DETERMINACIÓ D´ANTIBIÒTICS EN MOSTRES BIOLÒGIQUES PER ELECTROFORESI CAPIL·LAR

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Memoria presentada per

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El Dr. FRANCESC BORRULL I BALLARÍN, Professor Titular del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili, i

La Dra. MARTA CALULL I BLANCH, Professora Titular del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili,

CERTIFIQUEM:

Que la present Tesi Doctoral, que duu per títol: "DETERMINACIÓ D´ANTIBIÒTICS EN MOSTRES BIOLÒGIQUES PER ELECTROFORESI CAPIL·LAR", presentada per MARGARITA HERNANDEZ SOCIAS per optar al grau de Doctor en Química, ha estat realitzada sota la nostra direcció, a l´Àrea de Química Analítica del Departament de Química Analítica i Química Orgànica d´aquesta universitat, i que tots els resultats presentats són fruit de les experiències realitzades per la doctoranda esmentada.

I, per a que consti, expedim aquest certificat a Tarragona, 4 d´octubre de 2002.

Dr. Francesc Borrull i Ballarín Dra. Marta Calull i Blanch

Un cop acabada la redacció de la tesi es fa especialment agradable escriure unes línies per agrair a tota aquella gent que l'ha fet possible, a tota aquella gent que m'ha recolzat directa o indirectament en el seu desenvolupament.

En primer lloc vull agrair l'ajuda dels meus tutors, el Dr. Francesc Borrull i la Dra. Marta Calull. Ells m'han ajudat molt al llarg de tota la tesi, i especialment aquests últims mesos, durant la redacció de la mateixa, la qual per diverses raons ha resultat molt llarga i de vegades dura.

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ÍNDEX

OBJECTIU

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El principal objectiu de la present Tesi Doctoral ha estat aprofundir en l'aplicació de l'electroforesi capil·lar per a la determinació d'antibiòtics en mostres biològiques. Dins d'aquest objectiu principal se'n poden destacar de més específics com:

- El desenvolupament de mètodes analítics per a la determinació de diferents famílies d'antibiòtics per electroforesi capil·lar.

- L'aplicació de l'electroforesi capil·lar com a tècnica analítica per a la determinació de residus d'antibiòtics en mostres biològiques, estudiant diferents sistemes de tractament de mostra.

- L'estudi de sistemes de preconcentració *on-column* que permetin determinar els antibiòtics per electroforesi capil·lar a baixos nivells de concentració.

INTRODUCCIÓ

Els antibiòtics són substàncies que s'utilitzen per la seva acció antimicrobiana. Al principi només s'obtenien de manera natural, a partir de bacteris i fongs, però actualment també es poden obtenir per síntesi química. El terme antibiòtic inclou un grup divers de compostos químics diferents entre sí que l'únic que tenen en comú és la seva activitat antibacteriana. Hi ha una gran varietat de famílies d'antibiòtics, entre les quals es poden destacar les β-lactames, quinolones, tetraciclines i aminoglicòsids.

La inhibició microbiana dels diferents antibiòtics es realitza per mecanismes diferents. L'elecció d'un antibiòtic en el tractament d'una infecció depèn del microorganisme, de la seva sensibilitat o resistència, la gravetat de l'enfermetat, la toxicitat, els antecedents d'al·lèrgia del pacient i el cost. En infeccions greus de vegades és necessari combinar l'acció de dos antibiòtics [1,2].

Els antibiòtics han estat àmpliament utilitzats tant en medicina humana com en veterinària per tal de prevenir i tractar malalties. En medicina humana és important el control dels antibiòtics que s'administra als pacients. S'ha d'administrar l'antibiòtic dins del que es coneix com a dosi terapèutica. En dosis superiors l'antibiòtic pot ser tòxic per l'home produint-li al·lèrgies o d'altres efectes secundaris [3,4]. Com en medicina humana, els antibiòtics en veterinària s'utilitzen per prevenir i tractar enfermetats. Encara que principalment s'utilitzen per estimular el creixement dels animals destinats al consum humà, el que pot causar la creació d'antibiorresistència, o bé pot produir al·lèrgies en els individus que consumeixin aquests productes. Per altra banda, els residus no només de la droga administrada sinó també dels seus metabòlits poden ser tòxics o més tòxics que els antibiòtics dels quals provenen [5-7].
Degut a la importància que hi hagi un control en els productes farmacèutics, la Unió Europea (EU) va establir el que es coneixen com a límits màxims de residus (MRL) per a les drogues veterinàries, que són els valors que indiquen la quantitat màxima d'antibiòtic que pot romandre en un teixit o producte animal (com ous, llet, etc...) destinats al consum humà [7-10]. Els valors de MRL

normalment són valors baixos de concentració, de l'ordre de μg o mg d'antibiòtic per kg de teixit o altres productes animals destinats al consum humà [11].

Per poder controlar que se segueix la normativa establerta, en els últims anys s'han desenvolupat un gran nombre de metodologies analítiques per a determinar i establir la identificació i quantificació dels antibiòtics en diferents matrius. Les tècniques cromatogràfiques, sobretot la cromatografia de líquids, són les eines analítiques més utilitzades per a la separació i determinació d'aquests compostos en mostres biològiques [12-14]. Recentment s'ha desenvolupat l'ús de les tècniques electroforètiques com a alternativa a aquest tipus d'estudis [15,16].

Degut a la complexitat de les mostres biològiques generalment aquestes no es poden introduir directament en el sistema electroforètic o cromatogràfic, de manera que molts cops és necessari desenvolupar sistemes per extreure i purificar el compost que es vol analitzar. D'aquesta manera es disminueix la quantitat de substàncies interferents causades pels components de la matriu, els quals normalment es troben en concentracions molt més grans que els compostos d'interès. En funció del tipus i complexitat de la mostra es poden utilitzar diferents tractaments de la mostra, entre els quals es poden destacar la microdiàlisi, la ultrafiltració, la ultracentrifugació, la desproteïnització, l'extracció líquid-líquid (LLE) i l'extracció en fase sòlida (SPE) [16-18].
Com en aquestes mostres els antibiòtics normalment es troben a concentracions molt baixes, de vegades és necessari augmentar la sensibilitat dels sistemes analítics per dur a terme la seva anàlisi, ja sigui utilitzant detectors més sensibles com els detectors de fluorescència, electroquímics, etc... els quals poden augmentar la sensibilitat del sistema en diversos ordres de magnitud [19,20]. O bé, recentment el que s'han desenvolupat són sistemes de preconcentració on-column en electroforesi capil·lar, els quals fan que la preconcentració tingui lloc dins el mateix capil·lar [20-24].

L'estructura general de la memòria d'aquesta tesi es pot dividir en quatre parts ben diferenciades. La primera, corresponent al capítol I, on es comenten les principals característiques dels antibiòtics i la seva aplicació tant en medicina humana com en veterinària. També inclou la legislació establerta pel control del seu ús i es descriuen les característiques i aplicacions d'algunes de les famílies d'antibiòtics més utilitzades.

En la segona part, corresponent al capítol II, es comenten quines han estat les principals tècniques utilitzades en l'anàlisi dels compostos en estudi i les seves aplicacions. Es descriuen diferents sistemes per dur a terme el tractament de les mostres, així com sistemes que permeten augmentar la sensibilitat de l'electroforesi capil·lar, destacant l'ús de sistemes on-column.

La tercera part de la memòria, corresponent al capítol III, descriu la part experimental que s'ha dut a terme durant aquesta tesi doctoral i s'inclouen els diferents treballs que n'han sorgit. Cada treball està precedit d'una breu introducció en la que s'explica quin ha estat l'objectiu de l'esmentat treball així com les conclusions que se n'extreuen.

En la quarta i última part de la memòria, es recullen les conclusions sorgides arran del treball i dels resultats obtinguts en la present tesi.

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CAPÍTOL I. ANTIBIÒTICS

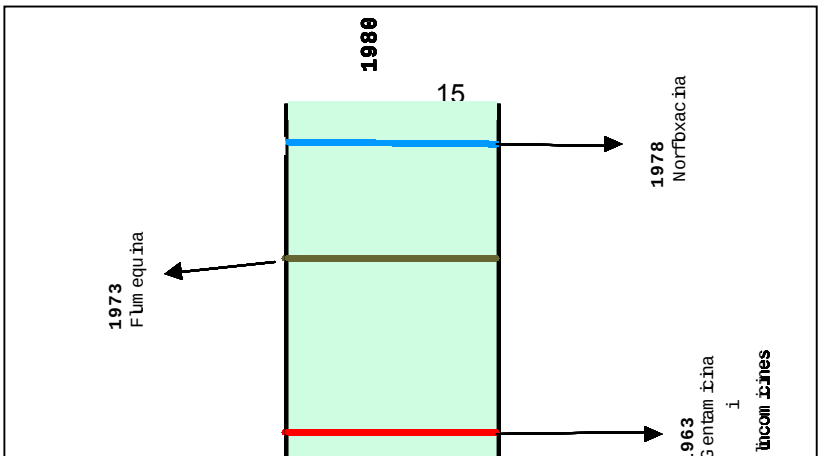
I.1. DEFINICIÓ I CLASSIFICACIÓ

Els antibiòtics són drogues que ataquen els bacteris, ja sigui destruint o inhibint la seva reproducció. El terme antibiòtic va ser proposat per Wasman, el descobridor de l'estreptomicina, ja que són substàncies amb activitat antimicrobiana i que van ser extretes d'estructures orgàniques vives.

L'elecció d'un antibiòtic en el tractament d'una infecció depèn del microorganisme, de la seva sensibilitat o resistència, la gravetat de l'enfermetat, la toxicitat, els antecedents d'al·lèrgia del pacient i el cost. En infeccions greus de vegades és necessari combinar l'acció de dos antibiòtics.

És important conèixer la resistència relativa dels microorganismes per a cada antibiòtic conegut per no administrar al malalt un antibiòtic que no tingui efecte sobre els gèrmens causants de la seva enfermetat. Els microbiòlegs i clínics utilitzen un índex per a determinar l'activitat antimicrobiana que es coneix com a concentració més baixa d'antibiòtic capaç d'inhibir el creixement d'un determinat germen [1].

Malgrat tot, els antibiòtics són capaços de prevenir el creixement i multiplicació de diferents tipus de bacteris. La inhibició microbiana pels diferents antibiòtics es realitza per mecanismes diferents. Això no és sorprenent ja que el terme antibiòtic inclou un grup divers de compostos químics diferents entre sí que l'únic que tenen en comú és la seva activitat antibacteriana. Entre els anys 60 i 80 es van estudiar extensament els mecanismes gràcies als quals els antibiòtics específics interactuen amb els components de les cèl·lules microbianes. A la Figura 1 es mostren alguns dels antibiòtics que han estat descoberts al llarg del segle XX.



Els antibiòtics es poden administrar oralment (càpsules, sobres), tòpica (coliris, gotes, etc.) o injectable (intramuscular o intravenosa). En infeccions greus normalment s'administren per via intravenosa [1,2].

A la bibliografia es poden trobar diferents classificacions dels antibiòtics depenent del criteri utilitzat. Així podem posar una classificació, segons el seu origen, segons l'acció bactericida, segons l'espectre bacterià i segons a quina part de la cèl·lula ataquen:

• *Segons el seu origen:*

Poden ser d'origen natural, semisintètic i sintètic:

- Els antibiòtics naturals són substàncies químiques produïdes per diverses espècies de microorganismes (bacteris i fongs) i que poden ser bactericides o bacteriostàtics. Un gran nombre d'antibiòtics naturals han estat identificats, i pràcticament una centena han estat desenvolupats pel tractament d'enfermetats infeccioses. Altres exemples d'antibiòtics naturals són: l'estreptomicina, el cloramfenicol, tetraciclines i macròlids.

- Els antibiòtics semisintètics deriven dels antibiòtics naturals. S'obtenen a partir de petites alteracions en l'estructura química d'aquests. Exemples d'aquest tipus d'antibiòtics són la fenoximetilpenicil·lina, la nafcil·lina i la cloxacil·lina.

- Els antibiòtics sintètics són sintetitzats químicament. El primer compost sintetitzat va ser el Prontosil. És el precursor de les sulfamides. Posteriorment es van desenvolupar les sulfamides que es caracteritzen per la seva activitat terapèutica en enfermetats infeccioses. Altres exemples més recents són els nitrofurans i les quinolones [2].

La distinció d'antibiòtics naturals i antimicrobians sintètics no és de vital importància, ja que per exemple el cloramfenicol originàriament era un producte natural i ara es produeix mitjançant síntesi química.

• *Segons l'acció bactericida:*

Es poden distingir dos grups:

- Antibiòtics bactericides els quals maten o provoquen la dissolució del bacteri invasor. Alguns exemples són: β-lactames, glicopèptids, aminoglicòsids, quinolones i polimixines.

- Antibiòtics bacteriostàtics que només inhibeixen el creixement bacterià i la duplicació. Els agents bacteriostàtics compten amb les defenses de l'hoste (és a dir en l'organisme on es troba) per a detenir la infecció i si es suspèn la teràpia antibiòtica el creixement bacterià es pot reprendre. Com per exemple: macròlids, tetraciclines, cloramfenicol, clindamicina, lincomicina i sulfamides.

Malgrat això, aquesta divisió no és molt estricte ja que els agents bacteriostàtics poden arribar a ser bactericides si s'incrementa la seva concentració i viceversa.

• *Segons l'espectre bacterià*

L'acció d'un antibiòtic es mesura en termes d'espectre bacterià. L'acció dels antibiòtics està restringida a una àrea petita de bacteris patògens, ja siguin grampositius o gramnegatius. Aquells antibiòtics que són eficaços contra bacteris grampositius i gramnegatius s'anomenen antibiòtics d'espectre ampli [3]. D'aquesta manera antibiòtics com la penicil·lina actuen en un sector restringit (bacteris grampositius), per això se'ls anomena d'espectre limitat. En canvi, d'altres antibiòtics com les tetraciclines i el cloramfenicol actuen contra bacteris grampositius i contra un gran número de bacteris gramnegatius, pel que se'ls coneix com antibiòtics d'espectre ampli.

• *Segons a quina part de la cèl·lula ataquen*

Els antibiòtics es poden agrupar d'acord al lloc de la cèl·lula on actuen. Els cinc punts més importants d'atac es mostren a la Figura 2.

A continuació es mostren alguns exemples d'antibiòtics per a cada mecanisme d'acció [2,3]:

→ Inhibició de la síntesi de la paret cel·lular:

- β-lactàmics: Són bactericides i poc tòxics. En aquest grup es poden incloure cinc subgrups: penicil·lines, cefalosporines, monolactàmics, carbapenems i inhibidors de β-lactamasses.
- Polipèptids: Poc importants, eficaços en gramnegatius i alteren la membrana bacteriana. La més utilitzada és la vancomicina.

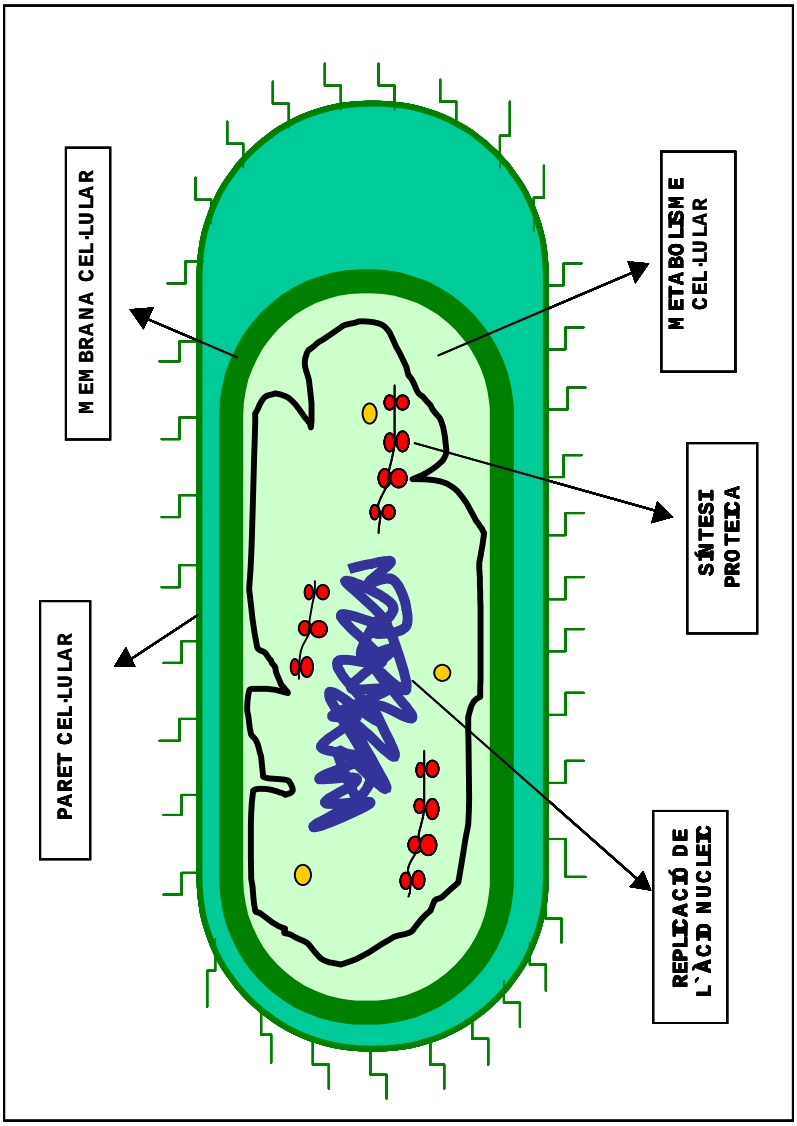


Fig. 2. Esquema representatiu dels punts diana de la cèl·lula on poden actuar els antibiòtics

- Glicopèptids: Actuen a nivell de la paret bacteriana, actiu en estalifococos.

