.9/11.5/12.0
	+7.1/6.8/7.2	-6.7/7.1/8.0	-8.8/9.2/8.7	-8.3/8.0/7.5
	+5.4/4.8/5.3	-4.2/6.3/6.7	-5.8/6.8/7.0	-5.0/6.3/5.2
Powdered whole egg	+9.2/8.0/7.8	-9.9/10.2/10.8	-12.8/11.7/10.3	-12.6/10.6/9.8
	+7.8/6.9/7.0	-8.3/8.5/8.0	-10.5/9.8/9.1	-9.2/8.7/7.9
	+5.6/5.8/5.3	-6.1/6.4/6.0	-6.9/7.4/8.0	-7.2/6.5/7.0
Omelette	+8.9/7.6/9.0	-11.0/10.8/11.6	-14.0/13.2/12.9	-13.6/10.3/12.5
	+6.8/8.5/7.5	-7.8/8.0/7.5	-10.9/10.3/9.5	-9.7/8.2/9.0
	+5.0/6.2/6.8	-5.8/6.5/5.8	-7.0/8.5/7.8	-7.8/6.9/7.5

3.3.6 Decision limit and detection capability

The meaning of these parameters, stated by the EU Commission Decision 2002/657/EC, has been extensively described in previous documents [33,38]. Both parameters were determined for each quinolone as indicated for substances that no established limit has been stated, separately for each kind of egg-derived foodstuff.

Decision limit (CCα), with a α=1%, was 3 times the standard deviation of the found concentrations obtained by the analysis of 20 blank samples. As the values were close to the MLOD, this value was taken as the CCα. Therefore, samples wherein the found concentration

was over the MLOD will be considered as non-compliant with the regulation, limiting to 1% the probability to reject a compliant sample.

The detection capability (CC_β) was calculated for β=5%, as the CC_α plus 1.64 times the standard deviation of the detected concentrations by the analysis of 20 blank samples fortified at the decision limit. The results can be seen in **Table 4.** Samples containing antibiotic concentration over these values have a low probability (<5%) to be accepted. The classification is barely affected to the uncertainty, due to the closeness of the detection capability to the MLOD (<40%).

According to the results, the method is able to correctly evaluate the compliance with the regulation of egg-derived foodstuff samples at even low concentrations of quinolone residues.

3.3.7 Stability

The stability of these antibiotics in working solutions kept in a fridge (+4ºC) in the darkness (at least two months) and bench-top in the micellar solution, obtained from a foodstuff sample pretreatment, at indoor conditions (a minimum of one day) has been proven in previously published studies [7,31,32]. Therefore, we examined the possible decay of the quinolones in the samples during storage at the usual conditions (in a freezer at -20ºC and in the darkness).

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For each kind of studied egg-derived foodstuff, a blank material was taken and divided into 20 parts, which were fortified at 0.5 mg/kg of OXO, CIPRO, ENRO and SARA. One aliquot was immediately analyzed, and the other ones stored. These were analyzed each half week. The stability of the antimicrobial group in the stored samples was monitored by comparing the peak area corresponding to each quinolone with that obtained in the analysis of the fresh one, as well as by the emergence of other peaks from decomposition products. No significant decay was noticed during the studied period, and then incurred samples can be kept up to two months before analysis.

Table 4. Detection capacity for each quinolone in the studied meats (concentrations in mg kg-1).

Sample	OXO	ENRO	CIPRO	SARA
Fresh-egg yolk	56	12	12	30
Fresh-egg white	57	11	12	29
Boiled-egg yolk	61	12	13	33
Boiled-egg white	60	13	13	33
Pasteurized egg yolk	58	13	12	28
Pasteurized egg white	57	12	12	29
Powdered egg yolk	63	13	13	34
Powdered egg white	62	12	13	33
Powdered whole egg	64	13	12	35
Omelette	63	13	14	34

3.4 Analysis of incurred samples

The analytical method was used for the screening of samples taken from fresh eggs (white and yolk) and industrially-made egg products (boiled-egg white and yolk; pasteurized egg white and yolk; powdered whole egg; powdered egg white and yolk; and omelette) (5 each one), purchased from local supermarkets, to detect OXO, CIPRO, ENRO and SARA residues. The analytes were not found in any sample, thus confirming they comply with the regulation about the absence of veterinary residues and can be ingested without risk by consumers.

The entire set of samples (20 liquid, 30 solid and 7 for the calibration curve) was analyzed in a one day by a single operator, which was able to simultaneously prepare the solutions, perform the experimental protocol, control the instrumentation and supervise the whole process. Certainly, the entire chromatographic sequence lasted <23 h. Liquid samples were treated in less than 1-2 min. Solid samples required a longer time (<90 min) but several samples can be simultaneously processed. Besides, the treatment of the samples can be performed while the previous ones are injected.

A large number of samples can be analyzed per day, resulting in a high sample throughput. In addition, the method can be executed with a relatively low disbursement (only common reagents, material and instrumentation are used), taking into account the charge *per* studied sample. The solutions used have a limited toxicity (far less than usual in hydro-organic-HPLC), and does not represent a risk for the environment and the workplace safety. Consequently, the method can be used for routine analysis in a quality-control laboratory with a high workload.

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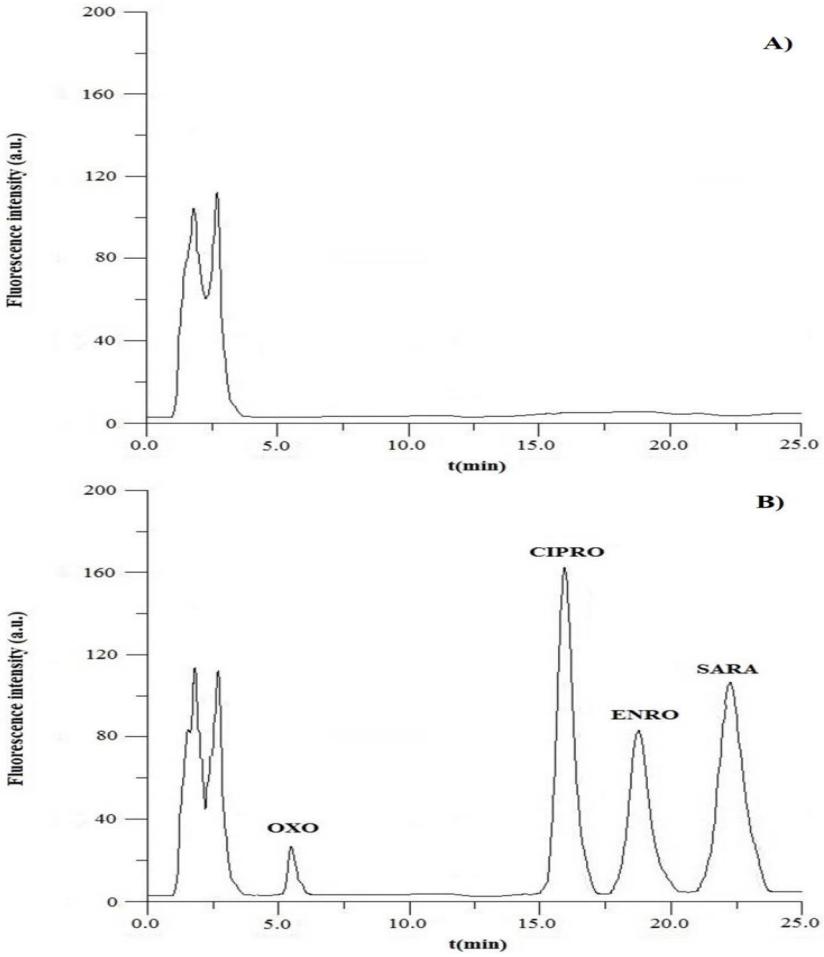

Figure 1. Chromatograms obtained by the analysis of a sample of fresh-egg yolk A) blank and B) fortified at 0.5 mg/kg of each quinolone.

4. Conclusions

The screening of samples from fresh eggs and the most common industrially-made egg-products to detect the antibiotics OXO, CIPRO, ENRO and SARA is feasible using micellar liquid chromatography coupled to fluorescence detector.

The main advantage is the sample pretreatment was easy-to-handle and requires a minimal participation from the operator, despite the physico-chemical complexity of food matrices. The main chromatographic responses were checked by a system suitability test, and were quite consistent. Analytical quality was evaluated by the guidelines of the EU Commission Decision 2002/657/EC (specificity, calibration range, linearity, trueness, precision, decision limit, detection capability, robustness and stability). According to the results, the method was found effectively able to reliably identify and quantify the analytes in the studied matrices, reaching relatively low concentrations (0.025 - 0.150 mg/kg). The procedure exhibited interesting practical features, like eco-friendly, safe, inexpensive, widely available and with a high sample throughput, which makes it useful for screening purposes. These outstanding practical and analytical performances were attained because of the particular properties of the micellar solutions, used as diluting/extracting solutions and mobile phases. Therefore, the procedure can be implemented in laboratories approved for official residue control to evaluate the compliance of egg-derived samples with the EU regulation 37/2010, regarding to the occurrence of the studied antimicrobials.

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Chapter 12

Analysis of Isoniazid and Pyridoxine in Plasma sample of Tuberculosis patients by MLC

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ABSTRACT

A simple Micellar Liquid Chromatographic procedure is described to determine Isoniazid and Pyridoxine in plasma patients with Tuberculosis. For the analysis, the antituberculosis compounds were diluted in water and samples were directly injected in chromatograph, avoiding pre-steps of extraction or purification procedures. The resolution from the matrix was performed using a mobile phase formed by 0.15 M SDS - 8%(v/v) Butanol / phosphate buffer at pH 3, running at 1 mL/min through a C18 column at 25ºC. Detection was carried out by UV setting the wavelength 265 nm. Under these conditions, the final chromatographic analysis time was less than 6 min. The analytical methodology was validated following the European Medicines Agency Guideline on Bioanalytical method validation (2011). The response of the drugs in plasma was linear in the 0.15 to 15 μg/mL for Isoniazid and 0.05 to 15 μg/mL for Pyridoxine range, with r2 > 0.99 in both compounds. Accuracy and precision were <10% for both substances. Limits of detection and quantification in plasma were 0.041 and 0.125 μg/mL for Isoniazid, and 0.013 and 0.041 μg/mL for Pyridoxine. The method developed does not show interferences by endogenous compounds. Finally, the analytical method was used to determine both compounds in plasma samples of Tuberculosis patients.

Chapter 12. Analysis of Isoniazid and Pyridoxine in Plasma samples of Tuberculosis patients by MLC

1. Introduction

Isoniazid (INH) is used for the treatment of tuberculosis (as part of combination therapy) or for latent tuberculosis infection [1]. Chemically, INH is known as isonicotinyl hydrazine or isonicotinic acid hydrazide and act by inhibiting the biosynthesis of mycolic acid [2]. Though it is very effective on tuberculosis treatment, can cause polyneuropathy (PNP) under treatment dose as a highly relevant adverse drug effect [3]. This fact is due to the conjugation of INH with vitamin B6 (pyridoxal and pyridoxal 5-phosphate) leads to the depletion of pyridoxal 5-phosphate, causing B6 vitamin deficiency [4] on plasma levels. Pyridoxine deficiency have been identified as a cause for peripheral neuropathy.[5]

Risk factors for developing neuropathy after isoniazid therapy include HIV infection old age, pregnancy, slow acetylator status, renal failure, alcoholism, malnourishment and chronic hepatic failure [6]. As well as patients taking medications which antagonize B6 effects like hydralazine, cycloserine, penicillamine and retroviral drugs are at higher risk of neuropathy due to INH.[7] INH preventive therapy is increasingly recommended for TB patients, specially those whit HIV infection [8]. This distinction is relevant due to the fact that new cases of TB mainly occur in Asia and Africa in association with HIV [9].

Vitamin B6, principally in its biologically active coenzyme form pyridoxal 5'-phosphate, is involved in a wide range of biochemical reactions, including the synthesis of neurotransmitters [10]. Due to structural similarity, pyridoxine (B6 vitamin) is the only antidote for INH toxicity [11]. Acute toxicity of INH lead on neuropathy and seizures, while chronic toxicity results in hepatotoxicity [12].

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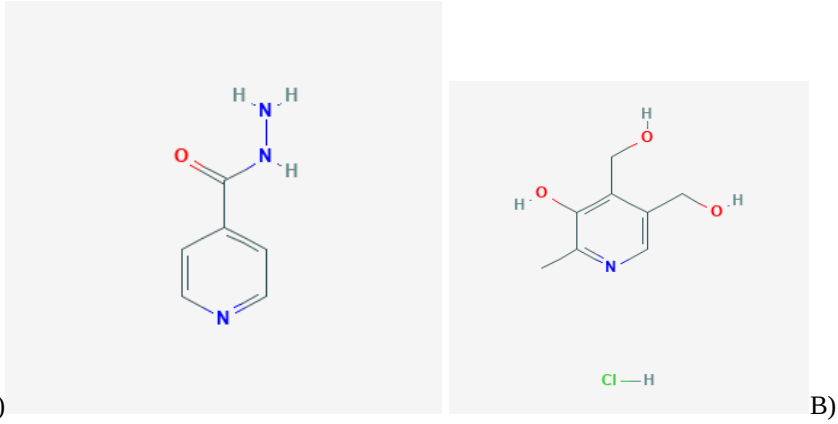
Figure.1. A) Isoniazid; B) Pyridoxine structure.