→ Danyar les funcions de la membrana cel·lular:

- Polimixines: La més important és la colistina.
- Tirocidina.
- Valinomicina.

→ Inhibició de les funcions de l'àcid nucleic:

- Quinolones: Inhibeixen la girasa del DNA, són molt actives, principalment les fluorades.
- Rifamicines: Bloquegen la polimerasa del RNA i tenen indicacions molt específiques.
- Nitroimidazols: Són útils en infeccions per anaerobis i en algunes parasitosi.
- Nitrofurans: El seu ús queda restringit a infeccions urinàries.

→ Inhibició del metabolisme cel·lular:

- Sulfonamides: Són poc actives, tenen certa toxicitat i poques indicacions.
- Derivats dels compostos diamino pirimidina.

→Inhibició de la síntesi proteica:

- Aminociclitols (o aminoglicòsids): Són alcohols cíclics aminats. Tenen potencial nefrotòxic.
- Tetraciclines: Són bacteriostàtiques.
- Macròlids: Poc tòxics i solen comportar-se de manera bacteriostàtica.
- Lincosamides: Són bacteriostàtics, eficaços en cocos grampositius i inductors de colitis pseudomembranosa.
- Pleuromutilines: Entre les quals destaquen la tiamulina i la valnemulina.

- Cloramfenicol: Inhibeix la síntesi proteica, és bacteriostàtic i tòxic per la medul·la òsea.

I.2. APLICACIONS

Si bé, tal com s'ha esmentat a la introducció els antibiòtics han estat àmpliament utilitzats tant en medicina humana com en veterinària per tal de prevenir i tractar malalties, la present tesi s'ha centrat sobretot en temes relacionats amb veterinària. Només s'inclou un treball en el qual es parla de la importància dels antibiòtics en medicina humana i es fa una aplicació de l'electroforesi capil·lar per analitzar un antibiòtic, la kanamicina, en mostres clíniques (sèrum humà). A partir d'aquest punt es fa sobretot referència de la importància dels antibiòtics en el camp de la veterinària.

En veterinària, tal com en medicina humana, els antibiòtics s'utilitzen per prevenir i tractar enfermetats. Malgrat això, molts cops la funció més important dels antibiòtics que són utilitzats en animals destinats al consum humà és que estimulin el seu creixement [4,5]. No es coneix exactament el seu mecanisme d'acció, però sembla radicar en una millora de l'absorció de nutrients, així com en el control del creixement de la població microbiana i els seus productes tòxics a nivell digestiu.

D'aquesta manera estimulen el creixement i s'augmenta el pes de l'animal entre un 5 – 15 %, disminuint el consum de pinso en proporció al quilogram

de guanys i, a més, hi ha una major homogeneïtat en els lots i menor mortalitat. Les concentracions que s'administren solen estar entre els 20 i 100 mg d'antibiòtic per Kg de pinso i dia.

Segons estudis que s'han realitzat a Estats Units s'estima que al 1979 quasi el 100 % d'aviram, 90 % porcs i vedells i un 60 % de bestiar vacu rebien menjar

amb antibacterians com a additius, i un 70 % de carn vacuna produïda provenia d'animals que havien estat alimentats amb pinso que contenia antibiòtics per augmentar el seu pes. Almenys un 80 % de les proteïnes animals consumides a la dieta americana provenien d'animals exposats a menjar medicat almenys part de les seves vides [5].

És important saber quin efecte pot tenir l'ús dels antibiòtics com a additius en l'alimentació animal ja que aquests compostos tenen el potencial de ser incorporats en els productes animals i teixits destinats al consum humà. Des del punt de vista clínic, les conseqüències de major interès són: la creació de antibiorresistència (fenomen que preocupa als microbiòlegs i als metges clínics) o el que puguin produir al·lèrgies a individus ja sensibilitzats a aquests tipus de productes i que no sospitin la seva presència en la carn que consumeixen. Els animals tenen la capacitat de biotransformar les drogues en un gran nombre de metabòlits amb diversitat d'estructures moleculars i toxicitat. Degut a això hi ha la possibilitat que el seu ús porti al desenvolupament de cadenes de bacteris resistents als antibiòtics i, d'aquesta manera inhibeixen l'habilitat d'antibiòtics específics a ser utilitzats en el tractament de malalties humanes. També pot conduir a resultats negatius en l'anàlisi bacteriològic dels aliments amb el risc posterior a toxiinfeccions alimentàries. Des de la perspectiva industrial poden crear interferències en la fabricació d'aliments fermentats (com són la llet, formatges,...) [5,6].

I.3. LEGISLACIÓ

Varis productes veterinaris s'utilitzen en animals de companyia (com gats, gossos, etc...) [7], si bé la majoria de medicines veterinàries són emprades en animals destinats al consum humà. Degut a això cal tenir en compte varis factors:

- Tant aquests animals com els productes que s'obtenen a partir d'aquests (llet, ous, mel) són destinats al consum humà i per tant és important

assegurar-se que no queden residus perillosos en aquests. Els residus, no només de la droga administrada, sinó també dels seus metabòlits poden ser tòxics o més tòxics que els antibiòtics dels quals provenen.

- Una altra cosa molt important és la necessitat de valorar l'efecte de les drogues antimicrobianes en la flora intestinal humana. En teoria, els antibiòtics poden alterar la composició de la flora intestinal fins i tot matant els bacteris susceptibles a aquests permetent la supervivència dels bacteris més resistents les quals poden proliferar creant unes condicions en l'organisme que permeten que fongs i llevats colonitzin el tractament gastrointestinal de l'organisme. S'han fet varis estudis in vivo i in vitro per predir els efectes de les drogues antimicrobianes en la flora intestinal però fins ara tots els estudis realitzats porten a resultats contradictoris [7].

- Gran part de la droga administrada pot donar lloc a contaminació del medi ambient, degut a la matèria fecal i l'orina del bestiar que poden contenir els esmentats residus.

D'aquesta manera, abans de posar a la venda un producte farmacèutic cal tenir en compte els següents factors:

1) *Eficàcia:* Com actua.
2) *Qualitat farmacèutica:* Química de la droga, ruta sintètica, puresa, temps de durabilitat abans de la venta i estabilitat, així com mètodes per a la seva anàlisi.
3) *Seguretat:* La seva toxicologia, seguretat en els assajos clínics, dades epidemiològiques (per drogues velles, per exemple).

Seguint tots aquests paràmetres s'assegura la seguretat del consumidor.

Degut a la importància que hi hagi un control en els productes farmacèutics la Unió Europea (EU) va establir que les drogues en veterinària segueixin la mateixa directriu que les drogues humanes pel que fa referència a les regulacions bàsiques i comuns per a tot tipus de medicaments. Aquesta normativa és el que es coneix com a Directriu 65/65/EEC.

A part d'aquesta hi ha dues directrius molt importants a seguir en els medicaments veterinaris, les directrius 81/851/EEC i 81/852/EEC. Aquestes dues estableixen les normes bàsiques per a la valoració i autorització de drogues veterinàries en la EU. Amb aquestes es va establir el que es coneix com a *Committee for Veterinary Medicinal Products* (CVMP). Estableix les normes bàsiques i essencials de la legislació en el control de les drogues veterinàries en la EU, i proporciona la pauta a seguir per controlar la seguretat, qualitat i eficàcia del medicament.

A la Taula 1 es mostren les normes establertes per la EU en productes veterinaris [7,8].

Taula 1

Directrius fixades per la EU per controlar l'ús d'antibiòtics en productes veterinaris

DIRECTRIUS	
65/65/EEC	Regulacions bàsiques dels medicaments
81/851/EEC	Regulacions bàsiques de les drogues veterinàries establertes pel CVMP
81/852/EEC	Assajos i dades requerides en els medicaments veterinaris
87/22/EEC	Control dels productes d'elevada tecnologia
2377/90/EEC	Requisits dels MRL (Maximum Residue Limits)
92/412/EEC	Principis per les Bones Pràctiques de Manufacturació pels productes en medicina veterinària
78/25/EEC	Agents colorants específics permesos per l'ús en medicaments
90/167/EEC	Disposicions pels aliments medicats

90/219/EEC	Ús controlat dels organismes modificats genèticament
90/220/EEC	Butlletí sobre el control dels organismes modificats genèticament
87/18/EEC	Disposicions de les Bones Pràctiques de Laboratori
88/320/EEC	Inspecció i verificació de les Bones Pràctiques de Laboratori
86/609/EEC	Protecció dels animals utilitzats en procediments científics
2309/93/EEC	Establiment d'una Agència Europea per a l'avaluació de productes mèdics

Algunes d'aquestes normatives han estat esmenades o modificades, donant lloc a noves normatives, que es recullen a la Taula 2.

Taula 2

Modificacions de les Directrius fixades per la EU per controlar l'ús d'antibiòtics en productes veterinaris

DIRECTRIUS	
90/676/EEC	Esmenes en 81/851/EEC. Afegeix nous requisits
87/20/EEC	Esmenes en 81/852/EEC sobre els assajos clínics
92/18/EEC	Més esmenes en 81/852/EEC. Completa actualització dels requisits en els assajos
90/677/EEC	Esmena de 81/851/EEC amb requisits addicionals en els productes inmunològics
92/74/EEC	Esmena de 81/851/EEC, extensió dels requisits per a productes homeopàtics
93/40/EEC	Esmena de 81/851/EEC i 81/852/EEC, dictar els procediments a seguir sota l'Agència Reguladora Europea

La directriu 2309/03/EEC estableix una Agència Europea per a l'avaluació dels productes mèdics. Els requisits generals de toxicitat són fixats en la directriu 92/18/EEC:

- Estudi d'una sola dosi aplicada.
- L'estudi de la toxicitat de repetides dosis.
- Efectes de la seva reproductibilitat.
- Estudis de mutagenicitat.
- Immunotoxicitat.
- Observació en humans.

Aquests resultats s'utilitzen per valorar quins són els riscos pel consumidor.

La directriu 2377/90/EEC és la que introdueix uns requisits formals per establir els valors que es coneixen com *Maximum Residue Levels* (MRL) per les drogues veterinàries. Els MRL indiquen els límits de màxim residu, és a dir, quina és la quantitat màxima que pot romandre d'un antibiòtic en un teixit o producte animal (com ous, llet, etc...) destinats al consum humà. Aquests MRL es basen amb el que es coneixen com a *Aceptable Daily Intake* (ADI) [7-10].

L'ADI fa referència a la quantitat màxima d'una substància química, com pesticides i additius alimentaris (per exemple antibiòtics), que pot ser ingerida diàriament per l'home durant tota la seva vida sense que li produeixi un efecte negatiu.

La Regulació 2377/90/EEC estableix els MRL [7-10]. D'aquesta manera, totes les drogues han de tenir establert un valor de MRL fixat per aquesta normativa europea. Totes aquelles drogues que no tenen establert aquest valor, o bé que no el compleixen, estan prohibides. Els valors de MRL són específics de cada espècie i cada teixit, això és degut a les diferències en les farmacocinètiques. Malgrat que l'ADI per a una droga és universal, quan es fixen els MRLs han de tenir-se en compte les farmacocinètiques específiques de cada espècie així com metabòlits específicament tòxics [7].

En establir-se els MRL per a drogues veterinàries és òbvia la necessitat d'estudiar l'eliminació o reducció de la droga en els teixits dels animals tractats. Les directrius de la EU, Estats Units (US) i japoneses tenen elements en comú, com és la necessitat que les companyies que sintetitzen els medicaments veterinaris realitzin estudis de residus en animals. Això implica l'administració de la droga en animals no medicats prèviament en la dosi recomanada i sacrificar seqüencialment els animals i analitzar els extractes dels teixits comestibles per a determinar la concentració de la droga. Són considerats teixits comestibles el múscul, fetge, ronyó, greix i en algunes espècies pell. Estudis similars inclouen els productes comestibles que produeixen aquests animals com són llet, ous i mel els quals poden ser usats en animals en període de lactància, aviram i abelles. Aquests productes són recollits cada cert període de temps després de l'administració de la droga.
Mitjançant els estudis de residus el que es pretén és fixar el que es coneix com a temps d'espera, que són els dies que s'ha d'esperar el granger abans de poder sacrificar l'animal després d'haver-l'hi administrat un cert medicament. D'aquesta manera el ramader s'assegura que després d'aquest període de temps l'animal ha eliminat l'antibiòtic que tenia en el seu organisme i que la

màxima quantitat que hi pot romandre en aquest és igual o inferior als MRL fixats per la normativa [7,8].

A la EU, la monitorització de residus en carn vermella és regulada per la directriu 86/469/EEC, i cada any els Estats Membres han de presentar plans de monitorització de residus a la Comissió Europea per la seva aprovació. Així per exemple, a Anglaterra, 40000 mostres de teixits van ser preses el 1992 i el 1993 per l'anàlisi de residus de diversos escorxadors escollits a l'atzar. Els compostos analitzats incloïen diverses hormones, antimicrobians i nitrofurans, entre d'altres. Els nivells positius, és a dir les concentracions de residus que es van trobar per sobre dels valors de MRL van ser del voltant del 0.5 %. En la majoria de casos eren residus de sulfonamides i tetraciclines en mostres de ronyó de porc. Problemes similars s'han observat en altres Estats Membres de la EU i en US [7].

Altres països que no pertanyen a la EU també apliquen plans de monitorització de residus. Per exemple en el cas d'USA la responsabilitat de la monitorització dels residus recau en tres agències governamentals, la US *Food and Drug Administration* (FDA), *the Food Safety and Inspection Service* (FSIS) del Departament d'Agricultura dels US, i the US *Environmental Protection Agency*. El programa nacional de residus consisteix en la monitorització i desenvolupament de tests ràpids i un programa d'eliminació de residus.

A nivell internacional, la *Joint FAO/WHO Expert Committee on Food Additives* (JECFA) estableix els valors ADI i MRL per residus de drogues veterinàries [8].

Altres aspectes importants a estudiar són els tests d'ecotoxicitat. Aquests són fixats per la directriu 92/18/EEC. En aquests tests es diferencien dues fases. La funció de la primera fase és fer una estimació de la probabilitat que la droga contamini el medi ambient. Es tenen en compte els següents paràmetres:

- S'administra la droga en espècies que no han estat medicades. Aquest estudi es pot fer mitjançant l'administració de la droga a un sol animal o bé en un ramat.
- El mètode d'administració i el potencial de la droga per entrar en el medi ambient.
- L'excreció de la droga i els seus metabòlits i la seva persistència d'aquests en els excrements.

En la segona fase es fan una sèrie d'estudis sobre la droga a determinar, com són:

- El seu destí i comportament en el sòl, en l'aire i en l'aigua.
- Efectes en els organismes aquàtics.
- Efectes en organismes que han estat medicats prèviament.

Aquests estudis són molt importants per les drogues que poden contaminar granges així com els medicaments que poden afectar als peixos, també són importants per a determinar la repercussió d'aquestes drogues en la contaminació del medi ambient.

I.4. COMPOSTOS ESTUDIATS

En la present tesi s'han desenvolupat metodologies analítiques per a la determinació de diferents antibiòtics, els quals pertanyen al grup de les β-lactames (penicil·lines i cefalosporines), les tetraciclines, les quinolones i els aminoglicòsids.

I.4.1. PENICIL·LINES

Les penicil·lines són els primers antibiòtics que es van descobrir. Es caracteritzen pel seu ampli ús en el tractament d'infeccions. Actuen inhibint la

formació de la paret bacteriana. S'utilitzen tant en veterinària com en medicina humana. S'han utilitzat àmpliament en el tractament d'infeccions respiratòries, gastrointestinals i urinàries. Pertanyen al grup de les β-lactames, juntament amb les cefalosporines. Tal com el seu nom indica inclouen un grup de substàncies fabricades per *Penicillium* i d'altres fongs afins [11]. Totes les penicil·lines posseeixen una estructura química comuna, dos anells heterocíclics, un de tiazolidina i un altre de β-lactama unit a un radical variable. A la Figura 3 es mostra l'estructura general de la penicil·lina.

La cadena lateral determina les propietats antibacterianes i farmacològiques de cada tipus de penicil·lina.

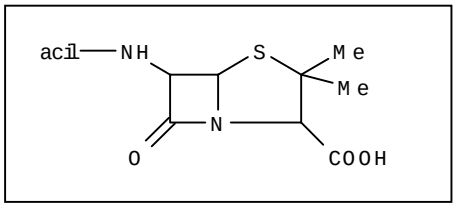


Fig. 3. Estructura general de la penicil·lina

N'existeixen diversos tipus:

- *Penicil·lina G:* S'utilitza per via intravenosa, intramuscular i oral. És de primera elecció en infeccions com les causades per estreptococos i en la sífilis. Molts bacteris, malgrat això, la inactiven produint un enzim (la β-lactamasa).

- *Penicil·lines resistents a la β-lactamasa (tipus cloxacil·lina)*: Poden actuar contra algun bacteri que produeix la β-lactamasa, com l'estafilococos.

- *Aminopenicil·lines*: Tenen més activitat front als microorganismes coneguts com a gramnegatius, i si s'associen amb substàncies com l'àcid clavulànic o el sulbactam, també poden actuar contra bacteris que

produeixen β-lactamasa, com l'estafilococos. L'amoxicil·lina i ampicil·lina són exemples d'aquest tipus de penicil·lina.

- *Penicil·lines antipseudomona*: Com el seu nom indica poden actuar contra la Pseudomona (un bacteri perillós que causa infeccions molt greus). Com per exemple la carbenicil·lina i la piperacil·lina.

Per tal de protegir al consumidor la CVMP (*Committee for Veterinary Medicinal Products*) estableix que es compleixen els valors de MRL que es mostren en la Taula 3 [12].

Taula 3
Valors de MRL per diferents penicil·lines

Substància farmacològicament activa	Espècies animals	MRL ($\mu g \cdot Kg^{-1}$)	Teixits diana	Llet ($\mu g \cdot Kg^{-1}$)
Penicil·lina G	Totes les espècies destinades al consum humà	50	Tots els teixits destinats al consum humà	4
Ampicil·lina		50		4
Amoxicil·lina		50		4
Oxacil·lina		300		30
Cloxacil·lina		300		30
Dicloxacil·lina		300		30

I.4.2. CEFALOSPORINES

Les cefalosporines pertanyen a la família de les β-lactames. Tenen una estructura i acció bactericida similar a les penicil·lines. Actuen com a bactericides, inhibint la síntesi de la paret bacteriana. Són antibiòtics que es caracteritzen pel seu ampli espectre, és a dir són actius contra un gran número de bacteris grampositius i gramnegatius.

S'utilitzen tant en veterinària com en medicina humana. Com en el cas de les penicil·lines s'utilitzen en el tractament d'infeccions respiratòries, gastrointestinals i urinàries [13-17].

L'estructura de les cefalosporines deriva de l'àcid 7-aminocefalosporànic, mentre que l'estructura de les penicil·lines deriva de l'àcid 6-aminopenicil·laic. Ambdues estructures contenen un anell β-lactama,

però l'estructura de les cefalosporines fa que tinguin una major activitat contra els bacteris gramnegatius [15].

A la Figura 4 es mostra l'estructura general de les cefalosporines.

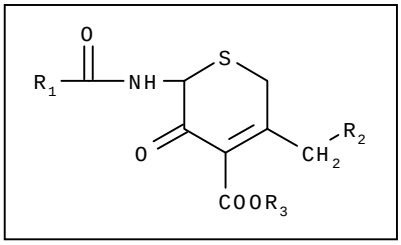


Fig. 4. Estructura general de les cefalosporines

Les cefalosporines es classifiquen per generacions, en funció de la seva activitat antibacteriana. Es poden diferenciar tres generacions:

- *Cefalosporines de primera generació:* Són aquelles que són actives contra bacteris grampositius, i actuen contra algun bacteri gramnegatiu. Són inactives contra els *enterobacteris* i les *Pseudomones aeruginosa*. Alguns exemples són: Cefalexina, cefazolina, cefapirina, etc...

- *Cefalosporines de segona generació:* Amplien una mica el seu espectre bacterià. Són actives contra els enterobacteris, però segueixen essent inactives contra *Pseudomones aeruginosa*, els *enterobacteris* i les *Pseudomones aeruginosa*. Aquest grup inclou: cefoxitin, cefamandole, cefotetan, etc...

- *Cefalosporines de tercera generació*: Augmenten la seva activitat respecte les anteriors generacions i algunes d'elles són actives contra

les *Pseudomones aeruginosa*. Alguns exemples són: Ceforaxima, cefsulodina, cefoperazona, cefotaxima, cefpiroma, etc...

Existeix una quarta generació de cefalosporines, però aquesta no s'inclou degut a l'absència de principi actiu d'ús veterinari.

A la Taula 4 es mostren els valors de MRL establerts per la normativa europea (aquests valors es donen en funció de la suma dels residus que es generen a partir de l'antibiòtic que s'ha administrat) [18-22].

Taula 4

Valors de MRL per diferents cefalosporines

Substància farmacològicament activa	**Residu**	**Espècies animals**	**MRL ($\mu g \cdot Kg^{-1}$)**	**Teixit diana**
Cefalexina	Cefalexina	Boví	200	Múscul
			200	Greix
			200	Fetge
			1000	Ronyó
			100	Llet
Cefapirina	Cefapirina + desacetilcefapirina	Boví	50	Múscul
			50	Greix
			100	Ronyó
			60	Llet
Cefazolina	Cefazolina	Boví, oví, caprí	50	Llet
Cefoperazone	Cefoperazone	Boví	50	Llet

I.4.3. TETRACICLINES

Les tetraciclines tenen un espectre d'activitat molt ampli. S'utilitzen contra bacteris grampositius i gramnegatius, així com protozous. Són antibiòtics bacteriostàtics que actuen inhibint la formació de proteïnes del bacteri. S'utilitzen pel tractament i control d'infeccions bacterianes tant en l'home com en animals. També s'utilitzen per conservar els fruits i vegetals conreats, així com per l'exterminació de plagues d'insectes i com a suplement alimentari del bestiar [23,24].

La primera tetraciclina que es va sintetitzar va ser la clortetraciclina (1948) com a producte del *Streptomyces aureofaciens*. Posteriorment van ser sintetitzades l'oxitetraciclina i la tetraciclina. Entre d'altres tetraciclines aparegudes posteriorment cal destacar la demeclociclina, la doxiciclina, la limeciclina i la minociclina. L'estructura química d'aquesta família d'antibiòtics deriva d'un nucli d'hidronaftracè que conté 4 anells. La seva estructura general es mostra a la Figura 5 [25].

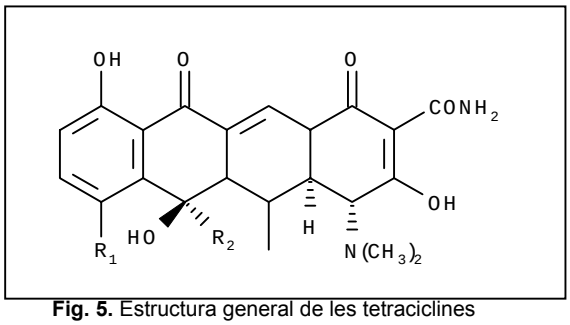


Fig. 5. Estructura general de les tetraciclines

Entre elles no difereixen gaire per la seva activitat antibacteriana, i es destaca sobretot les diferències en el seu comportament farmacocinètic. La

doxiciclina i la minociclina es caracteritzen perque, a diferència de les altres tetraciclines no empitjoren els problemes renals que pugui tenir un pacient, i a més la seva eliminació del sèrum dels pacients és el suficientment lenta com

per a que només sigui necessària la seva administració dos cops per dia [21,26-28]. Altres tetraciclines, com l'oxitetraciclina, la tetraciclina i la clortetraciclina, han estat àmpliament utilitzades en el tractament en el bestiar, tant per la prevenció i tractament d'enfermetats, i com a additiu alimentari en animals destinats al consum humà [21-25,28-31]. Degut a això, moltes tetraciclines estan regulades per la normativa europea. Els valors de MRL establerts es mostren a la Taula 5 (aquests valors es donen en funció de la suma dels residus que es generen a partir de l'antibiòtic que s'ha administrat) [32,33].

I.4.4. QUINOLONES

Les quinolones són antibiòtics de recent descobriment i ús. La primera quinolona que es va descobrir va ser l'àcid nalidíxic, que a més va ser el primer antibiòtic que es va utilitzar per les infeccions urinàries. Actuen inhibint la DNA girasa (o topoisomerasa II), un enzim sense el qual el DNA no es pot convertir en una molècula lineal i pugui replicar-se; al bloquejar la DNA girasa, s'inhibeix la replicació del DNA. S'utilitzen àmpliament en les clíniques per diversos tipus d'infeccions, així com pel tractament d'infeccions urinàries, intestinals i biliars [9,10,36]. S'utilitzen tant en medicina humana com en veterinària, especialment en animals de granja, com en l'aviram, bestiar vacu i porcí [37].

A la Figura 6 es mostra l'estructura general de les quinolones de 1ª generació i de les fluoroquinolones.

Taula 5

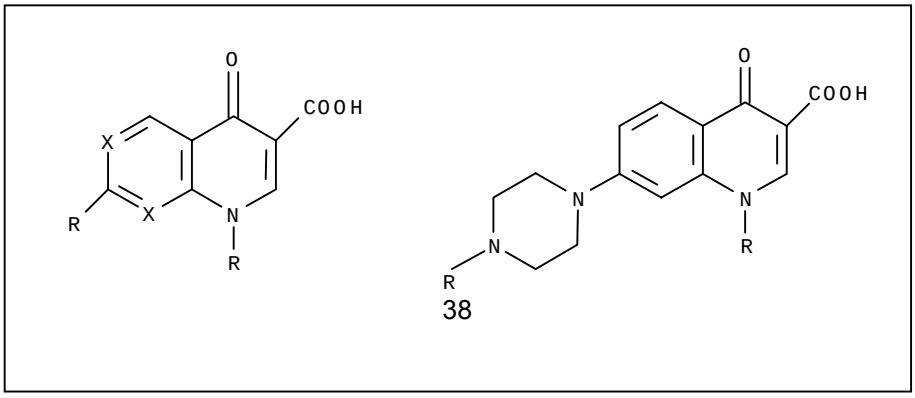
Valors de MRL establerts per diferents tetraciclines

Substància farmacològicament activa	Residu	Espècies animals	MRL ($\mu g \cdot Kg^{-1}$)	Teixit diana	Observacions
Tetraciclina	La suma dels	Totes les	600	Ronyó	

Oxitetraciclina **Clortetraciclina**	seus metabòlits 4-epímer	espècies destinades al consum humà	300 100 100 200	Fetge Múscul Llet Ous	
Doxiciclina	Doxiciclina	Boví	600 300 100	Ronyó Fetge Múscul	No aplicable en animals que produeixen llet destinada al consum humà
		Porcí, pollastres	600 300 300 100	Ronyó Fetge Pell+greix Múscul	No aplicable en animals que produeixen ous destinats al consum humà

S'utilitzen sobretot pel tractament d'infeccions respiratòries i pell, pel tractament d'enfermetats transmeses sexualment, així com pel tractament d'infeccions urinàries. Són actives contra bacteris grampositius i gramnegatius.

Tal com hem esmentat, les quinolones es divideixen en funció del seu espectre antibacterià, potència i farmacologia. Normalment s'utilitza la següent classificació [38-41]:



a) Quinolones de 1ª generació b) Fluoroquinolones

Fig. 6. Estructura general de quinolones de 1ª generació i de les fluoroquinolones.

• *Quinolones de primera generació*: Són actives contra microorganismes gramnegatius, amb excepció a les *Pseudomones* i d'altres bacils gramnegatius no fermentats. S'han utilitzat preferentment com antisèptics urinaris, intestinals i biliars. Alguns exemples són: l'àcid nalidíxic, l'àcid oxolínic, la flumequina i la cinoxacina, etc...

- *Fluoroquinolones*: Aquest grup inclou tres tipus de quinolones:

  - Les quinolones de segona generació: Són preferentment actives contra bacteris gramnegatius. També tenen bona activitat contra alguns gèrmens grampositius i micobacteris. Com és el cas de la ciprofloxacina, l'enrofloxacina, la marbofloxacina, la difloxacina, la sarafloxacina, la danofloxacina, etc...
  - Les quinolones de tercera generació: Mantenen l'activitat contra els bacteris gramnegatius i a més presenten millor activitat contra els grampositius, anaerobis i patògens *atípics*. Alguns exemples: gatifloxacina, esparfloxacina i tosufloxacina.

  - Les quinolones de quarta generació: Aquestes quinolones són les més recents. A part de ser actives contra els mateixos bacteris i gèrmens que els demés tipus de fluoroquinolones, tenen bona activitat contra cocs grampositius, patògens atípics (*Chlamydia, Mycoplasma, Legionella*). A més, són clínicament molt actives contra la majoria d'espècies d'anaerobis. Com a exemples hi ha la levofloxacina, trovafloxacina, clinafloxacina i moxifloxacina.

Les quinolones són un grup de compostos de gran interès clínic, i degut a la seva acció bactericida i a la seva favorable farmacocinètica poden ser considerats com una alternativa terapèutica dels antibiòtics β-lactàmics i aminoglicòsids [9,10].

L'administració simultània de quinolones i altres antibiòtics com cloramfenicol, eritromicina, rifamicina i tetraciclines està contraindicat. En canvi es poden administrar conjuntament amb altres antibiòtics com les β-lactames, els aminoglicòsids i els imidazols [9].

Com en el cas de les penicil·lines i tetraciclines hi ha vàries quinolones que estan regulades per la normativa europea i tenen valors de MRL fixats. Aquests valors es mostren a la Taula 6 (aquests valors es donen en funció

de la suma dels residus que es generen a partir de l'antibiòtic que s'ha administrat) [42-49].

Taula 6

Valors de MRL per diferents quinolones

Substància farmacològicament activa	Residu	Espècies animals	MRL ($\mu g \cdot Kg^{-1}$)	Teixit diana	Observacions
Enrofloxacina	Enrofloxacina + ciprofloxacina	Oví	100 100 300 200	Múscul Greix Fetge Ronyó	
		Boví	100 100 300 200 100	Múscul Greix Fetge Ronyó Llet	
		Conills	100 100 200 300	Múscul Greix Fetge Ronyó	
		Porcí	100	Múscul	

			100 200 300	Pell+greix Fetge Ronyó	
		Pollastres	100	Múscul	No aplicable a animals que produeixen ous destinats al consum humà
Marbofloxacina	Marbofloxacina	Boví	150 50 150 150 75		
		Porcí	150 50 150 150		

Substància farmacològicament activa	Residu	Espècies animals	MRL ($\mu g \cdot Kg^{-1}$)	Teixit diana	Observacions
Flumequina	Flumequina	Boví, oví	200 300 500 1500	Múscul Greix Fetge Ronyó	
		Boví	100	Llet	
		Porcí	200 300 500 1500	Múscul Pell + greix Fetge Ronyó	
		Gall dindi, pollastre	400 250 800 1000	Múscul Pell + greix Fetge Ronyó	
		Pollastre	400 250 800 1000	Múscul Pell + greix Fetge Ronyó	
		Salmonidae	600	Múscul I pell en proporcions naturals	
Difloxacina	Difloxacina	Gall dindi, pollastre	300 400 1900 600	Múscul Pell+ greix Fetge Ronyó	
		Boví	400 100 1400 800	Múscul Greix Fetge Ronyó	No aplicable a animals que produeixen llet destinats al consum humà
		Porcí	400 100 800 800	Múscul Pell + greix Fetge Ronyó	
Sarafloxacina	Sarafloxacina	Salmonidae	30	Múscul I pell en proporcions naturals	
	Metabòlits de la Sarafloxacina	Pollastre	100 10	Fetge Pell+ greix	

(Continuació de la Taula 6)

Substància farmacològicament activa	Residu	Espècies animals	MRL ($\mu g \cdot Kg^{-1}$)	Teixit diana	Observacions

Àcid Oxolínic	Àcid Oxolínic	Boví	100 50 150 150	Múscul Greix Fetge Ronyó	
		Porcí	100 50 150 150	Múscul Pell + greix Fetge Ronyó	
		Pollastre	100 50 150 150 50	Múscul Pell + greix Fetge Ronyó Ous	
		Ala de peix	300	Múscul I pell en proporcions naturals	
Danofloxacina	Danofloxacina	Boví	200 100 400 400 30	Múscul Greix Fetge Ronyó Llet	
		Pollastre	200 100 400 400	Múscul Greix Fetge Ronyó	No aplicable a animals que produeixen ous destinats al consum humà
		Porcí	100	Múscul	

(Continuació de la Taula 6)

I.4.5. AMINOGLICÒSIDS

Els aminoglicòsids són un important grup d'antibiòtics que es caracteritza perque són actius contra un elevat número de bacteris grampositius i gramnegatius. Es caracteritzen per la seva acció bactericida, actuen creant fissures en la membrana externa dels bacteris. S'utilitzen pel tractament d'una gran varietat d'infeccions bacterianes, sobretot pel tractament d'infeccions urinàries i a l'abdomen. S'utilitzen tant en medicina humana com en veterinària [50].