A)  B)

Literature reveals several methods for detection and quantification of Isoniazid from pharmaceuticals and biological samples. These methods include high performance Liquid Chromatography (HPLC) [13-16], Electrochemical [17,18], Ionic Liquids [19] or Liquid Chromatography coupled to Mass Spectrometry detectors (LC/MS) [20,21]. However, these techniques require sophisticated and delicate instrumentation, trained laboratory staff, toxic waste handling and are relatively expensive, considering the necessary reagents and maintenance.

The aim of the study is to develop a rapid, simple, reliable and cost-effective method for the detection and quantification of Isoniazid and Pyridoxine (B6 vitamin) in plasma and urine, that could be used to treatment monitoring on medical centers, specially on tuberculosis patients.

Is known that HPLC is wide extended to confirm the identity of a drug and provide quantitative results and also to monitor the progress of the therapy of a disease. For this purpose, we explored Micellar liquid chromatography (MLC) as a useful technique of detection and determination. MLC is a variation of HPLC and has proven to be useful for this purpose in many other drugs, previously [22]. Taking advantage of benefits offered by technique such as direct injection sample, non-toxic, non-flammable, biodegradable and relatively inexpensive

mobile phases and stable and reproducible signals, a method to determine isoniazid and B6 vitamin was developed.

The method must be able to quantify the drugs reliably in clinical concentrations and be useful for routine analysis in a clinical laboratory. To test the analytical performance, the procedure must be validated by European Medicines Agency Guideline on Bioanalytical method validation (2011) [23]. Finally, its reliability should be demonstrated by analyzing plasma samples from patients with tuberculosis after a treatment based on these drugs, provided by Government District Hospital, Sagar, India.

2. Experimental

2.1. Reagents and solutions

Isoniazid and Pyridoxine were supplied by Sigma (St. Louis, MO, USA). The other reagents used were sodium dodecyl sulphate (SDS) from Merck (Darmstadt, Germany), sodium dihydrogen phosphate, HCl, NaOH from Panreac (Barcelona, Spain), and methanol, butanol and pentanol from J.T. Baker (Holland). Ultrapure water was produced by Millipore S.A.S. (Molsheim, France) and used to prepare the aqueous solutions and mobile phases. The Isoniazid and Pyridoxine standard solutions were prepared dissolving the appropriate amount in ultrapure water, and the mixture was ultrasonicated for 2 min. Fresh solutions were prepared weekly. Blood samples were collected using a DB SST Tube (BD Vacutainer Systems, Plymouth, UK), and were centrifuged for 5 min at 756 Relative Centrifugal Force (RCF) or G-force at 4ºC; plasma was kept on the fridge.

2.2. Apparatus

The chromatograph used was an Agilent Technologies Series 1100 (Palo Alto, CA, USA) equipped with a quaternary pump, a degasser for the mobile phase, an autosampler and

a temperature controller for the column module coupled to a fluorescence detector. A Kromasil 5 C18 column with a 5 μm particle size and 150 mm_(4.6mm i.d.) (Scharlau, Barcelona) was used. Chromatographic signals were acquired and processed with the HP ChemStation Rev. B.01.01. An ultrasonic bath Ultrasons-H (Selecta, Abrera, Spain) was used to dissolve the standards and the pH of the solutions was measured with a Crison GLP 22 (Barcelona) potentiometer equipped with a combined Ag/AgCl/glass electrode. The analytical balance used was a Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland).

2.3. Chromatographic conditions

Separation and quantification of Isoniazid and Pyridoxine were performed using a mobile phase containing 0.15 M SDS - 8%(v/v) Butanol / phospate buffer at pH 3, running at a constant flow of 1.0 mL/min. The buffer was made adding 0.01 M of sodium dihydrogen phosphate, adjusting the pH by using suitable volumes of HCl 0.1 M. The mobile phase was filtered through 0.45 μm nylon membranes from Micron Separations (Westboro, MA, USA). Injection volume was set at 20 μL. Under these conditions, the Kromasil C18 column head pressure was 150 bars. The column temperature was kept constant at 25ºC. The wavelength for measurements was set at 265 nm.

3. Results and discussion

3.1. Mobile phase selection

The mobile phase selection was based on the resolution and a suitable analysis time of the two compounds including a third peak regarding to the band of plasma proteins eluted in first minutes, so as in the urine sample. The octanol–water partition coefficient (logPo/w) of Isoniazid and Pyridoxine are 0.64 and -0.8 respectively [Drugbank], which mean that can be expected they have high retention in MLC.

Chapter 12. Analysis of Isoniazid and Pyridoxine in Plasma samples of Tuberculosis patients by MLC

In the development of the MLC method for the determination of Isoniazid and Pyridoxine, eight factors were identified that influence the separation analytes: SDS and modifier concentrations, type of buffering species, running buffer pH, column length, column temperature, sample injection volume and flow rate. The criteria for the selection of the optimum separation conditions were the maximum separation of the analytes in the mixture within the minimum analysis time. Thus, peak width (i.e., resolution) and lower analysis retention time were parameters of primary importance to assess the goodness of the experimental result.

First of all, buffer selected was phosphate because it is useful to study the change in the chromatographic response over a wide range of pH, in this case from 3 to 7 due to it is the adequate for the column care. After studying pH range 3-7, the pH of 3 was selected due to was the most suitable pH in terms of lower retention time and better resolution, improving the retention time compared to pH 5 and 7. Second, molarity of the buffering agent was observed to have less influence on the separation and was therefore kept constant at 0.1 M. Third, the column length was studied making efficiency increase a 10% in a column of 25 cm compared to one of 15 cm, but retention time and analysis increased in a 35%, so a column of 15 cm was selected. Fourth, the influence of the temperature was checked in the range from 25 to 40ºC, result shows that this parameter does not improve the resolution or analysis time: 25ºC was selected for further studies. Fifth, the studies were focused in the injection volume, changing it between 0.5-30 µL, observing overloading at 25 µL: an injection volume of 20 µL was selected. Sixth, flow rate was experimented from 0.5 to 2 mL/min showing a decrease of analysis time, but decreasing the resolution dramatically, so 1 mL/min was selected. Finally, SDS and modifier concentrations have an important influence and were optimized using a factorial design.

Methanol, Propanol, Butanol and Pentanol are the most used short-chain alcohols as modifiers. All of them were checked. Using n-pentanol, Isoniazid and Pyridoxine elute too quickly and overlap with the protein band. As a retention time is a priority parameter, butanol was selected as the modifier for the analysis running. Finally, after factorial design selection,

0.15 M SDS - 8%(v/v) Butanol / phosphate buffer at pH 3 was selected as the optimum mobile phase in which Isoniazid eluted at 3.8 min and Pyridoxine at almost 5 min with a symmetric peak shape and clearly differentiables from protein band.

3.2. Mobile phase selection using factorial designs

The effects of SDS concentrations in the range of 0.05 to 0.15 M and butanol in the range 4 to 8% (v/v) were selected. Low values of both parameters could produce sub-micellar media, and upper the maximum level, microemulsions.

To evaluate the influence of SDS and butanol on the separation we used a three-level full factorial design (3^2). The runs of the design were carried out in a randomized sequence and the retention times and peak widths were measured. To estimate the experimental error, replications of factor combinations were necessary. The center point was run three times. Varying all factors simultaneously at a limited number of factor levels, and after the calculation of the function responses, a polynomial curve was obtained. As responses, two different functions were checked: first, the sum of resolutions and, second, the chromatographic resolution statistic (CRS) function. The resolution (R) of a pair of peaks was calculated using Eq. (1):

$$R = \frac{2(t_2 - t_1)}{(w_2 + w_1)}$$

where t_1 and t_2 are the migration times and w_1 and w_2 are the width at the peak base of two consecutive peaks, measured as time units. The numerator in Eq. (1) describes the separation process with regard to differential migration and the denominator expresses the dispersive

processes acting against it. The total resolution (Rs) was set as response and calculated as the sum of the resolutions of the all pairs of peaks,

$$Rs = \sum_{o}^{i} R,$$

where i is the number of analytes. To quantify and interpret the relationships between responses and factor effects a response surface method was used. The general empirical model is a second-order polynomial, where the response y is related to the variables (factors) x as:

$$y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{1 \leqslant i \leqslant j}^{k} b_{ij} x_i x_j + \sum_{i=1}^{k} b_{ii} x_i^2$$

where k is the number of variables (factors), b_0 is the intercept parameter, and b_i,b_{ij}, and b_{ii} are regression parameters for linear, interaction, and quadratic factor effects. The nonlinear regression analysis of the data was carried out using the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA. The data for the modeling are listed in Table 2, and the model obtained was:

$$\begin{aligned} Rs = & -23.03 - 16.72\mathrm{pH} + 44.12\mathrm{V} + 2.12\mathrm{pH}^2 \\ & - 2.70\mathrm{V}^2 - \mathrm{A09pHV} - 0.09\mathrm{pH}^2\mathrm{V} + 0.31\mathrm{pHV}^2 \end{aligned}$$

Chapter 12. Analysis of Isoniazid and Pyridoxine in Plasma samples of Tuberculosis patients by MLC

The three-dimensional plot of total Rs, as a function of SDS of the running buffer and pentanol. The surface plot allows the whole range of conditions to be explored, including combinations that were not experimentally demonstrated, indicating that the maximum resolution area corresponds to 0,15 M and to a pentanol concentration of 5% (v/v). Thus, these conditions were considered to be the optimum MLC conditions to separate the two antituberculosis drugs, including the band of proteins in the serum and urine samples. The same optimum was achieved when a second response function is used, which is the inverse of the chromatographic resolution statistic. The CRS is a mathematical function calculated with Eq.

(5):

$$\mathrm{CRS} = \left\{ \sum_{i=1}^{n-1} \left[\frac{(R_{i,i+1} - R_{opt})^2}{(R_{i,i+1} - R_{\min})^2 R_{i,i+1}} \right] + \sum_{i=1}^{n-1} \frac{R_{i,i+1}^2}{(n-1)R_{av}^2} \right\} \frac{t_n}{n}$$

where Ri, i+1 is the resolution between consecutive peaks, and R_{av} is the average resolution of all peaks, and R_{min} is the minimum acceptable resolution, which has a value of 1. Considering that peak width is 1 min, two peaks, which appear contiguous, have a difference in time of 1 min. Thus, Rs calculus shows that the result is 1 (minimum acceptable separation). R_{opt} is the desired resolution, the optimum resolution, in this case 1.5. Rs is 1.5 if the two peaks are separated by a time of 0.5 min. t_n is the migration time of the last-eluting solute and n is the number of compounds in the sample. The CRS considers the resolution of all solutes in the sample and incorporates three important aspects of the separation. The first term in Eq. (5), named the resolution term, evaluates the resolution between all adjacent solute pairs in comparison to defined values for optimum and minimum resolution. The second term in Eq. (5), named the distribution term, considers the relative spacing of the solute zones. The final multiplier term in Eq. (5) takes into consideration the analysis time and the number of analyte peaks to be separated. The CRS values obtained for each electrophoretic condition and also the inverse of CRS are shown in Table 2. The inverse of CRS was chosen because the maximum

of the function fits the optimal condition. The response surface of this response function (inverse of CRS) was compared with the results obtained with the Rs function. Fig. 1B shows the surface plot and the maximum of this function coincided with the optimum conditions obtained with the Rs function. The mathematical model obtained was Eq. (5).

$$CRS^{-1} = -0.16 + 0.04pH + 0.11V - 0.001pH^2 - 0.005V^2 - 0.03pHV + 0.001pH^2V + 0.001pHV^2. \quad (6)$$

To summarize, the conditions selected were as follows: SDS 0,15 M - Butanol 6% - $H_2PO_4^-$ 0.1 M at pH 3 in a C18 column of 15 cm thermostated at 25ºC, 20 µL of volume injection and a flow rate of 1 mL/min.

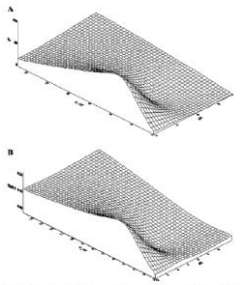


Fig. 1. Three-dimensional response surface of (A) resolution and (B) CRS^{-1} as a function of pH and applied voltage.

Table 2
3^2 Full factorial design and response obtained

Run	pH	V(kV)	*Rs*	CRS	CRS^{-1}
1	4.7	15	17.80	9.01	0.11
2	2	5	90.91	4.68	0.21
3	2	2	5.00	266.97	3.74E-3
4	9.5	5	9.80	465922.1	2.14E-6
5	4.7	2	1.93	154.49	6.47E-3
6	4.7	5	10.99	473090.6	2.11E-6
7	9.5	2	0.11	193.01	5.18E-3
8	2	15	15.87	8.48	0.12
9	9.5	15	30.06	7.98	0.13

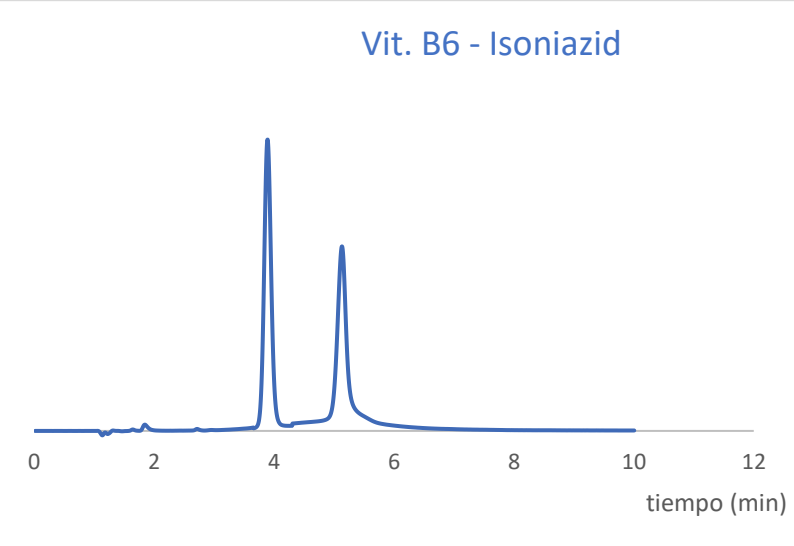


Figure 2. Chromatogram obtained for Vit. B6 (3.8 min) and Isoniazid (5 min)

3.3. Method validation

This methodology for the determination of isoniazid and pyridoxine in plasma of tuberculosis patients has been validated according to the European Medicines Agency Guideline on Bioanalytical method validation (2011) [23] which includes linearity, limits of detection (LOD) and quantification (LOQ), intra- and inter-day precisions, selectivity, recovery and robustness.

3.3.1. Selectivity

To study the matrix effects in plasma of the possible co-eluting compounds, ten blank samples were analysed**.** The protein band appeared before 3 min. A plasma sample spiked with 10 µg/mL of Isoniazid and another spiked with 10 µg/mL of Pyridoxine were analysed. The peaks of the two analytes can be observed as being sufficiently separated from each other, avoiding overlapping**.**

3.3.2. Linearity and sensitivity

A blank was simultaneously spiked at nine concentration levels of Isoniazid and Pyridoxine in the range 0.1-15 μg/mL. The slope and intercept were obtained by a correlation between the areas of the chromatographic peak of each analyte from a triplicate injection versus the concentration by least-square linear regression. Calibration was repeated five times (preparing the sample on each occasion) over a 2-month period, and each calibration level was analysed six times. The LODs and LOQs were set as three and ten times the standard deviation of the blank, respectively. The regression curves and limits were:

Isoniazid: A = (94.4644286 ± 3.1) [Isoniazid] - (14.2516101 ± 8.3), r2 = 0.99992367

LOD = 0.041 μg/mL; LOQ = 0.125 μg/mL

Pyridoxine: A = (70.5951851 ± 30) [Pyridoxine] - (34.7245055 ± 5.7) r2 = 0.99876608

LOD = 0.013 μg/mL; LOQ = 0.041 μg/mL

where A is the signal in arbitrary units and the concentration amount is provided in μg/mL.

3.3.3. Precision and accuracy

The intra- and inter-day accuracy and precision of the proposed methodology were determined for isoniazid and pyridoxine with plasma samples spiked at three concentration levels (0.1; 0.5; 1μg/mL). The intra-day analysis was determined by injecting aliquots of these samples six times on the same day, while the inter-day analyses correspond to the average of five measurements of the intra-day values taken over a 3-month period. The results, expressed as variation coefficients for accuracy and relative error for precision, are shown in Table 1. The

data show good accuracy (<10%) and adequate precision (2.7–8.5%) for both analytes, which are useful for routine analyses.

3.3.4. Robustness

The variation of the main chromatographic responses (retention factor, peak area and efficiency) was examined at small, but deliberate, changes in the main operational parameters. These were changed from their optimal value, and the tested oscillations were that we considered that may occur during the laboratory work and chromatographic run, in a usual situation. The studied factors and the studied interval were: A) detection wavelength ± 5 nm; B) SDS in mobile phase ± 0.05 M; C) pentanol in mobile phase ± 0.2%; D) pH in mobile phase ± 0.2; E) flow-rate ± 0.05 mL/min and F) injection volume ± 2 μL. A blank plasma sample spiked with 2.5 mg/L was analyzed by testing eight different set of experimental conditions, which value was fixed by an experimental design following a Youden approach [24]. Differences > 8.0% were judged significant, meaning that they may be especially controlled during the analysis. The retention factor was significantly affected by the concentration of SDS in mobile phase and the low-rate, the peak area by the concentration of SDS and the injection volume, and the efficiency by the flow and the injection volume. Therefore, these factors operational parameters must be especially controlled during analysis to achieve adequate analytical results.

3.3.5. Carry-Over Effect

A urine sample spiked with Isoniazid and Pyridoxine at 5 μg/mL was injected, and immediately afterwards, a blank urine sample were analyzed. In this last one, no peak was observed in the chromatogram at Rifampicin or Rifabutin retention time. Thus, the carry-over was considered negligible at concentrations within the calibration range.

3.3.6. Matrix Effects

The influence of the endogenous compounds of urine in the quantitative results, either by linking or any interaction interfering with the retention process, was evaluated. Working standard solutions containing the same concentration of Isoniazid and Pyridoxine as in Section 3.2.4 divided by five, were analyzed by following the same protocol to consider the dilution in the experimental procedure. The results for the intra-day accuracy and precision were similar to those obtained for urine samples. Therefore, no significant matrix effect was detected, regardless of the complexity of the chemical composition of urine. This was probably done by the interaction of the endogenous compounds of the urine and the SDS-micelles.

3.4. Application of the methodology to patients' plasma

Plasma studies were performed following the oral administration of a single dose of 300 mg of Isoniazid to several patients with tuberculosis disease. Samples were taken over a 24 h period. The chromatogram obtained by analysing the sample Isoniazid and pyridoxine could clearly be quantified without interferences.

3.5. Stability & decomposition studies

Stability means the capacity of the drug to remain unchanged throughout time. It can be studied under different conditions of light, temperature, oxidation – among others. It is determined by the monitoring of the analyte concentration in a stored solution or sample. The degradation is noticed by the diminishing of the concentration and the emergence of peaks from decomposition products. As reported in the literature, Isoniazid major products of decomposition are Isonicotinic acid, Isonicotinamide, Hydrazine, Isonicotinic Acid N´(pyridyl-4-carbonyl)-hydrazide, Isonicotinic acid ethylidene-hydrazide, Isonicotinic acid pyridine-4-methylene-hydrazide [25]. The stability was studied from solutions or spiked

samples containing 1.0 μg/ml of Isoniazid. Results were found similar for plasma and urine. The investigated conditions were:

- Fridge: Standard solution in a mobile phase kept in the fridge (2-5ºC) for one week. Under these conditions, Isoniazid was found stable up to 7 days

- Freeze: A Standard solution and biological fluid frozen at -20◦C for 2 weeks (the usual storage conditions in a hospital), a short decay (90%) was noticed at biological fluid and no variance was noticed on standard solution.

- Light exposition: The LC profiles of light exposed drug samples were similar to those in the dark, indicating that light had no particular influence on the drug in solution.

-Thermal exposition: The exposure of the solid drug to room temperature (20-23ºC) for 30 days did not show any evidence of decomposition, indicating that isoniazid is stable to dry heat.

4. Conclusion

Micellar liquid chromatography has proved a suitable technique to analyse Isoniazid and Pyridoxine in human plasma. The main advantage of the technique is the possibility of injecting a diluted sample directly into the chromatographic system, avoiding previous extractions or purifications steps. After sample irradiation to improve sensitivity, the analyte was satisfactorily resolved from the matrix under 20 min running analysis time, using a mobile phase of 0.15 M SDS - 8%(v/v) Butanol / phosphate buffer at pH 3. Validation was performed according to the European Medicines Agency Guideline on Bioanalytical method validation (2011) with satisfactory results in terms of linearity, selectivity, precision, accuracy and recovery. The limit of detection and the lineal range were sufficient to detect the usual amount of Isoniazid and Pyridoxine in patients's plasma. In addition, this method involves at sustainable chemistry due to it is relatively inexpensive and uses a smaller amount of toxic organic modifier.

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Chapter 12. Analysis of Isoniazid and Pyridoxine in Plasma samples of Tuberculosis patients by MLC

Chapter 13

Quantification of Rifampicin and Rifabutin in Plasma of Tuberculosis Patients by Micellar Liquid Chromatography

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ABSTRACT

A Micellar Liquid Chromatographic procedure is described to determine Rifampicin and Rifabutin in plasma on Tuberculosis patients. For the analysis, the two antituberculosis compounds were diluted in water and samples were then directly injected, avoiding extraction steps and experimental procedures. The resolution from the matrix was performed using a mobile phase containing SDS 0.15 M - 6%(v/v) n pentanol and phosphate buffer at pH 3, running at 1 mL/min through a C18 column at 25ºC. Detection was carried out by UV being the wavelength 270 nm. Under these conditions, the final chromatographic analysis time was 20 min. The analytical methodology was validated following the FDA 2018 Bioanalytical Method Validation Guidance for Industry. The response of the drugs in plasma was linear in the 0.5 to 15 μg/mL range, with r2 > 0.99. Accuracy and precision were <14% for both substances. Limits of detection and quantification (μg/mL) in plasma were 0,3 and 0,4 for Rifampicin, and 0,1 and 0,3 for Rifabutin. The developed method does not show interferences by endogenous compounds. Finally, the analytical method was used to determine both antituberculosis drugs in plasma samples of Tuberculosis patients.

Chapter 13. Quantification of Rifampicin and Rifabutin in Plasma of Tuberculosis Patients by Micellar Liquid Chromatography

1. Introduction

Tuberculosis remains a leading health issue worldwide and Rifampicin is the preferred first-line drug for its treatment [1-3]. Although it is quite well tolerated in a usual dose regime, but adverse effects could be developed including gastrointestinal reactions, exanthema, hepatotoxicity and immunological reactions, as thrombocytopenia, leukopenia, eosinophilia, hemolytic anemia, agranulocytosis, vasculitis, acute interstitial nephritis and septic shock [4,5]. While some adverse effects may be resolved with symptomatic treatment or spontaneously, others may require regimen changes because they are dose-dependent [6]. On the other hand, Rifabutin has activity against Mycobacterium tuberculosis similar to Rifampicin, in fact in most of the cases may be more effective [7]. Main difference between them is that rifabutin has lower incidence of severe adverse effects [8,9]. This point makes Rifabutin more attractive as a substitute in situations where Rifampicin may cause adverse effects or is not well tolerated. Furthermore, Rifampicin has more drug interactions than Rifabutin due to it is a potent inducer of the CYP450 system [10]. Due to this fact rifabutin has been used in patients coinfected with tuberculosis and HIV [11], trying to avoid possible difficulties with drug interactions and avoiding the disease spreading between susceptible people [12]. On the other hand, Rifabutin is the only frontline antituberculosis drug that has activity against an emergent disease as Mycobacterium Abcessus, an opportunistic pathogen causing dangerous pulmonary infections because are intrinsically multidrug-resistant [13].

Rifampicin (log K = 1,7 and log Po/w = 2,7) is the principal tool antituberculosis chemotherapy, however, Mycobacterium Tuberculosis develop resistance to this drug with high frequency restricting the utility of its use for treatment. Tuberculosis strains classified as multidrug-resistant (MDR) are those resistant at least to the two most potent first-line antituberculosis drugs, i.e. isoniazid and rifampicin [14-16]. The clinically significant resistance mechanism is mutation within a defined region of the rpoB gene, which encodes the target of RIF, the β subunit of bacterial RNA polymerase [17,18]. Most rifampicin-resistant Mycobacterium tuberculosis isolates are also resistant to rifapentine, while approximately

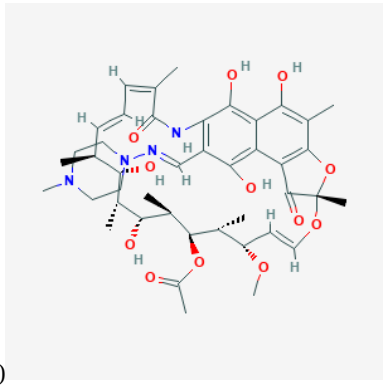
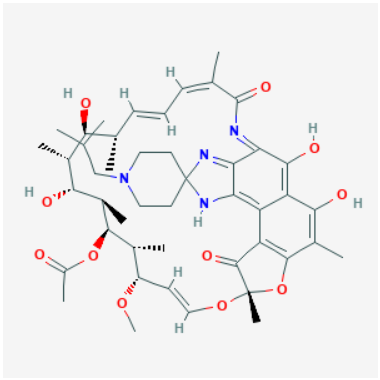
15-20% of them are susceptible to rifabutin [19]. Although that confers a significant advantage, Rifabutin (log K = 7,93 and log Po/w = 4,25) to treat patients with MDR tuberculosis is limited by its high cost and the restricted availability [20].