L'estructura bàsica dels aminoglicòsids consisteix en dos o més aminosucres units per un enllaç o-glicosídic a una hexosa. La gentamicina és l'aminoglicòsid més utilitzat, i l'amikacina es caracteritza per la seva efectivitat contra organismes molt resistents. Els aminoglicòsids també es caracteritzen perque es poden combinar en algun cas amb les β-lactames augmentant la seva activitat bacteriana. Aquestes combinacions s'utilitzen pel tractament d'infeccions molt greus.

Com a exemple en la Figura 7 es mostra l'estructura de la gentamicina, un dels aminoglicòsids més utilitzats [51].

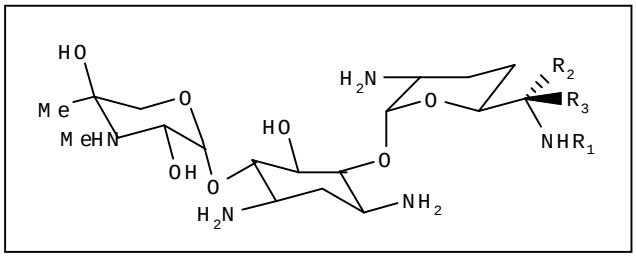


Fig. 7. Estructura general de la gentamicina

No existeix una classificació general com en els altres grups d'antibiòtics que s'han descrit. Aquesta família conté almenys 8 antibiòtics: amikacina,

gentamicina, kanamicina, neomicina, netilmicina, paromomicina, estreptomicina, dihidroestreptomicina i tobramicina [51,52].

A la Taula 7 es mostren els valors de MRL establerts per la normativa europea (aquests valors es donen en funció de la suma dels residus que es generen a partir de l'antibiòtic que s'ha administrat) [53-57].

Taula 7

Valors de MRL per diferents aminoglicòsids

Substància farmacològicament activa	Residu	Espècies animals	MRL ($\mu g \cdot Kg^{-1}$)	Teixit diana	Observacions
Gentamicina	Suma de gentamicina C1, gentamicina C1a, gentamicina C2 i gentamicina C2a	Boví	50 50 200 750 100	Múscul Greix Fetge Ronyó Llet	
		Porcí	50 50 200 750	Múscul Pell + greix Fetge Ronyó	
Kanamicina	Kanamicina	Boví, oví	100 100 600 2500 150	Múscul Greix Fetge Ronyó Llet	Valors de MRL provisionals. Expiren el 01.01.2002
		Porcí, pollastre	100 100 600 2500	Múscul Pell + greix Fetge Ronyó	
		Conill	100 100 600 2500	Múscul Greix Fetge Ronyó	

Substància farmacològicament activa	Residu	Espècies animals	MRL ($\mu g \cdot Kg^{-1}$)	Teixit diana	Observacions
Neomicina	Neomicina	Boví, oví, porcí, gall dindi, pollastre, ànec, caprí	500 500 500 5000	Múscul Greix Fetge Ronyó	Valors de MRL provisionals. Expiren el 01.06.2002
		Boví, oví	500	Llet	
		Pollastre	500	Ous	
Estreptomicina	Estreptomicina	Boví, oví	500 500 500 1000 200	Múscul Greix Fetge Ronyó Llet	
		Porcí	500 500 500 100	Múscul Pell + greix Fetge Ronyó	
Dihidroestreptomicina	Dihidroestreptomicina	Boví, oví	500 500 500 1000 200	Múscul Greix Fetge Ronyó Llet	
		Porcí	500 500 500 100	Múscul Pell + greix Fetge Ronyó	
Paromomicina	Paromomicina	Boví	500 1500 1500	Múscul Fetge Ronyó	No aplicable a animals que produeixen llet destinada al consum humà
		Porcí, conill	500 1500 1500	Múscul Fetge Ronyó	
		Pollastre	500 1500 1500	Múscul Fetge Ronyó	No aplicable a animals que produeixen ous destinats al consum humà

(Continuació de la Taula 7)

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CAPÍTOL II. ESTAT DE LA QÜESTIÓ

II.1. ESTUDI BIBLIOGRÀFIC DE LA DETERMINACIÓ ANALÍTICA

Tal com s'ha descrit en el capítol anterior, en els últims anys s'ha incrementat de manera molt important la necessitat de controlar la presència de residus en animals destinats al consum humà. D'aquí la necessitat d'establir metodologies analítiques que permetin determinar els antibiòtics a baixos nivells de concentració en diverses matrius biològiques [1].

II.1.1. TÈCNIQUES CROMATOGRÀFIQUES

En els darrers anys, hi ha hagut un enorme desenvolupament de metodologies analítiques per a determinar i establir la identificació i quantificació dels antibiòtics en diferents matrius. A part dels avenços d'equips analítics i computerització hi ha hagut avenços en els diferents passos del procés analític. Els mètodes cromatogràfics permeten identificar i quantificar els analits en una mostra determinada. Previ a la seva anàlisi és necessari extreure els analits dels constituents de la matriu.

Diferents tècniques analítiques han estat utilitzades per realitzar aquest tipus d'anàlisi, entre les quals cal destacar la cromatografia de capa fina, la cromatografia de gasos, la cromatografia de líquids, etc... De totes aquestes la més utilitzada ha estat la cromatografia de líquids en fase inversa [7-15,19]. Hi ha vàries aplicacions d'aquest sistema en l'estudi de residus com és la determinació d'àcid oxolínic i flumequina en mostres de teixit de peix [7], la determinació de danofloxacina en plasma [8], l'anàlisi de diverses quinolones en teixits de pollastre [9,10], l'anàlisi de diferents tetraciclines en teixits de porc i vedella [11-13], l'anàlisi d'oxitetraciclina i sulfadimidina en carn i ous [14,15], o bé la determinació de gentamicina en porcs i vedelles [19].
Quan els compostos a analitzar són iònics per dur a terme l'estudi de residus s'utilitza generalment la cromatografia de parells iònics [1,47,74-76]. Hi ha varis exemples on s'ha utilitzat aquest sistema, com és el cas de la determinació de

diverses β-lactames utilitzant decanosulfat sòdic com a parell iònic [77], l'anàlisi de diverses penicil·lines en diferents teixits de múscul utilitzant hidrogensulfat tetrabutilamoni com a parell iònic [78], l'anàlisi d'espectinomicina en diferents teixits bovins [79], la determinació de neomicina i gentamicina en diferents teixits animals [18], la determinació de diverses tetraciclines en llet i diferents teixits animals [81] i en teixits bovins i porcins [82], així com l'anàlisi [2] d'ampicil·lina i amoxicil·lina en llet.

En quant a la detecció el sistema més utilitzat és la detecció ultraviolada-visible. La gran majoria d'estudis utilitzen aquest tipus de detecció, com per exemple en la determinació de penicil·lines [2,85], quinolones [52,83,84] i tetraciclines [11-15,78,] en diferents mostres biològiques. De vegades pot ser que l'analit no absorbeixi en aquesta zona o bé que el sistema no sigui prou sensible utilitzant-se en aquests casos detectors de fluorescència. Per exemple en el cas de les quinolones és bastant habitual l'ús d'aquest tipus de detector, ja que es caracteritzen perque totes elles són fluorescents [7-10]. De vegades per a poder determinar un analit és necessari du a terme la seva derivatització, és a dir fer-lo reaccionar químicament de manera que pugui ésser detectat. La derivatització és bastant utilitzada en l'anàlisi de residus, un exemple força corrent és en l'anàlisi d'aminoglicòsids, els quals es caracteritzen perque no contenen grups cromòfors en la seva estructura i per tant és necessària la seva derivatització per a la introducció d'algun d'aquests grups. Lai i Sheenan [16] van desenvolupar l'anàlisi de la tobramicina en sèrum fent una derivatització amb o-ftaldialdèhid (OPA). Un estudi similar va ser aplicat per Stubbings et al. [17] en la determinació d'opramicina en teixit de ronyó. La derivatització també ha estat bastant utilitzada en la determinació de penicil·lines per tal d'augmentar la sensibilitat del sistema [3-5]. Un exemple és el sistema desenvolupat per Haginaka [3] que va utilitzar triazol i clorur de mercuri per dur a terme la derivatització de diverses penicil·lines, sistema que posteriorment va ser aplicat a l'anàlisi de benzilpenicil·lina i cloxacil·lina per Gee et al. [4] i en l'anàlisi d'amoxicil·lina i ampicil·lina per Rose et al. [5] en teixits animals.

En algun cas es du a terme la derivatització dels analits per donar lloc a compostos que siguin fluorescents per tal d'augmentar la sensibilitat del mètode. Hi ha varis exemples com l'anàlisi d'amoxicil·lina en sèrum i plasma utilitzant fluorescamina com a reactiu [6] i la determinació de gentamicina i neomicina utilitzant 9-fluorenilmetil [18]. Per a la determinació de les tetraciclines per fluorescència es formen complexos metàl·lics utilitzant com a reactiu alumini o zirconi, els quals sí que són fluorescents. Aquest sistema s'ha utilitzat per a la seva determinació en diferents teixits animals [86,87].

Un altre sistema de detecció que s'està implantant actualment en l'anàlisi de residus és l'ús de l'espectrometria de masses. Aquest sistema es caracteritza perque permet detectar un ampli rang de compostos amb gran sensibilitat i especificitat. Ja que la seva fonamental aplicació és la determinació de pesos moleculars permet la interpretació i identificació de les substàncies que s'estan analitzant. A més, la seva gran avantatge és que la matriu de la mostra no interfereix en el pic de l'analit, el que facilita la interpretació dels resultats. Aquest sistema va ser utilitzat en l'anàlisi de diferents penicil·lines i cefalosporines en llet per LC [88], gentamicina per LC en porcs i vedelles [19], de quinolones en llet, salmó i orina humana [89], de tetraciclina, oxitetraciclina i clortetraciclina en plasma [90], etc...

II.1.2. TÈCNIQUES ELECTROFORÈTIQUES

Com a tècnica alternativa a la LC en l'anàlisi d'antibiòtics en mostres biològiques s'està estudiant l'electroforesi capil·lar (CE). Aquesta tècnica ofereix vàries avantatges, especialment quan es disposa de poca quantitat de mostra. Les principals avantatges de la CE front les tècniques cromatogràfiques són la seva elevada eficàcia i resolució, els petits volums de mostra necessaris, i baixos costos degut al baix consum de solvents orgànics, així com el baix cost dels capil·lars que s'utilitzen, i a més és una tècnica fàcilment automatitzable

[24]. El principal inconvenient de l'electroforesi capil·lar és que en molts casos no és una tècnica el suficientment sensible per a la determinació de baixes concentracions. Malgrat això, actualment s'han desenvolupat molts sistemes de preconcentració *on-column* com és el cas de l'ús de la injecció de grans volums de mostra (FASI), o bé la isotacoforesi capil·lar (ITP), sistemes que permeten augmentar en diversos ordres de magnitud la sensibilitat del sistema [25,30,50,51,53-58,60-62], com es comentarà en l'apartat II.3 d'aquest capítol.

Diversos estudis mostren el potencial de la CE en l'anàlisi de residus en una gran varietat de mostres biològiques com són l'anàlisi de penicil·lines [25], cefalosporines [26,27], quinolones [28-34], tetraciclines [35-37] i aminoglicòsids [38].

II.1.2.1. Penicil·lines

L'estructura molecular de les penicil·lines consisteix en un anell de tiazolidina unit a un anell β-lactama amb una cadena lateral (tal com es mostra en la figura 3 de la Secció 3.4.1). Es caracteritzen perque són compostos neutres o difícilment ionitzables i, per tant generalment en CE s'utilitza la cromatografia capil·lar micel·lar electrocinètica **(**MEKC) com a mode per a dur a terme la seva separació. En molts casos s'ha utilitzat dodecil sulfat sòdic (SDS) per formar les micel·les [41-43].

Els primers estudis de penicil·lines per CE es van realitzar analitzant mostres comercials [41,42], demostrant el poder de separació per MEKC. En ambdós casos es va utilitzar dihidrogenfosfat com a electròlit, i SDS per formar les micel·les. Un estudi similar va ser desenvolupat per Hows et al. [43], els quals van separar diverses famílies de compostos (sulfamides, 2 inhibidors dihidrofolat reductasa i 11 β-lactames) per CE. En aquest cas també es va afegir SDS per donar lloc a les micel·les. Un cas d'aplicació de la CE en l'anàlisi

de penicil·lines en mostres reals va ser l'anàlisi d'amoxicil·lina en plasma de porc [25].

II.1.2.2. Cefalosporines

Les cefalosporines també pertanyen al grup de les β-lactames. Presenten una estructura i acció similar a les penicil·lines. Contenen un anell que deriva de l'àcid 7-aminocefalosporànic [80].

Les cefalosporines, a l'igual que les penicil·lines, són antibiòtics neutres o difícilment ionitzables. És per això que en la gran majoria de treballs s'utilitza el mode MEKC. Aquest per exemple és el cas de l'anàlisi de diferents cefalosporines en diferents teixits per microdiàlisi i també en plasma [26,45]. En aquests casos també es va utilitzar SDS per formar les micel·les.

En algun cas ha estat possible la separació per CZE de diversos d'aquests compostos optimitzant les condicions de pH, de manera que aquests estiguin parcialment ionitzats i es puguin separar. Un exemple és l'anàlisi de diverses cefalosporines en orina i bilis humà i de conills [39]. Un sistema semblant va ser l'estudi de cefazolin en sang humana [27].

II.1.2.3. Tetraciclines

Les tetraciclines contenen quatre anells benzènics fusionats, tal com es mostra en la Figura 5 de la Secció I.4.3 [36]. Hi ha vàries aplicacions per CE en la seva anàlisi en mostres biològiques. Per exemple en la determinació d'oxitetraciclina en filets de peix [37], o bé la determinació d'oxitetraciclina en diferents teixits de porc: ronyó, fetge i múscul [35].

Per altra banda, s'ha demostrat que l'ús de sistemes de CE en medi no aquós permet una bona eficàcia i resolució en la separació de diferents tetraciclines. Com per exemple la determinació d'oxitetraciclina, tetraciclina, 4-epioxitetraciclina i 4-epitetraciclina en plasma de porc [36], la determinació d'oxitetraciclina en una crema mitjançant un sistema de CE en medi no aquós (NACE) [48].

També s'ha estudiat la tendència de les tetraciclines a formar complexos amb metalls en medi no-aquós. Alguns d'aquests complexos metàl·lics són fluorescents el que permet desenvolupar sistemes CE - LIF, tal com mostra TjØrnelund et al. [49] en la determinació d'oxitetraciclina, tetraciclina i clortetraciclina en mostres de llet i plasma de vaca.

II.1.2.4. Quinolones

L'estructura bàsica de les quinolones és un esquelet de 4-oxo-1,4-dihydroquinolina (tal com es mostra a la figura 5.a de la Secció I.4.4). La seva activitat antibacteriana augmenta amb l'addició d'un grup fluoro en la posició 6 i un grup piperazinil en la posició 7 [32].

En el cas de les quinolones a la bibliografia s'han descrit diferents metodologies per a la seva anàlisi per CE. Hi ha varis exemples de la seva anàlisi per CZE com és el cas de la determinació de 10 quinolones per CZE en plasma de porc [31], l'anàlisi de moxifloxacina en plasma i en teixits per microdiàlisi en humans [28], l'anàlisi de ciprofloxacina i un dels seus metabòlits en plasma humà [29] o bé la determinació de marbofloxacina en plasma de porc per CZE [30]. En algun cas també s'ha dut a terme la seva determinació mitjançant un sistema MEKC, com és el cas de la determinació d'àcid nalidíxic i dos de les seves impureses en sèrum i orina [34].

Com en el cas de les tetraciclines, un estudi recent ha permès mostrar l'eficàcia d'utilitzar un sistema de CE en medi no aquós per tal de separar 5 quinolones [33]. Aquest sistema es va aplicar a la determinació d'aquestes quinolones en ronyó de porc.

II.1.2.5. Aminoglicòsids

L'estructura dels aminoglicòsids consisteix en dos o més aminosucres units mitjançant un enllaç o-glicosídic. Degut que aquests antibiòtics no contenen grups cromòfors tal com s'ha estudiat en l'apartat 4.1.1 és necessari, o bé derivatitzar aquests compostos per poder-los detectar per fluorescència o bé, la formació de complexos que continguin grups cromòfors per a la seva detecció per UV-visible.