Literature reveals several methods to determine this sort of compounds such as Liquid Chromatography (LC) and HPLC coupled with Mass Spectrometry (LC/MS) [21-24]. However, these methods require pre-steps on sample preparations, with several extraction and purification steps due to complex chemical composition of plasma. Besides, they need an expensive specific instrumentation, a large amount of toxic solvents and qualified staff, which means increment price per analysis result.

To carry out the study we use micellar liquid chromatography or MLC as an analysis technique. Due to its advantages, MLC has been proven as a useful technique for routine analysis of a wide range group of drugs based on physicochemical properties of substances. MLC use hybrid mobile phases of sodium dodecyl sulfate to drug determination in biological fluids. Micelles and monomers tend to bind proteins and other macromolecules competitively releasing bound drugs. Therefore, they are denatured and solubilized. In the column they are harmless and eluted at the front of the chromatogram instead of precipitating, allowing direct injection of the sample. Besides, the retention mechanism in the three-interaction environment is highly stable and reproducible, enabling its modeling by chemometrics. Another advantage is that the use of micellar mobile phases is non-toxic, non-flammable, biodegradable and relatively inexpensive if compared to aqueous-organic solvents [25].

The aim of the work is developing a reliable, rapid, practical, easy-to-handle and inexpensive procedure to determine Rifampicin and Rifabutin in plasma and urine. Another goal is analyse advantages of the method and its possible establishment as a reference method of analysis. The method will be fully validated through FDA 2018 Bioanalytical Method Validation Guidance for Industry to evaluate its analytical performance and used in incurred plasma and urine samples to ensure its suitability for routine analysis.

Figure 1. A) Rifampicin and B) Rifabutin structure.

A)  B) 

2. Experimental

2.1. Reagents and solutions

Rifampicin and Rifabutin were supplied by Sigma (St. Louis, MO, USA). The other reagents used were sodium dodecyl sulphate (SDS) from Merck (Darmstadt, Germany), sodium dihydrogen phosphate, HCl, NaOH from Panreac (Barcelona, Spain), and methanol, propanol, butanol and pentanol from J.T. Baker (Holland). Ultrapure water was produced by Millipore S.A.S. (Molsheim, France) and used to prepare the aqueous solutions and mobile phases. The rifampicin and rifabutin standard solutions were prepared by solubilizing the appropriate amount in ultrapure water, and the mixture was ultrasonicated for 2 min. Fresh solutions were prepared weekly. Blood samples were collected using a DB SST Tube (BD Vacutainer Systems, Plymouth, UK), and were centrifuged for 5 min at 756 Relative Centrifugal Force (RCF) or G-force at 4ºC; plasma was kept.

2.2. Apparatus

The chromatograph used was an Agilent Technologies Series 1100 (Palo Alto, CA, USA) equipped with a quaternary pump, a mobile phase degasifier, an autosampler and a temperature controller for the column module coupled to a fluorescence detector. A Kromasil 5 C18 column with a 5 μm particle size and 150 mm (4.6mm i.d.) (Scharlau, Barcelona) was used. Chromatographic signals were acquired and processed with the HP ChemStation Rev. B.01.01. An ultrasonic bath Ultrasons-H (Selecta, Abrera, Spain) was used to dissolve the standards and the pH of the solutions was measured with a Crison GLP 22 (Barcelona) potentiometer equipped with a combined Ag/AgCl/glass electrode. The analytical balance used was a Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland).

2.3. Chromatographic conditions

Separation and quantification of Rifampicin and Rifabutin were performed using a mobile phase containing 0.15 M SDS-6%(v/v)/n-pentanol/phosphate buffer at pH 3, running at a constant flow of 1.0 mL/min. The buffer was made adding 0.01 M of sodium dihydrogen phosphate, adjusting the pH by using suitable volumes of HCl 0.1 M. The mobile phase was filtered through 0.45 μm nylon membranes from Micron Separations (Westboro, MA, USA). Injection volume was 20 μL. Under these conditions, the Kromasil C18 column head pressure was 150 bars. The column temperature was kept constant at 25ºC. The wavelength for measurements was set at 270 nm.

3. Results and discussion

Experimental sequential and interpretative designs

The simultaneous separation and quantification of the analytes within the minimum analysis time and the maximum resolution and efficiency are the main objectives in the development of the MLC method for determination of solutes in biological samples. Thus, an optimization strategy might be considered helpful to find the best chromatographic conditions. The optimization strategy may be sequential or interpretative [26,27]. Sequential strategies have proved to have shortcomings when several local and/or secondary maxima exist; in fact, the optimum spot did not always correspond to the best maximum. Thus, the interpretative strategy may be much more efficient and reliable. Moreover, the interpretative strategy allows taking into account independent variables. In the interpretative strategy the experiments are designed before the optimization process and used to fit a model that allows prediction of the parameters in a wide range of space. An interpretative strategy was chosen for use in the present work.

One of the interpretative strategies is called factorial experiment design and is based on varying all factors simultaneously at a limited number of factor levels. This kind of experimentation is especially important at the beginning of experimental study, where the most influential factors, their ranges of influence and factor interactions are not yet known. Factorial experiments allow experiments to take place over the whole range of the factor space. They show a high degree of precision in exchange for a minimum experimental effort and they enable factor interactions to be detected.

3.1. Parameters selection

The mobile phase selection was based on the resolution and a suitable analysis time of the two compounds. The octanol–water partition coefficient (logPo/w) of Rifampicin and Rifabutin are 2,7 and 4.25 respectively [Drugbank], which means that it is hydrophobic in

nature and can be expected to have high retention in MLC.

In the development of the MLC method for the determination of Rifampicin and Rifabutin, eight factors were identified that influence the separation analytes: SDS and modifier concentrations, type of buffering species, running buffer pH, column length, column temperature, sample injection volume and flow rate. The criteria for the selection of the optimum separation conditions were the maximum separation of the analytes in the mixture within the minimum analysis time. Thus, peak width (i.e., resolution) and lower analysis retention time were parameters of primary importance to assess the goodness of the experimental result.

First of all, buffer selected was phosphate because it is useful to study the change in the chromatographic response over a wide range of pH, in this case from 3 to 7 due to it is the adequate for the column care. After studying pH range 3-7, the pH of 3 was selected due to was the most suitable pH in terms of lower retention time and better resolution, improving the retention time in more than ten minutes compared to pH 5 and no peak were observed at pH 7. Second, molarity of the buffering agent was observed to have less influence on the separation and was therefore kept constant at 0.1 M. Third, the column length was studied making efficiency increase a 10% in a column of 25 cm compared to one of 15 cm, but retention time and analysis increased in a 35%, so a column of 15 cm was selected. Fourth, the influence of the temperature was checked in the range from 25 to 40ºC, result shows that this parameter does not improve the resolution or analysis time: 25ºC was selected for further studies. Fifth, the studies were focused in the injection volume, changing it between 0.5-30 µL, observing overloading at 25 µL: an injection volume of 20 µL was selected. Sixth, flow rate was experimented from 0.5 to 2 mL/min showing a decrease of analysis time, but decreasing the resolution dramatically, so 1 mL/min was selected. Finally, SDS and modifier concentrations have an important influence and were optimized using a factorial design.

Methanol, Propanol, Butanol and Pentanol are the most used short-chain alcohols as modifiers. All of them were checked noting that Rifabutin is more susceptible to modifier concentration. Using pentanol acts reducing retention time in more than an hour in Rifabutin

and more than fifteen minutes in Rifampicin compared to methanol. As a retention time is a priority parameter, pentanol was selected as the modifier for the separation of Rifampicin and Rifabutin. Also, the use of pentanol improves resolution peak, significantly.

3.2. Mobile phase selection using factorial designs

The effects of SDS concentrations in the range of 0.05 to 0.15 M and pentanol in the range 2 to 6% (v/v) were selected. Low values of both parameters could produce sub-micellar media, and upper the maximum level, microemulsions.

To evaluate the influence of SDS and pentanol on the separation we used a three-level full factorial design (3^2). The parameter settings and the design are reproduced in Table 2. The runs of the design were carried out in a randomized sequence and the retention times and peak widths were measured. To estimate the experimental error, replications of factor combinations were necessary. The center point was run three times. Varying all factors simultaneously at a limited number of factor levels, and after the calculation of the function responses, a polynomial curve was obtained. As responses, two different functions were checked: first, the sum of resolutions and, second, the chromatographic resolution statistic (CRS) function. The resolution (R) of a pair of peaks was calculated using Eq. (1):

$$R = \frac{2(t_2 - t_1)}{(w_2 + w_1)}$$

where t_1 and t_2 are the migration times and w_1 and w_2 are the width at the peak base of two consecutive peaks, measured as time units. The numerator in Eq. (1) describes the separation process with regard to differential migration and the denominator expresses the dispersive

processes acting against it. The total resolution (Rs) was set as response and calculated as the sum of the resolutions of the all pairs of peaks,

$$Rs = \sum_{o}^{i} R,$$

where i is the number of analytes. To quantify and interpret the relationships between responses and factor effects a response surface method was used. The general empirical model is a second-order polynomial, where the response y is related to the variables (factors) x as:

$$y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{1 \leqslant i \leqslant j}^{k} b_{ij} x_i x_j + \sum_{i=1}^{k} b_{ii} x_i^2$$

where k is the number of variables (factors), b_0 is the intercept parameter, and b_i,b_{ij}, and b_{ii} are regression parameters for linear, interaction, and quadratic factor effects. The nonlinear regression analysis of the data was carried out using the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA. The data for the modeling are listed in Table 2) and the model obtained was:

$$\begin{aligned} Rs = & -23.03 - 16.72\mathrm{pH} + 44.12\mathrm{V} + 2.12\mathrm{pH}^2 \\ & - 2.70\mathrm{V}^2 - \mathrm{A09pHV} - 0.09\mathrm{pH}^2\mathrm{V} + 0.31\mathrm{pHV}^2 \end{aligned}$$

The three-dimensional plot of total Rs, as a function of SDS of the running buffer and pentanol. The surface plot allows the whole range of conditions to be explored, including combinations that were not experimentally demonstrated, indicating that the maximum resolution area corresponds to 0,15 M and to a pentanol concentration of 5% (v/v). Thus, these conditions were considered to be the optimum MLC conditions to separate the two antituberculosis drugs, including the band of proteins in the serum and urine samples. The same optimum was achieved when a second response function is used, which is the inverse of the chromatographic resolution statistic. The CRS is a mathematical function calculated with Eq. (5):

$$\mathrm{CRS} = \left\{ \sum_{i=1}^{n-1} \left[\frac{(R_{i,i+1} - R_{opt})^2}{(R_{i,i+1} - R_{\min})^2 R_{i,i+1}} \right] + \sum_{i=1}^{n-1} \frac{R_{i,i+1}^2}{(n-1)R_{av}^2} \right\} \frac{t_n}{n}$$

where $R_{i,\,i+1}$ is the resolution between consecutive peaks, and R_{av} is the average resolution of all peaks, and R_{min} is the minimum acceptable resolution, which has a value of 1. Considering that peak width is 1 min, two peaks, which appear contiguous, have a difference in time of 1 min. Thus, Rs calculus shows that the result is 1 (minimum acceptable separation). R_{opt} is the desired resolution, the optimum resolution, in this case 1.5. Rs is 1.5 if the two peaks are separated by a time of 0.5 min. t_n is the migration time of the last-eluting solute and n is the number of compounds in the sample. The CRS considers the resolution of all solutes in the sample and incorporates three important aspects of the separation. The first term in Eq. (5), named the resolution term, evaluates the resolution between all adjacent solute pairs in comparison to defined values for optimum and minimum resolution. The second term in Eq. (5), named the distribution term, considers the relative spacing of the solute zones. The final multiplier term in Eq. (5) takes into consideration the analysis time and the number of analyte peaks to be separated. The CRS values obtained for each electrophoretic condition and also the

inverse of CRS are shown in Table 2. The inverse of CRS was chosen because the maximum of the function fits the optimal condition. The response surface of this response function (inverse of CRS) was compared with the results obtained with the Rs function. Fig. 1B shows the surface plot and the maximum of this function coincided with the optimum conditions obtained with the Rs function. The mathematical model obtained was Eq. (5).

$$CRS^{-1} = -0.16 + 0.04pH + 0.11V - 0.001pH^2 - 0.005V^2 - 0.03pHV + 0.001pH^2V + 0.001pHV^2. \quad (6)$$

To summarize, the conditions selected were as follows: SDS 0,15 M-pentanol 6%-$H_2PO_4^-$ 0.1 M at pH 3 in a Kromasil C18 column of 15 cm thermostated at 25ºC, with 20 µL of injection, and a flow rate of 1 mL/min.

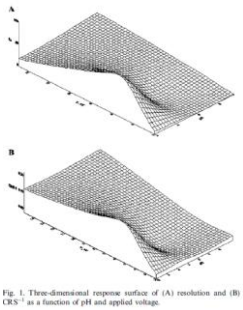


Fig. 1. Three-dimensional response surface of (A) resolution and (B) CRS^{-1} as a function of pH and applied voltage.

Table 2
3^2 Full factorial design and response obtained

Run	pH	V(kV)	*Rs*	CRS	CRS^{-1}
1	4.7	15	17.80	9.01	0.11
2	2	5	90.91	4.68	0.21
3	2	2	5.00	266.97	3.74E-3
4	9.5	5	9.80	465922.1	2.14E-6
5	4.7	2	1.93	154.49	6.47E-3
6	4.7	5	10.99	473090.6	2.11E-6
7	9.5	2	0.11	193.01	5.18E-3
8	2	15	15.87	8.48	0.12
9	9.5	15	30.06	7.98	0.13

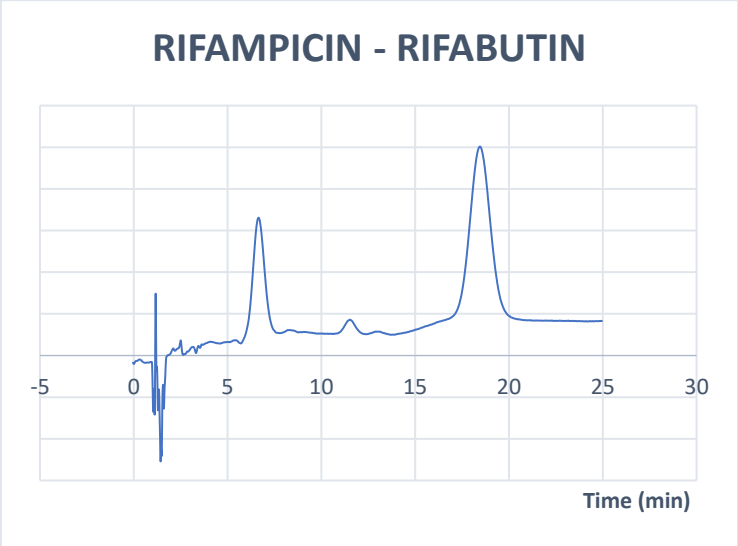


Figure 2. Chromatogram obtained for Rifampicin (6.6 min) and Rifabutin (18.4).

3.3. Method validation

This methodology for the determination of Rifampicin and Rifabutin in plasma of Tuberculosis patients has been validated according to the FDA 2018 Bioanalytical Method Validation Guidance for Industry which includes linearity, limits of detection (LOD) and quantification (LOQ), intra- and inter-day precisions, selectivity, recovery and robustness.

3.3.1. Selectivity

To study the matrix effects in plasma of the possible co-eluting compounds, ten blank samples were analysed. The protein band appeared before 5 min, and there were no peaks near the analytes time. A plasma sample spiked with 10 µg/mL of Rifampicin and another spiked with 10 µg/mL of Rifabutin were analysed. The peaks of the two analytes are sufficiently separated between them, which avoids overlapping.

3.3.2. Linearity and sensitivity

A blank was simultaneously spiked at nine concentration levels of Rifampicin and Rifabutin in the range 0.5-15 μg/mL. The slope and intercept were obtained by a correlation between the areas of the chromatographic peak of each analyte from a triplicate injection versus the concentration by least-square linear regression. Calibration was repeated five times (preparing the sample on each occasion) over a 2-month period, and each calibration level was analysed six times. The LODs and LOQs were set as three and ten times the standard deviation of the blank, respectively. The regression curves and limits were:

Rifampicin: A= (22.1958067 ± 150) [RIFAMPICIN] - (26.16404222 ± 250), r^2 = 0.999384217

LOD = 0,3 μg/mL; LOQ = 0,4 μg/mL

Rifabutin: A= (38.8366798 ± 30) [RIFABUTIN] - (21.21874863 ± 50), r^2 = 0.99933098

LOD = 0,1 μg/mL; LOQ = 0,3 μg/mL

where A is the signal in arbitrary units and the concentration amount is provided in μg/mL.

3.3.3. Precision and accuracy

The intra- and inter-day accuracy and precision of the proposed methodology were determined for Rifampicin and Rifabutin with plasma samples spiked at three concentration levels (0.5; 2.5; 5 μg/mL). The intra-day analysis was determined by injecting aliquots of these samples six times on the same day, while the inter-day analyses correspond to the average of five measurements of the intra-day values taken over a 3-month period. The results, are expressed as variation coefficients for accuracy and relative error for precision, are shown in The data show good accuracy (<14%) and adequate precision (2.7–8.5%) for both analytes, which are useful for routine analyses.

3.3.4. Carry-Over Effect

A urine sample spiked with Rifampicin and Rifabutin at 5 μg/mL was injected, and immediately afterwards, a blank urine sample were analyzed. In this last one, no peak was observed in the chromatogram at Rifampicin or Rifabutin retention time. Thus, the carry-over was considered negligible at concentrations within the calibration range.

3.3.5. Matrix Effects

The influence of the endogenous compounds of urine in the quantitative results, either by linking or any interaction interfering with the retention process, was evaluated. Working standard solutions containing the same concentration of Rifampicin and Rifabutin as in Section 3.2.4 divided by five, were analyzed by following the same protocol to consider the dilution in the experimental procedure. The results for the intra-day accuracy and precision were similar to those obtained for urine samples. Therefore, no significant matrix effect was detected, regardless of the complexity of the chemical composition of urine. This was probably done by the interaction of the endogenous compounds of the urine and the SDS-micelles.

3.3.6. Dilution Integrity

The effect of the introduction of another dilution step was investigated. Urine samples spiked at 15 μg/mL were 1/10 diluted and then processed as in Section 2.4. The intra-day accuracy and precision were determined. The results were inside the acceptance criteria, and the method allows a sample over ULOQ to be analyzed after the appropriate dilution.

3.3.7. Robustness

The variation of the main chromatographic responses (retention factor, peak area and

efficiency) was examined at small, but deliberate, changes in the main operational parameters. These were changed from their optimal value, and the tested oscillations were that we considered that may occur during the laboratory work and chromatographic run, in a usual situation. The studied factors and the studied interval were: A) detection wavelength ± 5 nm; B) SDS in mobile phase ± 0.05 M; C) pentanol in mobile phase ± 0.2%; D) pH in mobile phase ± 0.2; E) flow-rate ± 0.05 mL/min and F) injection volume ± 2 μL. A blank plasma sample spiked with 2.5 mg/L was analyzed by testing eight different set of experimental conditions, which value was fixed by an experimental design following a Youden approach [28]. Differences > 8.0% were judged significant, meaning that they may be especially controlled during the analysis. The retention factor was significantly affected by the concentration of SDS in mobile phase and the low-rate, the peak area by the concentration of SDS and the injection volume, and the efficiency by the flow and the injection volume. Therefore, these factors operational parameters must be especially controlled during analysis to achieve adequate analytical results.

3.4. Application of the methodology to the patient´s plasma

Plasma studies were performed following the oral administration of a single dose of 600mg of Rifampicin or 300mg of Rifabutin to several patients with tuberculosis disease. Samples were taken over a 24 h period. The obtained chromatogram from sample patient of Rifampicin and Rifabutin could be clearly quantified without interferences.

3.5. Stability & decomposition studies

Stability means the capacity of the drug to remain unchanged throughout time. It can be studied under different environmental conditions of light, temperature, chemical environment – among others. It is determined by the monitoring of the analyte concentration in a stored solution or sample. The degradation is noticed by the diminishing of the

concentration and the emergence of peaks from decomposition products. As reported in the literature, rifampicin is a highly unstable drug and readily decomposes in Rifampicin quinone due to oxidation [29] while Rifabutin is quite stable, being more affected by thermal and light conditions [30]. The stability was studied from solutions or spiked samples containing 1.0 μg/ml of Rifampicin and without Rifampicin quinone. Results were found similar for plasma and urine. The investigated conditions were:

- Standard solution in a mobile phase kept in the fridge for one week. Under these conditions, Rifampicin was found stable up to 4 days (90% decay), while Rifabutin was stable up to 7 days. Therefore, the standard solutions must be renewed after this period;

- Short term stability of the analyte in the processed biological fluid at room temperature for 1 day. The analyte decays in nearly 1 day, and then a prepared sample must be analyzed a maximum of 1 day after the dilution. This is a quite long period and to not hinder the normal work at the laboratory in routine conditions. The sample throughput is limited to 96 samples per day, a high value;

- In the biological fluid long term freeze at -20◦C for 2 weeks (the usual storage conditions in a hospital). A short decay (90%) was noticed at the end of the experiment. Therefore, the extracted sample can be stored until 15 days before analysis, without affecting the measured concentration of Rifampicin or Rifabutin.

An additional conclusion of the study was that the decomposition is accelerated as the temperature increased.

4. Conclusion

Micellar liquid chromatography has been proved as a suitable technique to analyse Rifampicin and Rifabutin in human plasma. One advantage of this procedure is the possibility of injecting a diluted sample into the chromatographic system, avoiding long and tedious extractions. After sample irradiation to improve sensitivity, the analyte was satisfactorily resolved using a mobile phase of 0.15 M SDS-6%(v/v)/n-pentanol/phosphate buffer at pH 3 from the matrix in an analysis time of under 20 min. Validation was performed according to the FDA 2018 Bioanalytical Method Validation Guidance for Industry with satisfactory results in terms of linearity, selectivity, precision, accuracy and recovery. The limit of detection and the lineal range were sufficient to detect the usual amount of Rifampicin and Rifabutin in patient´s plasma. Also, stability studies could be performed using the purposed method.

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Conclusion

Conclusion

Conclusion

Micellar liquid chromatography (MLC) is a reversed-phase liquid chromatographic (RPLC) mode with a mobile phase consisting in an aqueous solution of surfactant above its critical micellar concentration (CMC). The idea of using pure micellar solutions as mobile phases in RPLC is very attractive given their lower cost, less toxicity and poorer environmental impact. In practice, however, the addition of a small amount of organic solvent to the micellar solution is needed to achieve retention in practical time windows, and to improve peak efficiency and resolution.

Micellar mobile phases have been used with different bonded stationary phases (mostly C8, C18 and cyanopropyl). The most common surfactants are anionic sodium dodecyl sulphate (SDS), cationic cetyltrimethylammonium bromide (CTAB), and non-ionic Brij–35. Several organic solvents have been used as modifiers, of which short/medium chained alcohols and acetonitrile are the most suitable. The presence of micelles helps keep these organic solvents in solution at concentrations well above their solubility in water, and the risk of evaporation diminishes.

Above the CMC, a change in surfactant concentration leads to an increased concentration of micelles in the solution, whereas the number of monomers of the surfactant in the mobile phase remains constant. Adsorption of an approximately fixed amount of surfactant monomers in the stationary phase is also produced, giving rise to a stable modified column and to regular retention behaviour.

The retention mechanisms (e.g., solute-mobile phase and solute-stationary phase interactions) in MLC are fairly well understood, and there is a reasonable theoretical foundation on which to build. MLC is a fascinating example of the benefits of secondary equilibria in RPLC. The primary equilibrium is solute partitioning between bulk solvent (e.g., water or the aqueous-organic mixture) and the stationary phase. A secondary equilibrium is established with the micelles in the mobile phase. Both equilibria are affected by a variety of factors, such as the type and concentration of surfactant and additives (e.g., salts or organic modifiers), temperature, ionic strength and pH. The complexity of MLC is much greater than conventional RPLC with aqueous-organic mixtures.

Conclusion

MLC shares the basic components of RPLC systems, a non-polar stationary phase and a polar aqueous mobile phase. However, hydro-organic mobile phases in conventional RPLC are homogeneous, whereas micellar solutions are microscopically heterogeneous and composed of two distinct media: amphiphilic micellar aggregates (micellar pseudo phase), and the surrounding bulk water or aqueous-organic solvent that contains surfactant monomers in an approximate concentration equal to the CMC.

Retention behavior in MLC depends on the interactions established by the solute with the surfactant-modified stationary phase and micelles. Neutral solutes eluted with non-ionic and ionic surfactants, and charged solutes eluted with non-ionic surfactants, will only be affected by non-polar, dipole–dipole and proton donor–acceptor interactions. Besides these interactions, charged solutes will interact electrostatically with ionic surfactants (e.g., with the charged surfactant layer in the stationary phase and the charged outer layer of micelles). In any case, the steric factor is relevant.

The separation process in a micellar chromatographic system requires a structured approach in the development of practical applications. The resolution of complex mixtures should be made and optimised in a short time, and minimal consumption of reagents. Empirical and mechanistic models have been developed to model the retention behaviour of solutes.

Fundamental studies in MLC have focused mainly on surfactant adsorption, retention behaviour, measurement of partition coefficients, peak efficiency and selectivity. This knowledge has allowed a theoretical description of MLC, and a better understanding and use of the technique. Applications have greatly benefited from these research works.