D'aquesta manera, Kowalski et al. [46] va descriure l'anàlisi d'estreptomicina en ous per CZE. En aquest cas no va ser possible duu a terme la derivatització degut a la complexitat de la mostra, però mitjançant l'addició de borat en el medi es van formar complexos que es poden detectar per UV-visible. Es va utilitzar dihidrogenfosfat sòdic, àcid bòric i tetraborat com a electròlit.

En el cas de la determinació de l'amikacina en plasma humà per CE es va utilitzar IDA (1-metoxicarbonilindolizina-3,5-dicarbaldèhid) com a reactiu per donar lloc a un derivat que es pugui detectar per fluorescència [47]. En aquest cas per dur a terme l'anàlisi es va utilitzar MEKC amb tampó fosfat a pH 7 com a electròlit, i SDS per formar les micel·les. Aquest mètode es pot aplicar també a l'anàlisi d'altres aminoglicòsids en plasma humà, com són l'arbekacina, dibekacina i kanamicina.

II.2. SISTEMES DE PRETRACTAMENT DE LES MOSTRES

Degut a la complexitat de les mostres biològiques, molts cops és necessari desenvolupar sistemes de pretractament de les mostres per tal d'extreure els analits de la matriu. D'aquesta manera es disminueix la quantitat de substàncies interferents causades pels components de la matriu, els quals molts cops es troben en concentracions molt més grans que els compostos d'interès. Una important avantatge de l'electroforesi capil·lar front la cromatografia de líquids és la seva elevada eficàcia, és a dir que és un sistema que permet separar un nombre elevat de compostos per unitat de temps. És per això que de vegades no és necessari un tractament tan extens com en el cas de l'anàlisi per cromatografia de líquids.

Degut a que les mostres líquides (orina, bilis, sèrum, plasma, etc...) són més homogènies i menys complexes que les mostres sòlides, els tractaments de mostres líquides seran més simples que els de les mostres sòlides.

Hi ha matrius que no necessiten un tractament previ, com pot ser orina i bilis. En aquests casos, generalment, només és necessari diluir la mostra amb aigua Milli-Q o tampó i injectar-la directament a l'equip, com per exemple en l'anàlisi de cefalosporines en orina i bilis humanes [39] i la determinació de quinolones en mostres d'orina [40]. D'aquesta manera no hi ha pèrdues de la mostra degut a tractaments previs.

Malgrat això, generalment és necessari l'ús de sistemes per extreure els antibiòtics de les matrius biològiques. Les tècniques més utilitzades són la microdiàlisi, la ultrafiltració, la ultracentrifugació, la desproteinització, l'extracció líquid-líquid (LLE) i l'extracció en fase sòlida (SPE).

Un sistema molt utilitzat per l'anàlisi d´antibiòtics en teixits animals és mitjançant la microdiàlisi, sistema que consisteix en mostrejar les zones que hi ha entre diferents teixits utilitzant una membrana semipermeable. Aquest pretractament

s'ha utilitzat en la determinació de cefalosporines en múscul i en la capa subcutània de la pell [26,45], així com en l'anàlisi de quinolones en la capa subcutània de la pell [28].

El plasma és una matriu que conté diversos components com proteïnes albúmines, α-àcid glicoproteïnes, lipoproteïnes i τ-globulines, les quals s'enllacen fàcilment amb les drogues, i per això és necessari el desenvolupament d'un pretractament com pot ser la desproteinització, la ultrafiltració, la ultracentrifugació, etc... per la seva extracció. De vegades l'extracció de la droga no és del 100 % degut a que poden quedar ocluïdes en les proteïnes precipitades.

Els teixits són les matrius més complexes per l'extracció de les drogues. És necessari un primer pas en el qual s'homogeïnitza la mostra utilitzant un sistema tamponador en el qual els antibiòtics que es volen extreure hi són solubles i estables i posteriorment es realitza un tractament per tal d'eliminar les interferències presents en la matriu de la mostra. El tractament posterior és similar al que s'utilitza en diferents matrius líquides, com és el cas del plasma (com són els sistemes d'ultrafiltració, ultracentrifugació i desproteinització, entre d'altres).

Un sistema simple i ràpid pel tractament de mostres és la ultrafiltració. En aquest procés s'utilitza poca quantitat de mostra, a nivells de μl, i s'utilitzen membranes per tal d'eliminar part de la matriu de la mostra. És un sistema que consisteix en separar les substàncies en funció del seu pes molecular i tamany. S'utilitza sobretot per a la separació de sals i altres compostos de baix pes molecular. Aquest sistema ha estat aplicat a l'anàlisi de diversos aminoglicòsids en plasma humà [38]. Un altre sistema que es pot utilitzar per pretractar mostres de plasma és la ultracentrifugació. Aquest sistema permet determinar la massa, l'estructura i les interaccions entre macromolècules. Aquest sistema es pot utilitzar per dur a terme la separació dels antibiòtics de la matriu de la mostra. Aquest sistema ha estat aplicat a la determinació de cefalosporines en plasma

humà per CE [26,45]. En aquests casos es van obtenir recuperacions d'un 95 % aproximadament.

Una altra manera d'extreure els antibiòtics de les mostres biològiques és mitjançant la desproteinització de la mostra mitjançant l'ús d'agents que facin precipitar les proteïnes com són àcids o solvents orgànics. Els àcids són normalment més efectius en la precipitació de proteïnes. D'aquesta manera, es va utilitzar àcid tricloroacètic per a l'anàlisi de quinolones en mostres de sèrum [40] i en l'anàlisi de tetraciclines en mostres de plasma i llet [49]. El problema d'aquest tractament és que com els àcids que s'utilitzen són molt forts i de vegades poden produir la degradació dels analits d'interès. L'ús de solvents orgànics també pot ser efectiu en el procés de desproteïnització. Normalment s'utilitza acetonitril. També es pot utilitzar acetona, metanol i etanol, entre d'altres. Com els solvents orgànics són agents precipitants més suaus que els àcids la proporció que s'afegeix és més gran. El problema que implica això és la dilució de la mostra i també pot afectar a la resolució i eficàcia del sistema. Alguns exemples on s'utilitza l'acetonitril com a agent precipitant és en la determinació de cefalosporines en sèrum [27] i de quinolones en plasma [28,29] per CE, obtenint-se recuperacions de més del 95 %. Un altre exemple va ser la determinació d'aminoglicòsids en ous [46].

Els sistemes més utilitzats i efectius en el tractament de mostres biològiques són l'extracció líquid-líquid (LLE) i l'extracció en fase sòlida (SPE). Fins a principis de la dècada dels 80 el sistema més utilitzat per fer el *clean-up* de la mostra era la LLE, però posteriorment la SPE va guanyar terreny. La LLE consisteix en fer múltiples extraccions en un medi orgànic i una fase aquosa amb unes condicions adequades de pH i força iònica, de manera que s'extreuen els compostos desitjats de la matriu i, a la vegada es redueix la quantitat de substàncies interferents en l'extracte final. Diferents solvents orgànics es poden utilitzar per dur a terme aquest procés, com l'ús de cloroform en l'extracció de quinolones en mostres de sèrum i orina [34].

Actualment el sistema d'extracció més utilitzat és la SPE. Es caracteritza perque és un sistema més ràpid, reproduïble i s'obtenen extractes més nets que en LLE. Es poden utilitzar diferents tipus de sorbents per dur a terme aquest procés, com són els sorbents que contenen C_{18}, d'intercanvi iònic, polimèrics, etc. El sorbent convencional per excel·lència, són els sorbents de C_{18}. Es caracteritzen pels grups alquílics que contenen. S'han utilitzat en varis estudis, com la determinació de penicil·lines [25], quinolones [32] en plasma de porc i tetraciclines en filets de peix [37] analitzats per electroforesi capil·lar. En tots aquests exemples es van obtenir recuperacions d'un 90 %. També es va aplicar a la determinació de 5 quinolones, ciprofloxacina, enrofloxacina, danofloxacina, difloxacina i marbofloxacina, obtenint recuperacions d'un 80 % [33], i a la determinació d'oxitetraciclina en fetge i ronyó de porc, obtenint recuperacions d'entre un 69 i un 80 % [35]. El principal problema d'aquests sorbents és que és necessari que el procés d'extracció sigui continu. Si no és així el sorbent s'asseca amb facilitat i pot provocar pèrdues en la recuperació dels compostos, així com poca repetibilitat del procés [35].

Actualment s'han desenvolupat sorbents que contenen polímers. Aquests sorbents no presenten aquest problema. Encara que el procés d'extracció s'aturi per un període curt de temps el sorbent té característiques hidrofíliques que impedeixen que aquest s'assequi ràpidament. Per tant és un sistema més robust i repetitiu. Un exemple és l'estudi d'oxitetraciclina en múscul de porc per CE. Quan es va utilitzar un sorbent C_{18} per dur a terme la SPE, les recuperacions van ser d'un 30 % [35]. Quan es van utilitzar cartutxos Oasis HLB, les recuperacions van ser de 76.4 % [35]. S'han desenvolupat diferents estudis amb l'ús d'aquests sorbents, com en la determinació de quinolones en mostres de plasma [30,31] i en la determinació de tetraciclines en plasma de porc [35], per electroforesi capil·lar. En aquests casos les recuperacions van ser de l'ordre del 90 %.

II.3. SISTEMES DE PRECONCENTRACIÓ EN LÍNIA

Normalment les concentracions d'antibiòtics que s'administren tant en medicina humana com en veterinària són molt baixes, a nivell de μg d'antibiòtic per kg de teixit. A més, en relació amb HPLC, el volum de mostra injectat en CE és al voltant d'unes 1000 vegades menor i la cel·la del detector és uns 100 cops menor que la de HPLC. Degut a això el sistema de CE amb detecció ultraviolada-visible molts cops no és prou sensible per dur a terme aquest tipus d'anàlisi [24].

Una manera ràpida i senzilla d'augmentar la sensibilitat en electroforesi capil·lar és mitjançant l'ús de sistemes d'*stacking on-column*, és a dir, que la preconcentració tingui lloc dins el mateix capil·lar, com són l'ús de la injecció de grans volums de mostra, conegut com a *Field Amplified Sample Injection* (FASI) [25,30,50,51,60-62], o bé la isotacoforesi capil·lar (ITP) [53-58].

II.3.1. INJECCIÓ DE GRANS VOLUMS DE MOSTRA (FASI)

El sistema FASI permet la injecció de grans volums de mostra, sistema que permet augmentar la concentració de l'analit dins el capil·lar a la vegada que s'elimina part de la matriu de la mostra [25,30,50,51,60-62].

El sistema consisteix en dissoldre la mostra en una matriu poc conductora, com per exemple aigua Milli-Q o bé en el tampó de separació diluït. En el primer pas del procés s'injecta la mostra hidrodinàmicament durant un període llarg de temps. En el segon pas s'aplica un camp elèctric invers al que es desenvoluparà durant la separació dels compostos. En el moment en què el corrent que es genera és entre el 90-97 % del corrent que es genera en el capil·lar quan aquest només conté electròlit, s'inverteix la polaritat de manera que tingui lloc la separació i detecció dels analits. En aquest pas té lloc

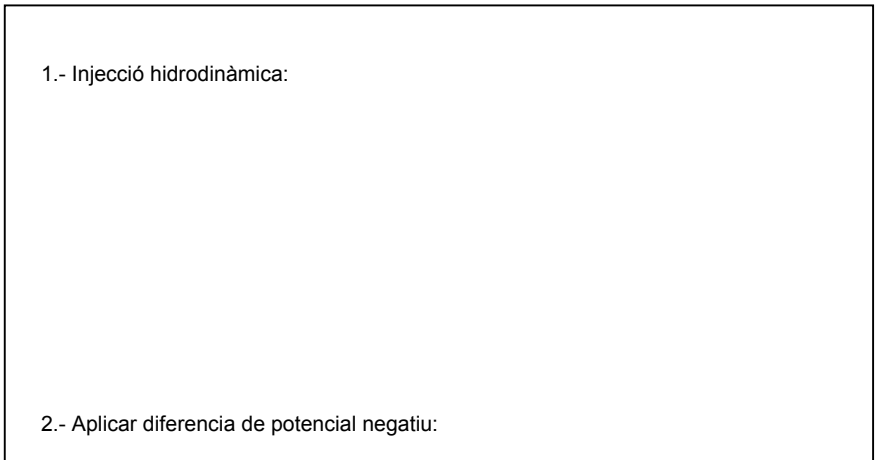
l'eliminació de la matriu de la mostra a la vegada que es preconcentren el/els analits. En l'últim pas té lloc la separació dels compostos. A la Figura 1 es mostren de manera gràfica aquests passos pel cas d'espècies carregades negativament.

Hi ha algunes aplicacions de FASI en mostres biològiques. Aquest sistema ha estat aplicat en l'anàlisi d'amoxicil·lina en plasma de porc [25]. Mitjançant aquest sistema FASI/CZE es va aconseguir augmentar la sensibilitat del sistema CZE convencional 4 cops, aconseguint límits de detecció de l'ordre de 280 $\mu g \cdot l^{-1}$ d'amoxicil·lina en plasma de porc. Aquest sistema FASI/CZE també va ser aplicat en l'anàlisi de marbofloxacina en plasma de porc [30]. En aquest cas l'augment del senyal va ser de 65 vegades, obtenint uns límits de detecció de 25 $\mu g \cdot l^{-1}$. Així doncs, l'efectivitat d'aquest sistema depèn dels analits a analitzar i de la mostra.

II.3.2. ISOTACOFORESI CAPIL·LAR (ITP)

La isotacoforesi és un sistema electrolític discontinu ja que s'utilitzen dos electròlits, el *leading* i el *terminating* [53-58,63-73]. Com en FASI els analits són dissolts en una matriu poc conductora. El *leading* conté ions d'elevada velocitat i el *terminating* de baixa mobilitat, i els ions de la mostra tenen mobilitat intermitja, de manera que es preconcentren isotacoforèticament entre el *leading* i el *terminating* [54,58].

L'acoblament d'un sistema d'ITP amb un de CZE es pot realitzar de diferents maneres [59,63-73]. Es poden acoblar dos capil·lars de manera que en primer té lloc la ITP, per tant, el procés de preconcentració i en segon té lloc la CZE, la separació dels compostos [64,65]. També hi ha la possibilitat de fer tot el procés ITP-CZE en un únic capil·lar.



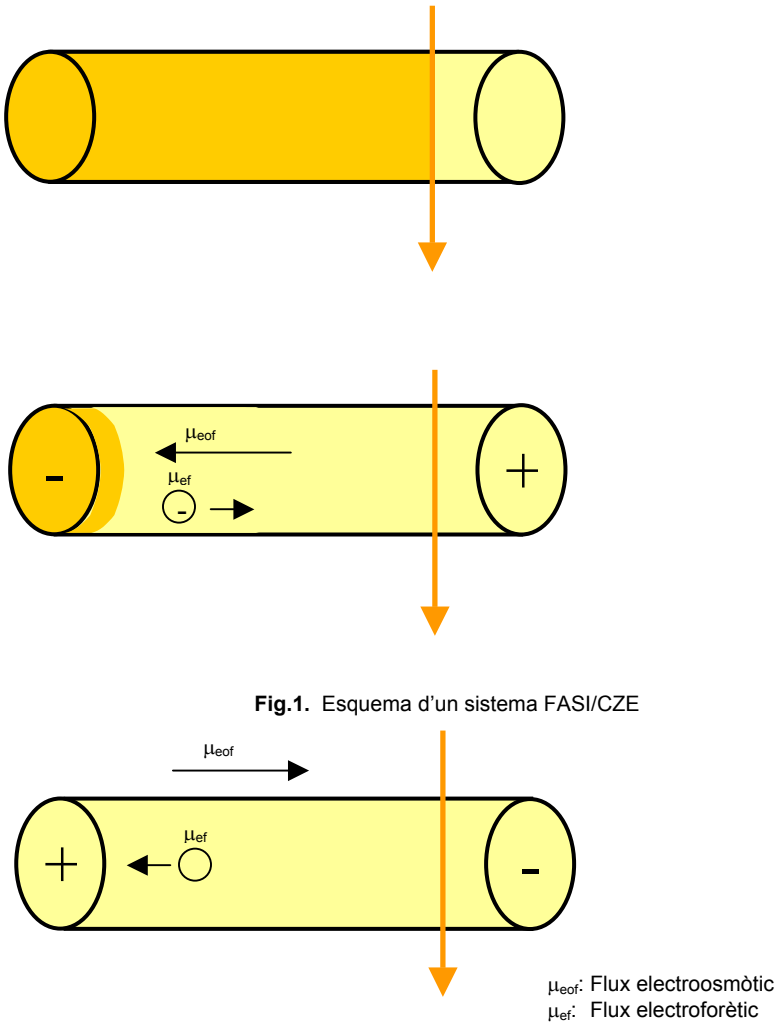


Fig.1. Esquema d'un sistema FASI/CZE

Fig.1. Esquema d'un sistema FASI/CZE

En aquest cas la quantitat de mostra que es pot injectar és menor, però la instrumentació és molt més simple que quan s'utilitzen dos capil·lars [59,63,69,73].

Per altra banda, tal com s'ha comentat anteriorment, l'*stacking* dels compostos té lloc en zones electroforètiques que es formen al llarg del capil·lar entre el

leading i el *terminating*. Per tant és important l'elecció dels electròlits que s'utilitzaran per dur a terme aquest procés ja que han de tenir la mobilitat adequada perque es doni l'*stacking* dels analits d'interès. Hi ha tres combinacions possibles de sistemes electrolítics que es poden utilitzar en els sistemes ITP/CZE. Es pot utilitzar el *leading* o el *terminating* per dur a terme la separació per CZE, o bé un electròlit diferent. Malgrat això, normalment s'utilitza per dur a terme la separació el *leading*.

En aquestes condicions, els passos que tenen lloc en isotacoforesi capil·lar són els següents:

1) Tant el vial d'entrada com el de sortida contenen el *leading*. S'omple el capil·lar amb el *leading*.
2) Se substitueix el vial d'entrada pel vial de la mostra i aquesta s'injecta hidrodinàmicament.
3) El vial de mostra se substitueix per un vial que contingui *terminating*. Llavors s'aplica un voltatge invers al de la separació. En el capil·lar els diferents analits es preconcentren en zones isotacoforètiques amb unes fronteres ben definides entre elles.
4) Se substitueix el vial de *terminating* per un que contingui *leading* i es torna a aplicar voltatge sense canviar la polaritat. Aquest pas serveix per eliminar completament el *terminating* del capil·lar.
5) S'inverteix la polaritat i té lloc la separació dels analits, és a dir, la CZE. En aquest cas s'utilitza com a electròlit el *leading* per donar lloc a la separació electroforètica.

Durant aquest procés s'eliminen els ions que tenen una major mobilitat que els ions del *leading* i els que tenen menor mobilitat que els ions del *terminating*. D'aquesta manera s'eliminen bastants interferències de la mostra [59].

A la Figura 2 es mostra un esquema del procés ITP/CZE en el cas de compostos aniònics i quan el sistema es caracteritza, perquè la velocitat del flux electroosmòtic és major que la del flux electroforètic.

És important escollir correctament els electròlits que s'utilitzaran per dur a terme l'ITP, de manera que les zones isotacoforètiques que es formin siguin estables i que els analits estiguin completament separats. Hi ha alguns estudis sobre la mobilitat de diferents ions i quins són els més adequats per l'ús com a *leading* (L) i terminating (T) [55,59].

Aquest sistema, ITP/CZE, ha estat aplicat en la determinació d'antibiòtics en mostres biològiques. Un cas va ser la determinació de tres quinolones, ciprofloxacina, enrofloxacina i flumequina en plasma de porc [31]. El sistema electrolític que es va utilitzar va consistir en 10 mM de monohidrogen fosfat sòdic i 5 mM de clorur sòdic a pH 9.0 com a *leading* i per dur a terme la CZE i 10 mM de β-alanina a pH 9.0 com a *terminating*. Mitjançant aquest sistema ITP/CZE es va aconseguir augmentar la sensibilitat per CZE uns 40 cops, aconseguint límits de detecció de 70, 85 i 50 $\mu g \cdot l^{-1}$ per ciprofloxacina, enrofloxacina i flumequina, respectivament. Un altre exemple va ser la determinació de marbofloxacina en plasma de porc [30]. En aquest estudi es

va realitzar una comparació dels resultats obtinguts en quant a sensibilitat mitjançant les tècniques d'ITP i FASI.

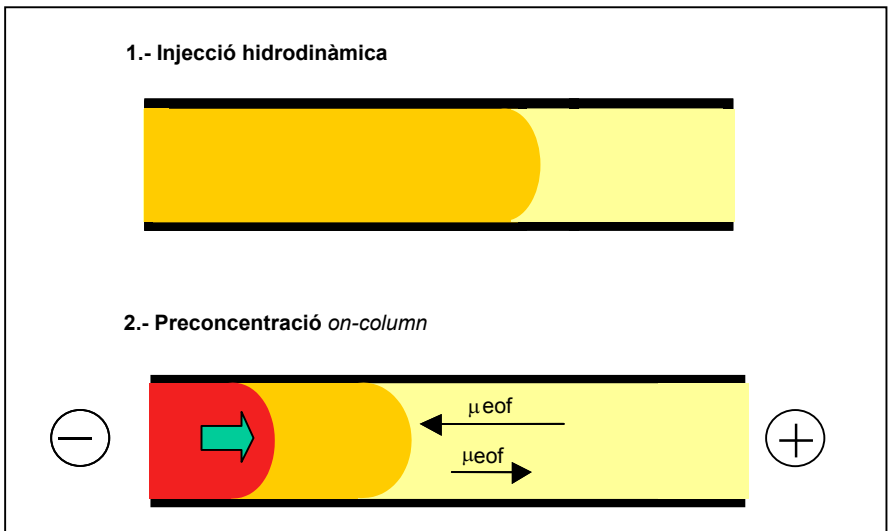


Fig. 2. Esquema d'un sistema ITP/CZE

S'utilitza com a electròlit per dur a terme la separació 10 mM de monohidrogen fosfat sòdic a pH 9.0. Per altra banda, dins de la ITP, es va estudiar quin electròlit era el més adequat utilitzar com a *terminating*. Es van estudiar tres de diferents, la β-alanina, la trietanolamina i tris(hidroximetil)aminometà (TRIS). Els millor resultat es van obtenir quan s'utilitzava la β-alanina, aconseguint

augmentar la sensibilitat del sistema uns 75 cops, obtenint límits de detecció de 25 ppb.

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Fins ara la cromatografia de líquids ha estat la tècnica analítica més utilitzada en l´anàlisi d´antibiòtics en mostres biològiques. Malgrat això, cal destacar el desenvolupament que ha sofert l´electroforesi capil·lar en aquests últims anys i que fa que sigui possible considerar aquesta tècnica com una alternativa real a la cromatografia de líquids.

L´electroforesi capil·lar en les seves diferents modalitats, tant l´electroforesi capil·lar per zones com la cromatografia capil·lar micel·lar, han incrementat considerablement l´aplicabilitat de les tècniques electroforètiques en la separació de diferents grups d´antibiòtics i la seva anàlisi en mostres biològiques [1-3]. Aquest tipus de mostres es caracteritzen per la seva complexitat i el gran nombre substàncies interferents, i és per això que és necessari desenvolupar sistemes de pretractament d´aquestes mostres perque sigui possible la seva anàlisi [4-9].

Per aquests motius, es va decidir realitzar un estudi bibliogràfic centrat en la discussió de l´aplicabilitat de sistemes de pretractament de la mostra per l´anàlisi de mostres biològiques per electroforesi capil·lar.

En el treball que es presenta a continuació es descriu quina és la importància de diferents grups d´antibiòtics, la seva estructura i les principals aplicacions de l´electroforesi capil·lar en les seves diferents modalitats, per l´anàlisi d´antibiòtics en mostres biològiques. Els antibiòtics que s´han estudiat són les β-lactames (penicil·lines i cefalosporines), aminoglicòsids, quinolones i tetraciclines. Aquestes són algunes de les famílies d´antibiòtics més utilitzades en el camp de la medicina i veterinària. S´ha fet una recerca bibliogràfica i es mostren treballs d´autors que mostren l´aplicabilitat de l´electroforesi capil·lar per la determinació d´aquests compostos en diferents mostres biològiques.
Aquest treball també inclou les diferents maneres amb que es pot pretractar la mostra abans de la seva anàlisi. Es diferencien dos tipus de mostres biològiques, els fluids biològics (com orina, serum, plasma, bilis, etc...) i els

teixits. En els cas dels fluids biològics de vegades no és necessari pretractar la mostra, simplement aquesta pot ser injectada directament [15-16].

Els sistemes de pretractament més utilitzats dels fluids biològics són la microdiàlisi, ultrafiltració, ultracentrifugació, extracció líquid–líquid i extracció en fase sòlida. Els sistemes que s´utilitzen són molts similars als utilitzats en cromatografia de líquids. En aquest treball es mostren vàries de les aplicacions que s´han desenvolupat a la bibliografia. En el cas dels teixits el pretractament consisteix en dues etapes. En la primera es talla et teixit en trossos petits i s´afegeix un tampó en el qual sigui soluble i estable l´antibiòtic que es vol analitzar, i a continuació es tritura. Així té lloc el que es coneix com homogenització de la mostra. La segona etapa és molt similar a la que es duu a terme en els fluids biològics. D´aquesta manera s´aconsegueix eliminar la matriu de la mostra així com un gran nombre de substàncies interferents [5-9].

L´altra part del treball inclou la discussió de diferents maneres d´augmentar la sensibilitat del sistema. Per una banda es parla de la possibilitat d´utilitzar diferents tipus de sistemes de detecció (fluorescència, detectors electroquímics, etc...), però es fa especial esment a l´ús de sistemes de preconcentració *on-column*, és a dir, dins el mateix capil·lar. Alguns dels sistemes de preconcentració més utilitzats són la injecció de grans volums i l´ús del sistema d´isotacoforesi capil·lar – electroforesi capil·lar per zones dut a terme dins un mateix capil·lar [10-14]. Aquests sistemes permeten la preconcentració de la mostra dins el capil·lar abans que tingui lloc la separació electroforètica. A través d´aquests sistemes es pot augmentar la sensibilitat del sistema varis ordres de magnitud.
A través dels sistemes de preconcentració presentats així com els diferents sistemes de tractament de les mostres, es demostra el gran potencial de separació i determinació que presenta l´electroforesi capil·lar en l´anàlisi d´antibiòtics en mostres biològiques. Per tant es tracta d´una bona alternativa a d´altres tècniques analítiques dintre d´aquest camp.

El treball presentat en aquest apartat ha estat enviat per a ala seva publicació a la revista internacional Trends Anal. Chem. 2002, una còpia del qual s´inclou a continuació.

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ANALYSIS OF ANTIBIOTICS BY CAPILLARY ELECTROPHORESIS IN BIOLOGICAL SAMPLES

Abstract

Antibiotics are widely used to treat and prevent diseases in humans and animals. It is therefore important to monitor their residues in biological samples. This is a review of some of the most important antibiotic groups used in medicine: □-lactam antibiotics (penicillins and cephalosporins), aminoglycosides, quinolones and tetracyclines. We describe how capillary electrophoresis (CE) is used to analyse these antibiotics in biological samples. We also discuss how the biological samples are pretreated before they are analysed and describe several ways in which the sensitivity of CE systems can be improved.

Keywords: Capillary electrophoresis, antibiotics, biological samples

Introduction

Antibiotics are chemical substances produced by microorganisms that kill or inhibit the growth of other microorganisms. They are used in human and animal medicine to prevent and treat diseases.

In veterinary medicine, antibiotics are used on companion animals, or pets, (mainly cats and dogs) to treat and prevent various infectious diseases such as mastitis, enteritis, peritonitis, pneumonia and septicemia [1].

Most veterinary medicines, however, are used to prevent diseases and promote growth in food-producing animals, to increase the rate of weight gain and reduce the amount of feed per unit of gain. They are administered in feed at subtherapeutic doses during the growth period. Widespread administration of antibiotics in veterinary medicine is a potential risk because residues of the drugs may be present in edible tissues. The main risk to human health from the use of antibiotics in animals is that animal bacteria may develop resistance to them. While antibiotics are used bacteria will develop resistance, either by mutation, gene acquisition or a combination of the two [1].

In several countries, consumer exposure to residues has been estimated through studies of total diet carried out by international agencies such as the WHO (World Health Organisation) and the FAO (Food and Agriculture Organization of the United Nations). These studies determined how far the population is exposed to certain residues in food. These data allow to calculated the average exposure of consumers to a range of chemicals in food and to interprete this average exposure in terms of the acceptable daily intake (ADI) for these chemicals [2]. ADI is an estimate of the amount of a substance, expressed on a body weight, that can be ingested on a daily basis over a lifetime without appreciable health risk.

All new and existing active substances used in veterinary medicinal products for food-producing animals require the establishment of a maximum residue limit (MRL) under Council Regulation of European Communities (EEC) 2377/90. MRLs are based on the concept of ADI, but this is matched against drug residue depletion in the target species so that values can be established for tissues and animal products.

Those that do not were banned in veterinary medicines from that date. Moreover, Member States cannot authorise a veterinary medicine that contains a new active ingredient until it has an EU MRL. Because of differences in pharmacokinetics, MRLs are specific to each species so, although the ADI for a drug is universal, MRLs must take into account species-specific pharmacokinetics and specific toxic metabolites [2].

Residue analysis is concerned with food safety, e.g. it involves establishing whether food is safe or unsafe for human consumption. For chemicals used in farming, residue analysis is part of the monitoring programmes of regulatory agencies. Such testing is designed to ensure that residues are at levels that respect the established MRLs. For prohibited substances, residue testing is designed to monitor whether they comply with the regulations. It is therefore important to develop analytical procedures for determining antibiotics in biological matrices at low levels, e.g. below MRLs [1,2,3].

Residue analysis methods have taken enormous strides since the early 1980s. We have seen advances in analytical equipment and computerisation and at every stage of the analytical process. Many of these advances have been driven by the increased sensitivity and specifity of the determination techniques. Regulatory demands for the control of chemical contaminants in food have increased dramatically over the last decade, to such an extent that regulations are now an important consideration in the international trade of food commodities. This has led to an increase in the demand for analytical methods

for detecting residues in foodstuffs. Chromatographic techniques have played a key role, especially high-performance liquid chromatography (HPLC). This versatile technique has been widely studied and is commonly used to analyse antibiotic residues in biological samples [4,5]. It determines low concentration levels, which is fundamental to residue analysis. However, HPLC is limited by sometimes poor separation efficiencies, expensive columns, and the consumption of relatively high amount of buffer solutions amd organic solvents.

Capillary electrophoresis (CE) is a good alternative to chromatographic techniques, especially when only small amounts of samples are available [6]. The main advantages of CE are that separation efficacy is greater, that smaller sample volumes are needed, and that it is less expensive because organic solvent consumption is lower and because the capillaries are cheaper. CE is also suitable for automation, high sample throughput, and multiple detection modes. CE is therefore suitable for monitoring antibiotics in a variety of matrices such as plasma and tissue samples and has been used in several studies with a wide variety of biological samples by capillary electrophoresis [7-20,45]. Due to space limitation this review only includes some of antibiotic groups (penicillins, cephalosporins, aminoglycosides, quinolones, and tetracyclines), which we have studied more deeply. Section 2 includes a description of each antibiotic group and some examples of their analysis by CE.

Residue analysis is very strongly related to the sample. Biological samples are characterised in terms of their complexity. They contain many components that can interfere and make their analysis more difficult. It is therefore necessary to pretreat the samples so that they can be obtained in a suitable medium for their injection. In Section 3 we describe several types of pretreatment.

One important inconvenient of CE is that sometimes it is not enough sensible to determine low concentrations. This is caused by the low sample injection volume and the short optical pathlength for on-capillary detection. Also, one of the main

problems with analysing traces of residues is their low concentration. They are most often present in mg/kg concentration, or even lower, in biological matrices (e.g. serum, plasma, urine, muscle, kidney, liver, etc.). Ways of increasing the sensitivity of the method are therefore needed. In Section 4 we describe several ways of doing this.

Study of antibiotic classes

β-Lactams

Penicillins

Penicillins work against bacterial infections, inhibiting the formation of the cell wall in susceptible bacteria. They are used to treat diseases in humans and animals.

The basic structure of penicillins is a thiazolidizine ring connected to a β-lactam ring, to which a side chain is attached. These compounds are therefore neutral and weakly ionic molecules, so micellar electrokinetic capillary chromatography (MEKC) is often the mode of CE used to separate them. In penicillin analysis, sodium dodecyl sulphate (SDS) is generally used to form micelles and phosphate and borate solutions are often used as the background electrolyte (BGE).

Li et al. [21] separated amoxicillin and 14 of its impurities in a commercial sample of amoxicillin sodium salt. These compounds were practically resolved in a micellar buffer made of 70 mM sodium dihydrogenphosphate, 125 mM sodium dodecyl sulphate (SDS) and 5 % acetonitrile adjusted to pH 6.0. In another study, ampicillin and 15 of its impurities were analysed and separated in a buffer

system consisted of 40 mM sodium dihydrogen phosphate and 100 mM SDS at pH 7.5 [22].

Hows et al. [23] then developed the separation of a complex mixture of 17 sulphonamides, 2 dihydrofolate reductase inhibitors and 11 β-lactam antibiotics (9 of which were penicillins) dissolved in deionised water in a MEKC system. The buffer system consisted of 20 mM sodium tetraborate, 100 mM SDS and 0.5 mM EDTA adjusted to pH 8.5. This system practically separated all the compounds since 25 peaks were produced from 30 total compounds.