This thesis describes several methods for the separation and determination of different groups of antibiotics, antitumoral, as well as related compounds (rivaroxaban and pyridoxine) in a wide variety of matrices (pharmaceuticals, urine, plasma and food samples), by direct injection and isocratic elution in micellar mobile phases that contain sodium dodecyl sulphate (SDS) and an organic solvent (short-chain alcohol):

Conclusion

1. Food contaminant: Melamine.
2. Antitumoral group Tyrosine Kinase Inhibitors (TKIs): Afatinib, Axitinib, Dabrafenib, Lapatinib, Pazopanib, Regorafenib.
3. Oral anticoagulant: Rivaroxaban.
4. Antibiotic group of Fluoroquinolones: Flumequine, Marbofloxacin, Difloxacin, Sarafloxacin, Oxolinic acid, Ciprofloxacin, Enrofloxacin, Sarafloxacin.
5. Antibiotic antituberculotic group: Isoniazid, Rifampicin, Rifabutin, Pyridoxine and Rifampicin Quinone.

Analytical method validation

Validation of an analytical method should enable the verification of its suitability, as well as the capacity of both staff and the laboratory, which is based on the procedure's statistical parameters. The methods developed by a laboratory must be fully validated before use. The procedures and scope of validation are not always the same and should be set individually.

Validation requires guaranteeing the suitability of the results obtained, so laboratories should work in accordance with proper guidelines which are established by Good Laboratory Practices (GLP). Analytical methods should be validated or re-validated by the laboratory: prior to routine use, each time the original validated conditions are changed, or when the method is modified without this being planned.

Validation is arranged in three blocks: (1) establishment of the conditions to be performed, (2) determination of the procedure's statistical parameters, and (3) evaluation of the validation results by comparing the statistical parameters obtained under the established conditions, and the decision making on the validity of the procedure for the intended purpose. This process ends when the method can fulfill the established purpose.

The quality parameters of an analytical method to be checked are: selectivity, linearity, limits of detection and quantification, accuracy, precision, recovery, and robustness, whose definition can be found in several IUPAC or ISO documents, among others. Guidelines differ

depending on the validation matrix to be analyzed. This thesis is focused on the following guidelines: International Conference on Harmonization (ICH), Food and Drug Administration (FDA), European Medicines Agency (EMA), and the European Commission Decision 2002/657/EC.

Analysis in biological fluids and pharmaceuticals samples

Antitumoral TKIs (Afatinib, Axitinib, Dabrafenib, Lapatinib, Pazopanib and Regorafenib), antibiotics antituberculosis (Rifampicin, Rifabutin, Isoniazid), and Pyridoxine were analyzed in plasma and urine samples using UV detection. Samples were directly injected into the chromatographic system after dilution, which allows using the same column to analyze a large number of samples, thus avoiding the possibility of poor reproducibility of extraction procedures. Micelles tend to bind proteins competitively, thereby releasing proteinbound drugs and proteins. Thus, proteins are solubilized in micellar media and washed harmlessly away to elute with the solvent front rather than precipitating into the column. The analysis revealed the relevance of urine samples dilution before injection to avoid an unnecessary exposure of the column to samples containing high concentrations of proteins, and to reduce the wide protein band at the beginning of the chromatograms, as well as the peaks of endogenous compounds. For most drugs, the sensitivity achieved after dilution was adequate for urine detection at 24 hours after its ingestion. On the other hand, the retention times remained unchanged under these conditions after approximately 250 injections (maximum number of injections performed with the same mobile phase without cleaning the column). It should be noted that the experiments included in this thesis were done by recycling the micellar mobile phase in order to reduce procedure cost. Columns Kromasil C18 were used in all the studies.

The direct injection procedures herein developed can be applied in routine control analyses in plasma and urine samples. The chromatographic conditions selected are shown in **Table 1**. The values of relative standard deviation (RSD) for intra-and interday precisions were less than 8.5%.

Conclusion

The low efficiency of the chromatographic peaks in MLC compared to that obtained using the hydro-organic mobile phases has been widely discussed in the literature. However, this is not a general behavior since it has been demonstrated that the efficiencies of amino compounds are better in MLC. On the other hand, the peak width limits the possibility of systematic drugs research. It should be noted that all the studies herein presented were performed with an isocratic elution. MLC allows the analysis of compounds with a very different hydrophobicity using a single mobile phase. The number of compounds that can be resolved in an adequate analysis time should be evaluated under these conditions. The use of a gradient elution would enhance this prospect, although this has not been investigated in this thesis.

One of the advantages of MLC is the high reproducibility of compounds, retention time and the possibility of their prediction using simple models, which have a chemical-physical justification. In the procedures outlined in this section, the retention time error prediction was always lower than 3%. Modelling retention allows the use of interpretive optimization through factorial design strategies to resolve mixtures of compounds. These strategies entail a lower number of experiments than sequential strategies, whose application is difficult when there are changes in the elution order of compounds when varying the mobile phase composition. In addition, the results obtained with interpretive strategies are more reliable. Chromatographic separation quality was measured by the application of the elemental criterion of the overlapped fractions, which takes into account the position and shape of the chromatographic peaks. The individual values of the overlapped fractions, obtained for each peak, should be combined to quantify the global resolution of the compounds mixture in a chromatogram. In all cases, the normalized product of elementary resolutions was used which provides a more realistic and global resolution.

After selecting the pH of the mobile phase, an interpretative optimization procedure of mobile phase composition was performed considering the surfactant and organic solvent (short-chain monoalcohol) concentrations. TKIs group have an octanol/water partition coeffcient in the range of 3.6-5.5 and pka in the range of 4.8-8.8. This means that this group need a neutral or basic pH to be neutral charged and can it be detected. For this reason, pH 7 was selected as

optimum chromatographic parameter. In other hand, pH 3 was selected to antituberculosis drugs in order to ensure the positive charged form, enhancing the resolution and retention time.

The methods were validated according to the EMA guidelines (TKIs, Isoniazid, Rifampicin and Pyridoxine), ICH (Rivaroxaban in pharmaceuticals), and FDA (Rifampicin, Rifabutin, Rivaroxaban in plasma and urine). The parameters studied were: selectivity, linearity, limit of detection and quantification, accuracy, precision, recovery, and robustness; which obtained good results in all cases.

The plasma and urine samples analysis showed that the developed methods could be applied in a pharmacokinetic study due to the limits of quantification were adequate considering that the sample was diluted before analysis.

Rivaroxaban also was analyzed in pharmaceuticals tablets. The label claim percentage values were in the 95–105% range and the RSD fell in the 0.1–2.4% range. In all cases, the results fall within the contents declared by the manufacturers.

Analysis in food samples

The aim of the European food surveillance programme is law enforcement antibiotic residues in products of animal origin, and they strongly depend on the availability of rapid, precise, and economic methods for analytical determination. The European Food Safety Authority (EFSA) has introduced legislation on the authorization of veterinary medicines and has established maximum residual levels (MRLs) for these drugs in different tissues of animal origin for human consumption. Thus, there is a continuing need to develop new reliable analytical methods for determining compliance with national and international regulations in all quality and food safety areas.

The antibiotic fluoroquinolone group was analyzed in different kind of meats, eggs and egg products, following the European Commission Decision 2002/657/EC. In these matrices it was possible to resolve mixtures of seven (fluoroquinolones), with log Po/w values in range:

1.1–2.9. The column used in all cases was a Kromasil C18, while optimization was carried out as described for biological fluid samples.

The developed method was applied to spiked eggs and meat samples, where recoveries fell in the 83.7–103.0% range. Pointing out that the sample preparation procedure is suitable for this type of analysis. Flumequine, Marbofloxacin, Difloxacin and Sarafloxacin were determined by fluorescence detection in meat (pork, beef, chicken, turkey, duck, sheep, goat, rabbit, and horse), using the programme time and wavelengths of excitation/emission: 0.0–8.5 min, 240/370 nm for Flumequine; 8.5–11.5 min, 300/488 nm for Marbofloxacin; 11.5–20 min, 280/455 nm for Difloxacin and Sarafloxacin.

Oxolonic acid, Ciprofloxacin, Enrofloxacin and Sarafloxacin were determined by fluorescence detection in egg and egg products, using the programme time and wavelengths of excitation/emission: 0–10 min, 260/366 nm for Oxolinic acid, and 10.01-30: 280–455 nm for Ciprofloxacin, Enrofloxacin, and Sarafloxacin

In the robustness study, five factors were selected to examine variation on the retention time of antibiotics: a) SDS (M), b) propanol/butanol (%), c) pH, d) flow rate, and e) TEA (%). Most remained sufficiently unaffected by slight changes to these parameters (RSD < 6%), except flow rate and the variation of SDS concentration, which have more influence on the retention of the studied compounds (RSD < 10%). In addition to the validation parameters previously specified for biological samples, the decision limit (CC_α) and detection capability (CC_β) were also determined. The chromatographic conditions selected are shown in **Table 2**.

The method was applied to determine the seven quinolones in meat and eggs samples, which were obtained with satisfactory recoveries for all the compounds (94.3-113.7%). The sample treatment consisted in homogenization, extraction and filtration prior to injection into the chromatographic system. The advantage over usual extraction procedures is that no organic solvent was used. Briefly, samples were finely ground using a mincer. Then, an amount was weighed, homogenized and mixed with a solution of 0.05 M SDS–pH 3, and stirred for 1 h. Finally, the supernatant was filtered and injected into the chromatographic system.

Conclusion

The methods were enough accurate and precise, (RSD < 9% and precision <14%). The recoveries obtained fell in the 84–107% range. The results point out that the proposed chromatographic methods can be applied to a residue control programme for fluoroquinolones in meat, egg and egg products.

Conclusion

Drug	Optimum mobile phase	Detection	Wavelength (nm)	Analysis time (min)	Sample	Guideline
Afatinib	0.07 M SDS-6%(v/v) 1-pentanol phosphate buffered at pH 7	UV	260	15.2	plasma	EMA
Axitinib	0.07 M SDS-6%(v/v) 1-pentanol phosphate buffered at pH 7	UV	260	4.2	plasma	EMA
Dabrafenib	0.12 M SDS-2%(v/v) 1-pentanol phosphate buffered at pH 7	UV	260	8.8	plasma	EMA
Lapatinib	0.07 M SDS-6%(v/v) 1-pentanol phosphate buffered at pH 7	UV	260	12.5	plasma	EMA
Pazopanib	0.12 M SDS-2%(v/v) 1-pentanol phosphate buffered at pH 7	UV	260	3.4	plasma	EMA
Regorafenib	0.12 M SDS-2%(v/v) 1-pentanol phosphate buffered at pH 7	UV	260	15.9	plasma	EMA
Rifampicin	0.15 M SDS-6%(v/v) 1-pentanol phosphate-buffered at pH 7	UV	337	5.8	plasma urine	EMA
Rifampicin	0.15 M SDS-6%(v/v) n-pentanol phosphate-buffered at pH 3	UV	270	6.6	plasma urine	FDA
Rifampicin quinone	0.15 M SDS-6%(v/v) 1-pentanol phosphate-buffered at pH 7	UV	337	11.8	plasma urine	EMA
Rifabutin	0.15 M SDS-6%(v/v) n-pentanol phosphate-buffered at pH 3	UV	270	18.4	plasma urine	FDA
Rivaroxaban	0.05 M SDS-12.5%(v/v) 1-Propanol phosphate-buffered at pH 7	UV	250	4.6	plasma urine	FDA
Rivaroxaban	0.05 M SDS-12.5%(v/v) 1-Propanol phosphate-buffered at pH 7	UV	250	4.6	pharma-ceuticals	ICH
Isoniazid	0.15 M SDS-6%(v/v) 1-Pentanol phosphate-buffered at pH 7	UV	265	4	urine	EMA
Isoniazid	0.15 M SDS-8%(v/v) Butanol phosphate-buffered at pH 3	UV	265	3.8	plasma	EMA
Pyridoxine	0.15 M SDS-8%(v/v) Butanol phosphate-buffered at pH 3	UV	292	5	plasma	EMA

Table 1. Summary of the chromatographic conditions of the developed methods in biological fluids.

Conclusion

Drug	Optimum mobile phase	Detection	λex/λem (nm)	Analysis time (min)	Sample	Guideline
Ciprofloxacin	0.05 M SDS 7.5%(v/v) 1-propanol 0.5% triethylamine buffered at pH 3	FLD	280/455	16	egg	EU 2002/657/ EC
Difloxacin	0.05 M SDS 8%(v/v) 1-butanol 0.5% triethylamine buffered at pH 3	FLD	280/455	13.6	meat	EU 2002/657/ EC
Enrofloxacin	0.05 M SDS 7.5%(v/v) 1-propanol 0.5% triethylamine buffered at pH 3	FLD	280/455	18.8	egg	EU 2002/657/ EC
Flumequine	0.05 M SDS 8%(v/v) 1-butanol 0.5% triethylamine buffered at pH 3	FLD	240/370	7.3	meat	EU 2002/657/ EC
Marbofloxacin	0.05 M SDS 8%(v/v) 1-butanol 0.5% triethylamine buffered at pH 3	FLD	300/488	10.2	meat	EU 2002/657/ EC
Oxolinic acid	0.05 M SDS 7.5%(v/v) 1-propanol 0.5% triethylamine buffered at pH 3	FLD	260/366	5.5	egg	EU 2002/657/ EC
Sarafloxacin	0.05 M SDS 8%(v/v) 1-butanol 0.5% triethylamine buffered at pH 3	FLD	280/455	16.9	meat	EU 2002/657/ EC
Sarafloxacin	0.05 M SDS 7.5%(v/v) 1-propanol 0.5% triethylamine buffered at pH 3	FLD	280/455	22.3	egg	EU 2002/657/ EC

Table 2. Summary of the chromatographic conditions of developed methods in food.