Recently, amoxicillin in pig plasma samples was analysed by CE [7]. In this study, the samples were pretreated by solid-phase extraction (SPE) with C_{18} cartridges and analysed by CZE. MEKC was not necessary because a single component was studied. The buffer system consisted of 20 mM sodium tetraborate at pH 9.0. Fig. 1 shows the electropherogram for the analysis of amoxicillin in pig plasma samples by SPE/CZE. Bossuyt et al. [39] developed a CZE method to determine piperacillin in human serum. After its administration, at different period of time the samples are collected and analysed. The running buffer was a borate buffer.

Some papers show CE with mass spectrometry detection (MS) as alternative to analyse penicillins [40,41]. Samskog et al. [40] used CE-MS for analyzing a standard mixture of peptides and penicillins. The buffer consisted of 16 mM acetic acid-ammonium acetate buffer (pH 3.0) with 20 % 2-propanol as organic modifier. Hilder et al. [41] determined penicillin V and several of its impurities by CE-MS. In this case a CE in nonaqueous medium (NACE) was developed. The buffer system consisted of 20 mM ammonium acetate (pH 6.5) in 8:2 (v/v) 2-propanol/water. This system was applied in the analysis of these penicillins in a real fermentation broth.

Table 1 lists several applications of CE for analysing antibiotics in biological samples.

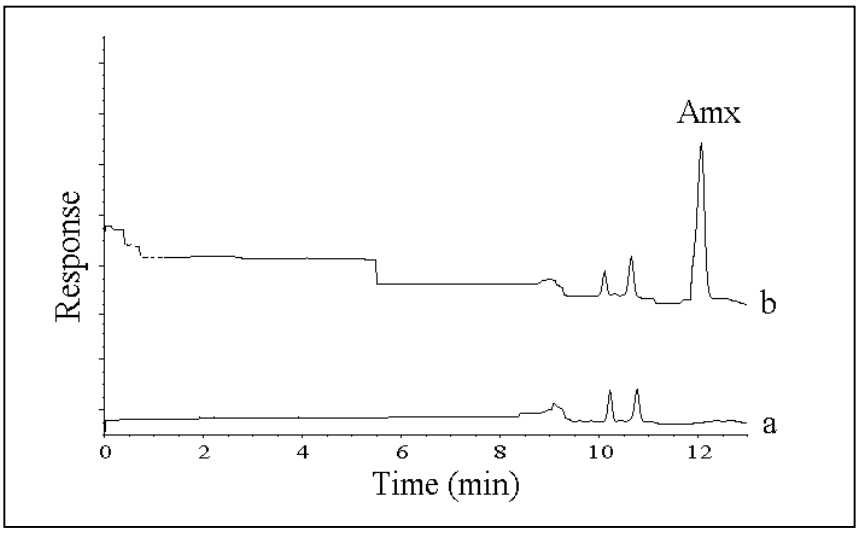

Fig. 1. Electropherograms of a blank pig plasma sample (a) and a spiked plasma sample (20 $mg \cdot l^{-1}$ of amoxicillin added to the original sample of plasma) (b) after solid-phase extraction (from [7], reproduced with permission from Elsevier Science).

Table 1. Analytical determination of antibiotics in biological samples by CE

Compounds	Sample	CE mode	Pretreatment	Recovery	Detection	Detection limit	Ref.
Amoxicillin	Plasma	CZE	SPE (C_{18} cartridges)	90 %	UV	0.28 mg·l^{-1}	[7]
Piperacillin	Serum	CZE	Direct injection	100 %	UV	--	[39]
Cefpirome	Microdialysate, plasma	MEKC	Ultracentrifugation	95 %	UV	1 and 0.3 mg·kg^{-1} in plasma and microdialysate, respectively	[8,26]
Cefazolin	Serum	CZE	Deproteinisation (with acetonitrile)	95 %	UV	5 mg·l^{-1}	[9]
Several cephalosporins	Urine, bile	CZE	Direct injection	100 %	UV	2.5 and 5 mg·l^{-1} in urine and bile, respectively	[27]

Compounds	Sample	CE mode	Pretreatment	Recovery	Detection	Detection limit	Ref.
Nalidixic acid and two impurities	Serum, urine	MEKC	LLE (with chloroform)	95-100 %	UV	0.8 $\mu g \cdot l^{-1}$	[16]
Moxifloxacin	Plasma, microdialysate	CZE	Ultrafiltration	95 %	Fluorescence	2.5 and 5 $mg \cdot Kg^{-1}$, in plasma and microdialysate, respectively	[10]
Ciprofloxacin, desethyleneciprofloxacin	Plasma	CZE	Deproteinisation (with acetonitril)	95 %	Fluorescence	20 and 10 $\mu g \cdot kg^{-1}$ for ciprofloxacin and desethyleneciprofloxacin, respectively	[11]
Ciprofloxacin, enrofloxacin, flumequine	Plasma	CZE	SPE (Oasis HLB cartridges)	90 %	UV	70, 85 and 50 $\mu g\ l^{-1}$ for ciprofloxacin, enrofloxacin and flumequine, respectively	[13]
Marbofloxacin	Plasma	CZE	SPE (Oasis HLB cartridges)	90 %	UV	20 $\mu g\ l^{-1}$	[12]
Several quinolones	Kidney	NACE	SPE (C_{18} cartridges)	80 %	UV	$< 120\ \mu g\ l^{-1}$	[15]
Pipemidic acid	Serum	CZE	Direct injection	100 %	Amperometric detection	0.35 $mg \cdot l^{-1}$	[43]
Oxolinic acid, piromidic acid	Urine	CZE	Direct injection	100 %	Amperometric detection	8.5 and 0.8 $mg \cdot l^{-1}$ for oxolinic acid and piromidic acid, respectively	[44]

(Table 1 continued)

Compounds	Sample	CE mode	Pretreatment	Recovery	Detection	Detection limit	Ref.
Oxytetracycline	Raw and cooked catfish	CZE	SPE (C_{18} cartridges)	89.6 – 94.5%	UV		[19]
Oxytetracycline	Kidney, liver and muscle	CZE	SPE (C_{18} cartridges for kidney and liver and Oasis HLB cartridges for muscle)	69.80 % for kidney and liver, 76.4 % for muscle	UV	160, 120 and 85 μg Kg^{-1} for kidney, liver and muscle, respectively	[17]
Oxytetracycline and three impurities	Plasma	NACE	SPE (Oasis HLB cartridges)	74-100%	UV	0.2-0.3 μg l^{-1}	[18]
Several tetracyclines	Milk, plasma	NACE	Deproteinisation (with trichloroacetic acid)	95 %	Fluorescence	100 $\mu g \cdot kg^{-1}$	[25]

(Table 1 continued)

Cephalosporins

Cephalosporins are a group of β-lactam antibiotics whose structure and action are similar to those of penicillins. The cephalosporin ring structure is derived from 7-aminocephalosporanic acid (7-ACA) while the penicillins are derived from 6-aminopenicillanic acid (6-APA). Both structures contain the basic β-lactam ring but the cephalosporin structure allows for more gram-negative activity than the penicillins and aminocillins. Substitution at the "R" sites (different side chains) allows for changes in the spectrum of the activity and the duration of the action [24].

As with penicillins, MEKC is the mode of CE that is most often used to analyse cephalosporins. As before, SDS is generally used to form micelles in these systems [8,24,25]. Li et al. [24] determined cefadroxil and 10 of its known related substances in an artificial mixture. In this mixture, the amounts of all impurities were much higher than in commercial samples, which means that the method may be used for commercial samples. The compounds were completely separated in a micellar buffer made of 50 mM sodium acetate buffer and 110 mM SDS adjusted to pH 5.2.

Today, β-lactam antibiotics are widely used in clinical applications. Some studies have developed the analysis of cefpirome in microdialysate and centrifuged plasma human samples using MEKC [8,26]. Fig. 2 shows the MEKC separation of cefpirome in plasma samples. This figure compares the results of the analysis of blank plasma sample with the analysis of a plasma sample after cefpirome administration and shows the efficacy of using CE to analyse this kind of samples. The running buffer was made of 20 mM citrate and 50 mM SDS at pH 2.5 [8].

In some studies, CZE is used to analyse cephalosporins. Mrestani et al. [27] developed a CZE method for determining 9 cephalosporins in urine and in the

bile of humans and rabbits. 50 mM citrate buffer at pH 6.0 was used as BGE. Meter et al. [9] developed a CZE method for determining the effect of intraoperative blood loss on the serum levels of cefazolin in patients undergoing total hip arthroplasty. They established a CZE method using sodium bicarbonate buffer at pH 10.0 as BGE.

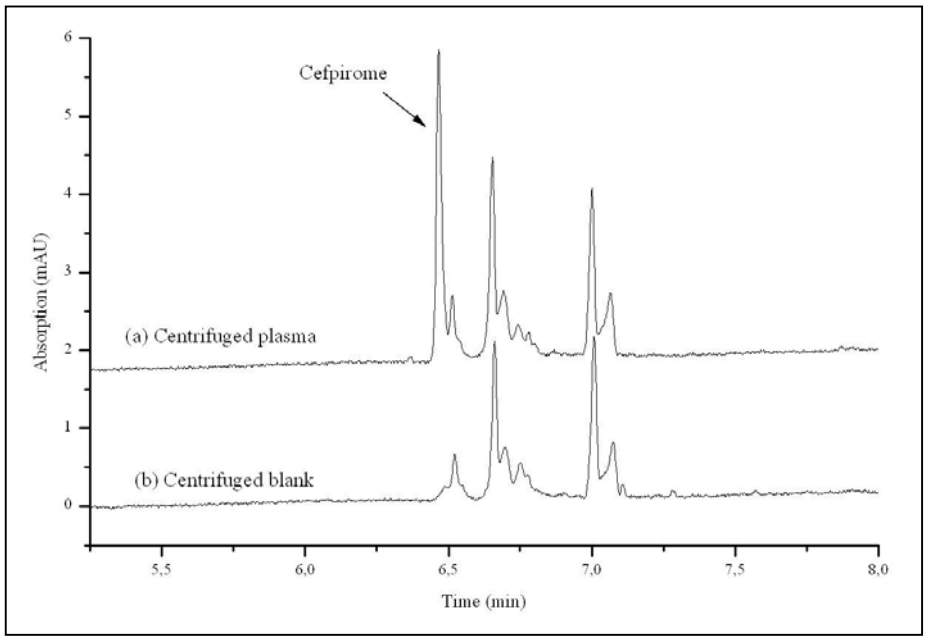

Fig. 2. (a) Electropherogram of a centrifuged plasma sample obtained after i.v. administration of 2 g cefpirome to a healthy volunteer as a bolus infusion over 10 min. Blood was collected 8 h after drug administration. (b) Electropherogram of a centrifuged plasma blank before drug administration. Plasma samples were subjected to ultracentrifugation procedure (from [8], reproduced with permission from Wiley-VCH).

Aminoglycosides

Aminoglycosides are an important class of antibiotics that are active against both gram-positive and gram-negative bacterial infections and have found wide

used in both human and veterinary medicine. They act by creating fissures in the outer membrane of the bacterial cell [28]. Aminoglycosides enhance the bactericidal activity of β-lactam antibiotics and make a useful combination for treating serious infections.

Aminoglycosides consist of two or more aminosugars joined via a glycoside linkage to a hexose nucleous of the drug. As most of these antibiotics lack chromophores, they need to be chemically derivatised, or to form complexes spectrometically actives [20,29-31].

Several studies used CZE mode to analyse aminoglycosides [29-31]. For example, Kaale et al. [30] developed a CZE system to analyse gentamicin using pre-capillary derivatisation with 1,2-phthalic dicarboxaldehyde (OPA) and mercaptoacetic acid (MAA) and UV detection. This mixture was resolved in a buffer made of 30 mM sodium tetraborate, 7.5 mM β-cyclodextrin and 12.5 % (v/v) methanol at pH 10.0. This method separated the components quickly, selectively and sensitively and could be used to analyse gentamicin in commercial preparations.

Sometimes when borate buffers are used, borate complexes are formed. These are UV-absorbing complexes that enable aminoglycoside antibiotics to be directly detected [29,31]. Flurer et al. [31] separated twelve aminoglycosides by CZE system in this way. The running buffer was 185 mM sodium tetraborate at pH 9.0 and the sample buffer was 140 mM sodium tetraborate at pH 9.0. This system can be used to analyse pharmaceutical preparations such as injectables and ointments.

Kowalski et al. [29] described a rapid and sensitive CZE assay for determining streptomycin in eggs. It is especially difficult to analyse antibiotics in eggs because they contain very low density lipoprotein, phospholipids and proteins, which makes most derivatisation techniques impossible. It was possible to

determine them because adding borates to the aqueous solutions of mono- and oligosaccharides results in the complex formation between tetrahydroxybirate and polyols. Borate complexes which are directly detected by UV – visible were therefore obtained. The analysis was performed with a buffer solution of 30 mM sodium dihydrogenphosphate, 5 mM boric acid and 5 mM sodium tetraborate.

Oguri et al. [20] developed a MEKC system for analysing amikacin in human plasma samples. 1-methoxycarbonylindolizine-3,5-dicarbaldehyde (IDA) was used as fluorescence derivatisation reagent. Fig. 3 compares the electropherograms obtained by analysing a blank human plasma and a plasma sample spiked with amikacin at 10 and 100 $mg \cdot l^{-1}$ levels. It shows that MEKC can be used to analyse amikacin in biomatrices. The analysis was performed using 20 mM phosphate-borate buffer as BGE and 40 mM SDS adjusted to pH 7.0.

This method may have wider applications for determining other aminoglycoside antibiotics such as arbekacin, dibekacin and kanamycin in human plasma without altering the method too much.

Kane et al. [42] show the application of CE-MS-MS system for determining amikacin and its acetylated derivatives. 25 mM tris-192 mM glycine buffer at pH 8.4 were used as BGE.

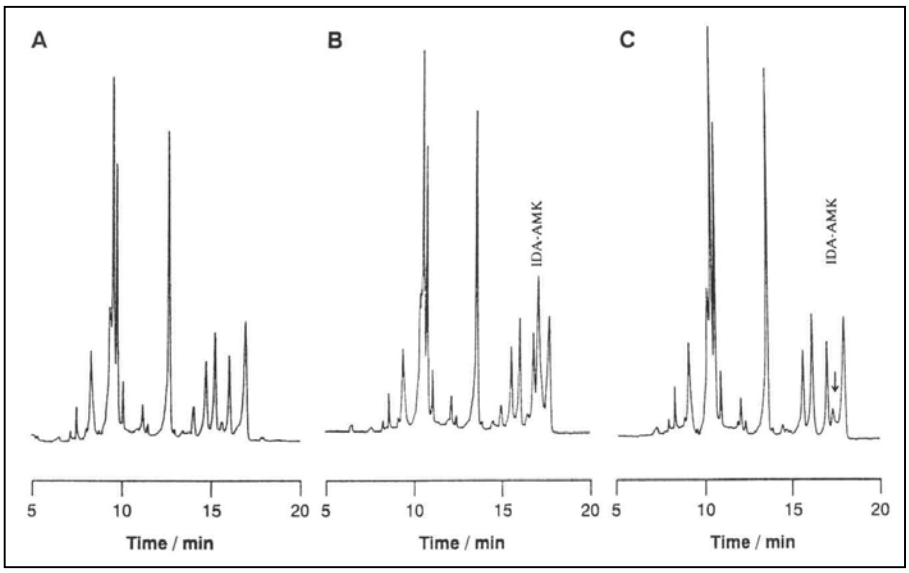

Fig. 3. Typical electropherogram of human plasma (A) blank, (B) spiked with amikacin at 100 $mg \cdot l^{-1}$ and (C) at 10 $mg \cdot l^{-1}$ (from [20], reproduced with permission from Elsevier Science).

Quinolones

Quinolones are an important group of synthetic antibiotics with bactericidal action that results from the selective inhibition of bacterial DNA synthesis. They are active against many gram-positive and gram-negative bacteria. Numerous structurally related quinolones have been synthesised, and several of them are in routine clinical use all over the world. Their antibacterial activity is greatly increased when 6-fluoro and 7-piperazinyl groups are added to their 4-oxo-1,4-dihydroquinoline skeleton. The more recent introduction of fluorinated quinolones is a particularly important therapeutic advance, since these agents have broad antibacterial activity and are an effective way to treat a wide variety of infectious diseases. They are also widely used to treat and prevent veterinary diseases in food-producing animals [14].

CE with both operation modes (CZE, MEKC) has proved useful for analysing quinolones [10-14,16]. Pérez-Ruiz et al. [16] developed a CE system for analysing nalidixic acid and 2 major impurities in serum and urine samples using UV – visible detection. A MEKC system was needed to completely separate all the compounds. The electrophoretic electrolyte consisted of 50 mM borate buffer at pH 9.0 containing 25 mM SDS and 10 % acetonitrile.