Annex 1. Original contributions

The studies have been carried out thanks to the funding received from the Jaume I University (project: UJI-B2018-20) and Generalitat Valenciana (project: AICO/2017/063). The work carried out has been disseminated through the following original contributions as articles in leading scientific journals in the area of Bioanalytical Chemistry and as papers in prestigious scientific conferences:

Scientific Research Journals Articles

1) **Advances on melamine determination by micellar liquid chromatography**. J Peris-Vicente, J Albiol-Chiva, P Roca-Genoves, J Esteve-Romero. (2016). Journal of Liquid Chromatography & Related Technologies 39 (7), 325-338

2) **A review on development of analytical methods to determine monitorable drugs in serum and urine by micellar liquid chromatography using direct injection**. J Esteve-Romero, J Albiol-Chiva, J Peris-Vicente. (2016). Analytica Chimica Acta 926, 1-16

3) **Development and validation of a micellar liquid chromatographic method to determine three antitumorals in plasma**. JE Romero, JA Chiva, J Peris-Vicente, E Ochoa-Aranda. (2017). Bioanalysis 9 (9), 799-812

4) **Development of a method to determine axitinib, lapatinib and afatinib in plasma by micellar liquid chromatography and validation by the European Medicines Agency guidelines**. J Albiol-Chiva, J Esteve-Romero, J Peris-Vicente. (2018). Journal of Chromatography B 1074, 61-69

5) **Optimization and Validation of a Chromatographic Method for the Quantification of Isoniazid in Urine of Tuberculosis Patients According to the European Medicines Agency Guideline**. Mishra, P., Albiol-Chiva, J., Bose, D., Durgbanshi, A., Peris-Vicente, J., Carda-Broch, S., & Esteve-Romero, J. (2018). Antibiotics, 7(4), 107

6) **A rapid and reliable assay to determine flumequine, marbofloxacin, difloxacin, and sarafloxacin in commonly consumed meat by micellar liquid chromatography**. J Peris-Vicente, JJ Iborra-Millet, J Albiol-Chiva, S Carda-Broch, ... (2019). Journal of the science of food and agriculture 99 (3), 1375-1383

7) **Stability studies of rifampicin in plasma and urine of tuberculosis patients according to the European Medicines Agency Guidelines**. P Mishra, RP Pawar, D Bose, A Durgbanshi, J Albiol-Chiva, ... (2019). Bioanalysis 11 (08), 713-726

8) **Micellar liquid chromatography determination of rivaroxaban in plasma and urine. Validation and theoretical aspects**. J Albiol-Chiva, J Peris-Vicente, D García-Ferrer, J Esteve-Romero. (2019). Journal of Chromatography B 1120, 8-15

9) **Procedure for the Screening of Eggs and Egg products to detect Oxolonic acid, Ciprofloxacin, Enrofloxacin and Sarafloxacin using Micellar Liquid Chromatography**. Peris-Vicente, J., García-Ferrer, D., Mishra, P., Albiol-Chiva, J., Durgbanshi, A., Carda-Broch, S., ... & Esteve-Romero, J. (2019). *Antibiotics*, *8*(4), 226.

10) **An Assay to Determine Rivaroxaban in Pharmaceutical Formulations by Micellar Liquid Chromatography** (in publication process)

11) **Analysis of Isoniazid and Pyridoxine in Plasma samples of Tuberculosis patients by MLC** (in publication process)

12) **Quantification of Rifampicin and Rifabutin in Plasma of Tuberculosis Patients by Micellar Liquid Chromatography** (in publication process)

Scientific International Research Congress Communications

49th International Symposium on High Performance Liquid Phase Separations and Related Techniques. HPLC Kyoto 2019. Kyoto, Japan.

1) Development and Validation of a method to determine Rivaroxaban in Plasma and Urine by Micellar Liquid Chromatography. Study of the associated retention mechanism. Jaume Albiol Chiva; Juan Peris Vicente; Samuel Carda Broch; Daniel García Ferrer; Josep Esteve Romero; Diego Enrique Kassuha.

2) Micellar Liquid Chromatography to quantify Axitinib, Lapatinib and Afatinib in Plasma. Validation of the method by the European Medicines Agency guidelines. Jaume Albiol Chiva; Josep Esteve Romero; Daniel García Ferrer; Juan Peris Vicente; Samuel Carda Broch.

3) An assay to determine Pazopanib, Dabrafenib and Regorafenib by direct injection – Micellar Liquid Chromatography. Optimization and Validation. Josep Esteve Romero; Jaume Albiol Chiva; Juan Peris Vicente; Samuel Carda Broch; Enrique Ochoa Aranda.

4) Preparation, Characterization and potential therapeutic effect in Hypertension treatment of Anandamide/Polycaprolactone nanoparticles. Virna Margarita Martín Giménez; Carlos Gamarra Luques; Marcos Russo; Griselda Narda; Lucía Fuentes; Luciana Mazzei; Diego Kassuha; Josep Esteve Romero; Walter Manucha; Jaume Albiol Chiva; Samuel Carda Broch; Juan Peris Vicente.

5) Analysis of Rifampicin and Rifabutin in Plasma of Tuberculosis Patients by Micellar Liquid Chromatography. Jaume Albiol Chiva; Juan Peris Vicente; Josep Esteve Romero; Samuel Carda Broch; Abhilasha Durgbanshi; Devasish Bose; Pooja Mishra.

6) Determination of Isoniazid and B6 vitamin in Tuberculosis patients by Micellar Liquid Chromatography. Jaume Albiol Chiva; Juan Peris Vicente; Josep Esteve Romero; Samuel Carda Broch; Abhilasha Durgbanshi; Devasish Bose; Pooja Mishra; Daniel García Ferrer.

7) Stability of Rifampicin and Quinone derivative in Plasma samples of Tuberculosis Patients by MLC. Jaume Albiol Chiva; Juan Peris Vicente; Josep Esteve Romero; Samuel Carda Broch; Abhilasha Durgbanshi; Devasish Bose; Pooja Mishra; Mar Esteve Amorós.

8) Antibiotic Quinolone Surveillance in most Consumed Meats. Juan Peris Vicente; Jaume Albiol Chiva; Josep Esteve Romero; Samuel Carda Broch; Pau Esteve Amorós; Diego Kassuha.

32nd International Symposium on Chromatography. ISC 2018. Cannes-Mandelieu, France.

9) Chemometrically Treatment of Optimisation by Factorial Design. Josep Salvador Esteve Romero; Juan Peris Vicente; Jaume Albiol Chiva; Samuel Carda Broch; Misericordia Jiménez; Jose Vte. Gimeno Adelantado; José Vte. Gómez; Andrea Tarazona; Rufino Mateu Castro; Eva Mateo.

10) Determination of Tyrosine Kinase Inhibitors in Human Plasma of Cancer Patients by Micellar Liquid Chromatography. Jaume Albiol Chiva; Josep Salvador Esteve Romero; Juan Peris Vicente; José Vte. Gimeno Adelantado; Diego Enrique Kassuha; Devasish Bose; Abhilasha Durgbanshi; Samuel Carda Broch; Rufino Mateu Castro; Eva Mateo.

11) Optimization and Validation by HPLC Method for Quantification of Rifampicin in Plasma and Urine Samples from Tuberculosis Patients. Jaume Albiol Chiva; Devasish Bose; Pooja Mishra; Rajendra Prasad Pawar; Abhilasha Durgbanshi; Josep Salvador Esteve Romero; Juan Peris Vicente; Samuel Carda Broch; Rufino Mateo Castro; Eva Mateo.

12) Determination of deoxynivalenol in oat grains by liquid chromatography triple quadrupole-mass spectrometry. Josep Salvador Esteve Romero; José Vicente Gómez; Andrea Tarazona;

Juan Peris Vicente; José Vicente Gimeno Adelantado; Jaume Albiol Chiva; Samuel Carda Broch; Misericordia Jiménez; Eva Mateo.

13) MLC as a Useful Method for Amoxicillin, Ampicillin, Cloxacillin and Dicloxacillin Determination in Pharmaceuticals and Urine. Jaume Albiol Chiva; Josep Salvador Esteve Romero; Juan Peris Vicente; Jesús Javier Iborra Millet; Diego Enrique Kassuha; Micaela Flores; Gerardo Castro Ocampo; Debasish Bose; Samuel Carda Broch; Eva Mateo.

14) Monitoring of Ochratoxin an Occurrence in The Plasma of Healthy Blood Donors by HPLC. Josep Salvador Esteve Romero; Jose Vte. Gómez; Andrea Tarazona; Juan Peris Vicente; José Vte. Gimeno; Jaume Albiol Chiva; Samuel Carda Broch; Misericordia Joménez; Eva Mateo.

15) Pharmacogenetic of Polymorphisms in HIV and Renal Transplanted Patients. Jaume Albiol Chiva; Diego Enrique Kassuh; Micaela Flores; Gerardo Castro Ocampo; Josep Salvador Esteve Romero; Juan Peris Vicente; Samuel Carda Broch; Jesús Javier Iborra Millet; Eva Mateo.

16) Accelerated solvent extraction of H-2 and HT-2 toxins in oat grains for HPLC analysis. Josep Salvador Esteve Romero; José Vicente Gómez; Andrea Tarazona; Juan Peris Vicente; José Vicente Gimeno Adelantado; Jaume Albiol Chiva; Samuel Carda Broch; Misericordia Jiménez; Eva Mateo.

17) Monitoring of Quinolones Use in Livestock Farming by MLC. Jaume Albiol Chiva; Josep Salvador Esteve Romero; Juan Peris Vicente; Devasish Bose; Pooja Mishra; Rajendra Prasad Pawar; Rufino Mateo Castro; Abhilasha Durgbanshi; Samuel Carda Broch; Abhishek Jain; Jesús Javier Iborra Millet; Eva Mateo.

18) Simulated Biological Conditions for Allopurinol Derivatives by MLC. Josep Salvador Esteve Romero; Jaume Albiol Chiva; Juan Peris Vicente; José Vicente Gimeno Adelantado; José Vicente Gómez; Andrea Tarazona; Samuel Carda Broch; Rufino Mateo Castro; Misericordia Jiménez; Eva Mateo.

International Conference on Chemistry & Material Science. 2017. Roma, Italy.

19) Micellar liquid chromatography determination of sildenafil. Jaume Albiol Chiva; Pascual Roca Genovés; Juan Peris Vicente; Samuel Carda Broch; Josep Salvador Esteve Romero; D. Kassuha; A. Garcia.

20) Polyphenols determination from olive oil industry wastes and correlation with its antioxidant activity. Pascual Roca Genovés; Jaume Albiol Chiva; Juan Peris Vicente; Josep Salvador Esteve Romero; Samuel Carda Broch; D. Kassuha; Khaled Tayeb Cherif.

21) Development of micellar liquid chromatographic method to evaluate the quality of tamoxifen citrate tablets. Jaume Albiol Chiva; Pascual Roca Genovés; Juan Peris Vicente; Samuel Carda Broch; Josep Salvador Esteve Romero; E. Ochoa Aranda; D. Bose.

22) Analysis of tamoxifen and its main matabolites in plasma from breast cancer patients by micellar liquid chromatography to compare the effect in women and men. Jaume Albiol Chiva; Pascual Roca Genovés; Juan Peris Vicente; Samuel Carda Broch; Josep Salvador Esteve Romero; E. Ochoa Aranda; D. Bose.

23) Development of a method to detect four fluoroquinolones in honey by micellar liquid chromatography and validation by eu regulation 2002/657/EC. Pascual Roca Genovés; Jaume Albiol Chiva; Juan Peris Vicente; Josep Salvador Esteve Romero; Samuel Carda Broch.

24) Determination of thiabendazole, o-phenylphenol, pyrimethanyl and imazalil in environmental water by micellar liquid chromatographic. Pascual Roca Genovés; Jaume Albiol Chiva; Juan Peris Vicente; Josep Salvador Esteve Romero; Samuel Carda Broch; D. Kassuha.

25) Determination of the most prescribed B-lactam antibiotics by HPLC. Pascual Roca Genovés; Jaume Albiol Chiva; Juan Peris Vicente; Josep Salvador Esteve Romero; Samuel Carda Broch; E. Ochoa Aranda; D. Kassuha.

26) Advantages of direct injection in bioclinical analysis. Jaume Albiol Chiva; Pascual Roca Genovés; Juan Peris Vicente; Josep Salvador Esteve Romero; Samuel Carda Broch; A. García; D. Bose.

27) Determination of tamoxifen and endoxifen in plasma samples from breast cancer patients. Jaume Albiol Chiva; Pascual Roca Genovés; Juan Peris Vicente; Josep Salvador Esteve Romero; Samuel Carda Broch; D. Bose; E. Ochoa Aranda; A. Garcia.

28) Human clinical genetic tests: validation procedure. Pascual Roca Genovés; Jaume Albiol Chiva; Juan Peris Vicente; Samuel Carda Broch; Josep Salvador Esteve Romero; E. Ochoa Aranda; A. García.

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29) Quantification of Thiabendazole and O-phenyphenol in water by direct injection – micellar liquid chromatography - fluorescence detection. Jaume Albiol Chiva; Juan Peris Vicente; Khaled Tayeb Cherif; Josep Salvador Esteve Romero; Pascual Roca Genovés; Iris Garrido Cano; Samuel Carda Broch.

30) Reverse phase high performance liquid chromatography method to quantify of acyclovir in rat plasma. Jaume Albiol Chiva; Enrique Ochoa Aranda; Pascual Roca Genovés; Juan Peris Vicente; Josep Salvador Esteve Romero; Samuel Carda Broch; Aurelio Garcia Garcia.

31) Direct injection - micellar liquid chromatography to determine seven antiretroviral drugs in human plasma. Jaume Albiol Chiva; Josep Salvador Esteve Romero; Aurelio Garcia Garcia; Samuel Carda Broch; Juan Peris Vicente; Diego Kassuha; Pascual Roca Genovés.

32) Determination of Citalopram, Paroxetine and Fluoxetine in Biological Fluids by Direct Injection - Micellar Liquid Chromatography - Fluorescence Detection. Jaume Albiol Chiva; Pascual Roca Genovés; Aurelio Garcia Garcia; Juan Peris Vicente; Samuel Carda Broch; Khaled Tayeb Cherif; Josep Salvador Esteve Romero.

33) Micellar liquid chromatography -Fluorescence detection to quantify four quinolones in honey. Jaume Albiol Chiva; Khaled Tayeb Cherif; Juan Peris Vicente; Enrique Ochoa Aranda; Samuel Carda Broch; Pascual Roca Genovés; Josep Salvador Esteve Romero.

34) A Green Analytical Method to Determine Melamine in Swine Kidney based on Micellar Liquid Chromatography. Jaume Albiol Chiva; Josep Salvador Esteve Romero; Juan Peris Vicente; Samuel Carda Broch; Iris Garrido Cano; Pascual Roca Genovés; Enrique Ochoa Aranda.

European Symposium on the Practical Applications of Analytical Technologies in the Biopharmaceutical Industry (ATEurope 2016). Viena, Austria.

35) Development and Validation of a Method to Detect Eight Fluoroquinolones in Honey Using Micellar Liquid Chromatography - Fluorescence Detection. J. Albiol Chiva; K. Tayeb Cherif; J. Peris Vicente; P. Roca Genovés; Josep Salvador Esteve Romero; Samuel Carda Broch.

36) Use of Micellar Liquid Chromatography to Analyse Thiabendazole, Tert-octylphenol and

Chlorpyrifos in Wastewater. J. Albiol Chiva; J. Peris Vicente; Josep Salvador Esteve Romero; Samuel Carda Broch; P. Roca Genovés.

37) Determination of Melamine in Several Matrices by Micellar Liquid Chromatography. J. Albiol Chiva; Samuel Carda Broch; P. Roca Genovés; J. Peris Vicente; Josep Salvador Esteve Romero.

38) Use of Micellar Liquid Chromatography to Quantify Several Quinolones in Porcine and Bovine Flesh. J. Albiol Chiva; K. Tayeb Cherif; Samuel Carda Broch; P. Roca Genoves; Josep Salvador Esteve Romero; J. Peris Vicente.

39) Quantification of the Antidepressants Citalopram, Paroxetine and Fluoxetine in Plasma, Urine and Tablets by Micellar Liquid Chromatography. J. Albiol Chiva; Josep Salvador Esteve Romero; J. Peris Vicente; A. García García; Samuel Carda Broch; P. Roca Genovés.

40) Analytical Determination of Paracetamol in Serum and Urine by Micellar Liquid Chromatography with Electrochemical Detection. J. Albiol Chiva; Josep Salvador Esteve Romero; S. Carda Broch; A. Garcia Garcia; J. Peris Vicente; P. Roca Genoves.

41) Determination of Antibiotics in Pharmaceuticals and Physiological Samples by Micellar Liquid Chromatography. J. Albiol Chiva; Samuel Carda Broch; K. Tayeb Cherif; A. García García; J. Peris Vicente; P. Roca Genovés; Josep Salvador Esteve Romero.

42) Comparison of the Concentration of Antiretroviral in Serum Samples of Men and Women Using Micellar Liquid Chromatography. J. Albiol Chiva; P. Roca Genoves; Samuel Carda Broch; Josep Salvador Esteve Romero; J. Garcia Garcia; J. Peris Vicente; Enrique Ochoa de Aranda.

43) Comparing the Effect of the Improvement of Tamoxifen Treatment Efficiency in Breast Cancer Patients with a Poor Metabolizer Genotype in Men and Women. J. Albiol Chiva; Enrique Ochoa de Aranda; J. Peris Vicente; A. Garcia Garcia; P. Roca Genovés; Josep Salvador Esteve Romero; Samuel Carda Broch.

44) Relationship Between Tamoxifen/Endoxifen Concentration Ratio and CYP2D6 Genotipe in Men and Women. J. Albiol Chiva; P. Roca Genovés; Enrique Ochoa de Aranda; Josep Salvador Esteve Romero; J. Peris Vicente; Tayeb-Cherif; A. Garcia Garcia.

Annex 1. Original contributions

Annex 2. Future research lines

The main research line of the Bioanalytical Chemistry group is focus on the development on fast, simple, economic, safe, practical, and reliable analytical methods for the determination of endogenous drugs and metabolites in biological matrices. These methods must be applicable for clinical, forensic and toxicological analysis. Furthermore, pursue obtaining a sustainable chemistry on working routines laboratories.

In the future, I will collaborate on research projects with the Bioanalytical Chemistry group of the Jaume I University, which are related to the clinical and health field. I plan to develop and validate analytical methods for the drugs studied in other biological fluids and tissues (urine, gastric juices, organs, feces, etc.). To do this, the chromatographic behavior of the matrix should be studied and experimental conditions variation in case of interference. A method of leaching analytes for solid or very viscous matrices should be optimized. The methods could be used together for a complete study of the pharmacokinetics of each drug (bioavailability, distribution, metabolization and elimination). The retention of analytes and matrices should be previously modeled, depending on the composition of the mobile phase, to solve them in the shortest possible time. The detection conditions will also be optimized, depending on their physicochemical properties, to maximize the selectivity and the signal-to-noise ratio. The most prescribed, innovative and most interesting drugs will be selected, in collaboration with the Hospital´s guidance. Thus, doctors could apply them to patients and use the information obtained for individualization dose treatment, with the aim of increasing therapeutic efficacy.

Anexo 3. Aceptación de los coautores de las publicaciones que integran la tesis, que el doctorando presenta el trabajo como tesis y renuncia expresa de éstos presentarlos como parte de otra tesis doctoral (según Art. 23 de la NORMATIVA DELS ESTUDIS DE DOCTORAT, REGULATS PEL RD 99/2011, EN LA UNIVERSITAT JAUME I (Aprovada pel Consell de Govern núm. 19 de 26 de gener de 2012)

Josep Esteve Romero, director de la presente tesis, declara que los coautores de las publicaciones que se presentan en esta tesis, y que paso a enumerar: S. Carda Broch, J. Peris Vicente, E. Ochoa Aranda, P. Roca Genovés, J. Iborra Millet, D. García Ferrer, D. Bose, A. Durgbanshi, P. Mishra, i R. Pawar no utilitzaran el material que aquí se presenta para formar parte de otra tesis. Para que conste dónde convenga, se aporta la firma de los coautores.

Josep Esteve Romero

Juan Peris Vicente

Enrique Ochoa Aranda

Pascual Roca Genovés

Jesus Iborra Millet

Daniel García Ferrer

Samuel Carda Broch

Devasish Bose

Abhilasha Durgbanshi

Pooja Mishra

Rajendra Pawar

Universitat Jaume I, 2 de Octubre de 2019

Anexo 4. Abreviaturas y acrónimos

Analitos

AFA: Afatinib

AXI: Axitinib

B_6: Pyridoxine

CIPRO: Ciprofloxacin

DABRA: Dabrafenib

DIFLOX: Difloxacin

ENRO: Enrofloxacin

FLUME: Flumequine

ISON: Isoniazid

LAPA: Lapatinib

MEL: Melamine

MARBO: Marbofloxacin

OXO: Oxolinic acid

PAZO: Pazopanib

REGORA: Regorafenib

RIFAB: Rifabutin

RIFAMP: Rifampicin

RIVAROX: Rivaroxaban

SARA: Sarafloxacin

Reactivos de laboratorio

CTAB: bromuro de cetiltrimetilamonio (*Cetyl Trimethyl Ammonium Bromide*)

HCl: ácido clorhídrico

NaH_2PO_4: dihidrogenofosfato de sodio

NaOH: hidróxido de sodio

SDS ($C_{12}H_{25}SO_4Na$): dodecil sulfato sódico *(Sodium Dodecyl Sulfate)*

TEA: Trietanolamina

Terminos médicos

AUC: área bajo la curva (*area under the curve*)

ATC: acrónimo de Anatomical, Therapeutic, Chemical classification system

Cl: Aclaramiento renal

$C_{mín}$: concentración plasmática mínima

$C_{máx}$: concentración plasmática máxima

Frecuencias: MF, muy frecuente; F, frecuente; PF, poco frecuentes; R, raros; MR, muy raros;

NACO: nuevos anticoagulantes orales / new anticoagulants oral

LD_{50}: dosis mediana letal (*Median Lethal Dose*)

MRLs: maximum residual levels

$t_{1/2}$: vida media

TDM: Monitorización terapéutica de fármacos (*therapeutic drug monitoring*)

TKIs: Tirosine Kinase Inhibitors

$t_{máx}$: tiempo para alcanzar la Cmáx tras administración

Instituciones

AEMPS: *Agencia Española de Medicamentos y Productos Sanitarios*

EMA: *Agencia Europea de Medicamentos / European Medicines Agency*

EFSA: The European Food Safety Authority

FDA: *U.S. Food and Drug Administration*

ICH: *International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use*

IUPAC: *Unión Internacional de Química Pura y Aplicada / International Union of Pure and Applied Chemistry*

ISO: *Organització Internacional de Normalització / International Organization for Standardization*

OMS: Organización Mundial de la Salud / World Health Organization

Técnicas analíticas

DAD: Detector de matriz de diodos (*Diode Array Detection*)

ECD: Detección electroquímica (*electrochemical detector*)

HILIC: Cromatografía líquida de interacción hidrofílica (*hydrophilic interaction liquid chromatography*)

HPLC o RP-HPLC: cromatografia líquida en fase inversa (*High Performance Liquid Chromatography- Reversed Phase*)

LC-ECD: HPLC acoplada a un detector electroquímico

HPLC-UV: HPLC acoplada a un detector de absorbancia UV-visible de onda variable

IPC: Cromatografía de pares de iones (*ion-pairing chromatography*)

MLC: Cromatografía líquida micelar (*micellar liquid chromatography*)

UPLC: Cromatografía líquida de ultrarresolución (*ultra-high performance liquid chromatography*)

MS: Espectrómetro de masas (*mass spectrometry*)

UV: Ultravioleta

Parámetros químicos y cromatográficos

CMC: concentración micelar crítica (*critical micellar concentration)*

K_a: constante de desprotonación de un ácido

K_{AS}: producto del coeficiente de reparto soluto (A)-fase estacionaria (S) por la relación de fases

K_{AM}: constante de asociación soluto-micela (M)

K_{MS}: constante de reparto de soluto entre las micelas y la fase estacionaria

K_{AD}, K_{AD1}, K_{AD2}: variación de la concentración de soluto en la fase móvil a causa del disolvente orgánico

K_{MD}, K_{MD1}, K_{MD2}: variación de la concentración de soluto en las micelas a causa del disolvente orgánico

K_{SD}: variación de la concentración de soluto en la fase estacionaria a causa del disolvente orgánico

Po/w : coeficiente de reparto octanol-agua *(octanol-water partition coefficient)*

Parámetros cromatográficos

α_{ij}: selectividad cromatográfica

α'_{ij}: selectividad modificada

φ: proporción de disolvente orgánico (v/v)

A: área de pico

B/A: Asimetría

k: factor de retención

H_0: altura máxima de pico

h (t): señal del cromatograma al tiempo *t*

N: número de platos teóricos (eficacia)

R o *Z*: resolución global

r_i o $r_{i;i+1}$: resolución individual y elemental

t: tiempo transcurrido desde la inyección

t_0: tiempo muerto

t_R: tiempo de retención

Parámetros de validación

LOD: límite de detección (*Limit of Detection*)

LLOQ: límite mínimo de cuantificación (*Lower Limit of Quantitation)*

r^2: coeficiente de determinación (*determination coefficient*)

RSD: desviación estándar relativa (*Relative Standard Derivation)*

QC: control de calidad (*Quality Control*)

ULOQ: límite máximo de cuantificación (*Upper Limit of Quantitation*)