In the literature the separation and quantification of 10 quinolones by CZE in pig plasma samples is described [14]. The buffer consisted of 40 mM sodium tetraborate at pH 8.1 containing 10 % (v/v) methanol. Moller et al. [10] developed a CZE method to measure moxifloxacin in human plasma and microdialysate samples in clinical studies. The running buffer was made of 60 mM phosphoric acid at pH 2.2 for plasma samples and 80 mM phosphoric acid at pH 2.0 for microdialysate samples. Another CZE system was used to analyse ciprofloxacin and its metabolite desethyleneciprofloxacin in human plasma samples [11]. Separation was carried out in a 1:1 mixture of 100 mM phosphoric acid and 100 mM boric acid.

Another application was the analysis of ciprofloxacin, enrofloxacin and flumequine in pig plasma samples by capillary isotacophoresis – zone electrophoresis (ITP-CZE) in a single capillary [13]. The BGE consisted of 10 mM sodium monohydrogen phosphate and 5 mM NaCl at pH 9.0. An ITP-CZE system was also used in the analysis of marbofloxacin in pig plasma samples by CZE [12].

Recently, 5 quinolones in pig kidney samples were analysed by a nonaqueous capillary electrophoresis (NACE) system [15]. The effects of the composition of the organic media were studied. The BGE was 20 mM ammonium acetate, 0.004 % polycation hexadimethrine bromide (HDB) and 4 % acetic acid at pH* 5.4 in methanol/acetonitrile (50:50,v/v) medium. (In nonaqueous systems, the pH is generally defined as apparent pH or pH*) [18,25,32].

Pipemidic acid was determined in human serum samples by CZE with end-column amperometric detection [43]. The buffer consisted of 0.12 mM sodium acetate - 0.88 M acetic acid. Zhang et al. [44] developed a CE with on-column amperometric detection for determining ofloxacin and parimiazid in human urine.

The BGE consisted of 40 mM borax-phosphoric acid (pH 4.0) for ofloxacin and 40 mM borax (pH 9.2) for parimiazid.

Tetracyclines

Tetracyclines (TCs) are a group of clinically important natural products and Tetracyclines (TCs) are a group of clinically important natural products and semi-synthetic derivatives characterised by a broad spectrum of activity against pathogenic micro-organisms including Gram-positive and Gram-negative bacteria and protozoa. These therapeutic compounds are bacteriostatic antibiotics that act by inhibiting the formation of proteins within bacteria. They are used to control bacterial infections in humans and animals and have also found applications in preserving harvested fruits and vegetables, exterminating insect pests, and supplementing animal feed. The chemical structures of this group of antibiotics are closely related and are derived from a common hydronaphthacene nucleus containing four fused rings [18].

Several studies have analysed TCs by CZE and obtained an efficient separation [17,19]. Huang et al. [19] analysed oxytetracycline (OTC) in raw and cooked channel catfish by CZE using 200 mM phosphate buffer at pH 2.0 as electrolyte. Fig. 4 compares the analyses of OTC in a standard solution, blank catfish, untreated catfish spiked with OTC, and treated raw catfish.

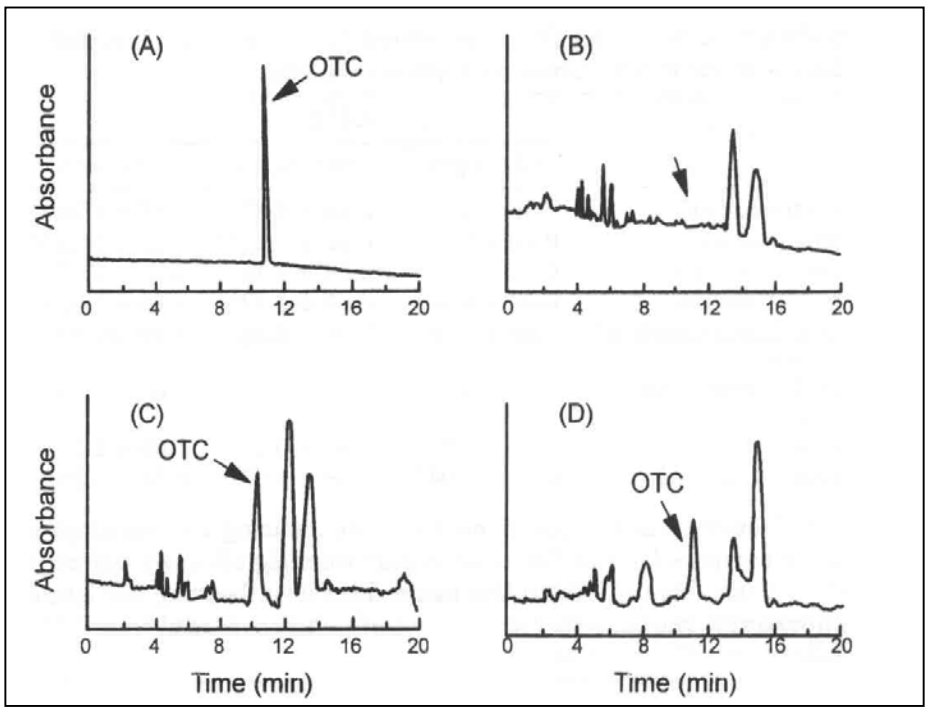

Fig. 4. Typical electropherograms of (A) 1.0 mg·Kg^{-1} of OTC standard solution, and muscle extracts of (B) blank catfish, (C) untreated catfish spiked with 1 mg OTC/g, and (D) OTC-treated raw catfish (from [19], reproduced with permission from American Chemical Society).

There is no interference with the OTC peak in treated catfish muscle. Another application in tissue samples involved the determination of OTC in pig kidney, liver and muscle samples by CZE [17]. The running buffer was 20 mM sodium carbonate and 1 mM EDTA adjusted to pH 11.2. This method determined OTC in pig tissue samples with LOD below MRL values, which are 600, 300 and 100 μg·kg^{-1} for kidney, liver and muscle, respectively. CZE becomes a complementary tool to current HPLC methods for analysing residual TCs in tissue samples.

TCs are known to form chelates with different metal ions. These have recently been used to obtain high efficiency and selectivity with NACE system. TjØrnelund et al. [32] used NACE system to analyse the widely used antibiotic oxytetracycline (OTC), which is contained in the drug “hydrocortisone with terramycin ointment”. The electrophoresis medium consisted of 500 mM magnesium acetate tetrahydrate in N-methylformamide (NMF). Another NACE system with UV-visible detection was used to analyse OTC and 3 of its impurities in pig plasma samples [18].

The running buffer consisted of 25 mM sodium acetate, 1 mM EDTA and 26 mM methanesulfonic acid at pH* 4.0 dissolved in methanol / acetonitrile (50:50,v/v).

This system showed that NACE has great potential for analysing OTC and its impurities in pig plasma samples.

The same authors analysed several TCs by NACE system using LIF detection [25]. Metal complexes are known to form in nonaqueous solvents. The chelates formed with Mg^{2+}, Ca^{2+} or Eu^{2+} are fluorescent under neutral or alkaline conditions. Fluorescent quelates of the TCs may also form in acidic solutions of Zr^{4+} or Al^{3+}. NACE – LIF system was therefore established to determine several TC antibiotics in milk and plasma samples. 500 mM magnesium acetate tetrahydrate in NMF was used as BGE.

METHODS FOR EXTRACTING ANTIBIOTICS FROM BIOMATRICES

Biological samples are contain several interference and proteinaceous components, which can make them difficult to analyse. It is therefore necessary to develop sample-pretreatment systems to remove interference, proteins and particulate matter. CE has two features that may make it better than HPLC for reducing the extent of sample pretreatment. Firstly, separation is generally performed in solution in an open tube. So there are no problems with fouling a stationary phase: there is only the problem of fouling the capillary surface. Secondly, CE is very efficient; so as a large number of components can be separated per unit of time, extractions to remove interference may be less needed [33].

However, the matrix of the sample can affect the quality of the separation strongly and can also affect the reproducibility and the quantitative results. In particular, large concentrations of salt or proteins in the sample solution can deform the shape of the peak and/or shift migration time [34].

We can differentiate two different kinds of samples: biological fluids and tissue samples. Biological fluids, such as urine, bile, saliva, blood -plasma or serum- or milk, are generally easier to handle than solid samples and residues are more homogeneously dispersed throughout the sample.

Under certain conditions, biofluid samples such as serum, plasma, bile and urine can be injected directly into CE capillaries. Fig. 5 shows the analysis of a blank human urine standard and a urine sample containing several cephalosporin compounds by direct injection [27].

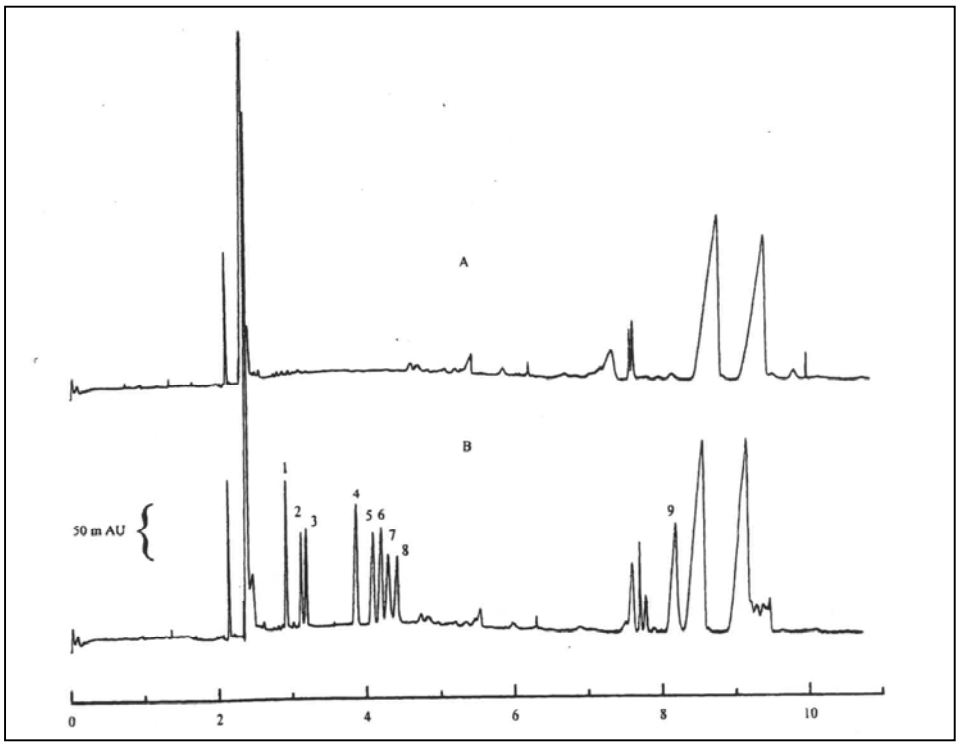

Fig. 5. Electropherogram of a blank human urine standard (A) and urine sample containing 20 $mg \cdot l^{-1}$ of a mixture of cephalosporins (B): 1 cephalexin; 2 cefadroxil; 3 cefaclor; 4 ceftazidim; 5 cefsulodin; 6 cefuroxim; 7 cefamandol; 8 cefotaxim; 9 cefodizim (from [27], reproduced with permission from Elsevier Science).

The human urine samples were diluted with standard solutions containing a known amount of cephalosporins. This can reduce the analysis time and cut the cost of consumables considerably. Direct injection of the sample solution also guarantees 100 % recovery as the entire sample solution is introduced into the capillary. In such cases, the samples are only diluted with buffer or deionised water and are introduced into the instrument with no further sample preparation [33,35]. This was used to analyse penicillins in serum [7], cephalosporins in urine and bile samples [27] and quinolones in serum [44] and in urine samples [36,43] with CE.

The usual techniques for extracting and cleaning-up antibiotics from biological fluids involve microdialysis [8,10,26], ultrafiltration [20], ultracentrifugation [8,26], sample deproteinization [9-11,25,29,36], liquid-liquid extraction (LLE) [16], solid-phase extraction (SPE) [7,12-15,17,18], etc.

Most bacterial infections are located in the interstitial space of peripheral tissues rather than in the blood stream. The clinical efficacy of antimicrobial chemotherapy is therefore determined by antibiotic concentrations at the target site of drug action, e.g. the interstitial space. Microdialysis is a tool for directly measuring free interstitial tissue. It is based on sampling analytes from interstitial space using a semipermeable membrane at the tip of a microdialysis probe.

Ultrafiltration is usually used in the pretreatment of plasma samples. This system provides quick and easy sample preparation of microlitre volumes of solution prior to instrumental analysis. Ultrafiltration systems are generally designed for microlitre volumes (up to 400 µL) with low-binding membranes and low hold-up

volumes for improved recoveries. Oguri et al. [20] used this simple and highly efficient system as a sample pretreatment for analysing aminoglycosides in human plasma samples by CE.

Another way to pretreat plasma samples is to use ultracentrifugation. This system uses a centrifugal filter device with a small cut-off and is suitable for separating free and bound analytes. Only the free form of the drug can pass through the centrifugal filter device. Examples of this system are shown in the analysis of cephalosporins in human plasma samples by CE [8,26], where recoveries were around 95 %.

Deproteinisation is one of the most useful sample pretreatment techniques. Several deproteinisation methods used for HPLC sample preparation can also be used with CE. Acids are quite effective precipitating agents; e.g. 0.4 ml 10 % w/v trichloroacetic acid per ml of plasma precipitates more than 95 % of sample proteins. Pérez-Ruíz et al. [36] used this system to analyse quinolones in serum samples and Tjørnelund et al. [25] used it to analyse tetracyclines in plasma and milk samples by CE. With this system, many interference were removed and recoveries were good. However, this system uses extreme acid conditions, which can sometimes degrade the analytes. Organic solvents can also be effective, but a 2:1 organic solvent/plasma ratio is needed for acetonitrile and acetone to achieve 95 % precipitation of proteins. Ethanol and methanol are even less efficient [33]. In these cases, the proportion of organic solvent is very high, dilution may be excessive and alterations in resolution and peak-shape may take place in CE analysis. However, several studies have used acetonitrile as a precipitating agent, e.g. to analyse quinolones in plasma samples [10,11] and cephalosporins in serum samples [9], with recoveries of over 95 %. Another example is the analysis of aminoglycosides in egg yolk [29], where CE was used to separate the compounds. In this case, recoveries were 71.8 % because of the complexity of the sample.

Both solid-phase extraction (SPE) and liquid-liquid extraction (LLE), therefore, can effectively remove proteinaceous components from biological fluids. A typical residue laboratory in the 1970s and early 1980s frequently used LLE as a clean-up step. However, LLE is time-consuming because it requires lengthy solvent evaporation steps and it is difficult to apply the method to large batches of samples. The risk of cross-contamination is high because it is difficult to use disposable materials. Glass separatory funnels are generally used. LLE is more laborious than other extraction/purification methods. Intractable emulsions may form when shaking. It is also expensive because large volumes of organic solvents are often used. Also, some of these solvents are hazardous environmental pollutants. LLE requires expensive glassware and distillation or evaporation apparatus. The properties of the extracting solvent are limited by those of the sample solvent because the two liquids must be immiscible. In the literature there are some applications of LLE for pretreating biological samples before CE analysis e.g. when chloroform was used in the analysis of quinolones in serum and urine samples [16] as an extracting agent, obtaining recoveries between 95-100 %.

SPE has now become prominent as an integral part of most antibiotic extraction procedures. Its advantages over LLE are that it is faster and more reproducible, obtains cleaner extracts, prevents the creation of emulsion and needs smaller sample sizes. It can use several kinds of sorbents e.g. sorbents of silica, alumina, various polymeric beds, ion-exchange media, etc.

C_{18} cartridges are the most common sorbents. They are surface-bonded porous silica particles characterised by their hydrophobic alkyl groups. There are several applications in the literature, e.g. in the CE analysis of penicillins [7] and quinolones [14] in pig plasma samples and in the analysis of tetracyclines in raw and cooked channel catfish [19]. Recoveries were around 90 % in all cases.

Therefore, tissue samples are usually edible tissues such as muscle, fat, liver, kidney or eggs. These samples are a complex mixture of water, collagen, muscle proteins, elastin, fat, other lipids and mineral constituents. Unlike in biological fluids, therefore, a large portion of tissue must be homogenised before analytes are extracted from the samples. Fat is generally cut into pieces and either melted or dissolved in a lipophilic solvent. Homogenisation is sometimes performed concurrently with extraction (maceration). The sample is cut and added to water or, preferably, to a buffer and the sample is homogenised using blade homogenisers (mixers, Utra-Turrax, etc.) [5]. Analytes must be soluble and stable in the buffer. For example, hydrochloric acid 0.1 N was used in the extraction of several quinolones from pig kidney samples [15], and McIlVaine-EDTA solution, which contains citric acid, phosphate buffer and EDTA at pH 4.0, was used in the extraction of oxytetracycline from kidney, liver and muscle sample [17]. Other paper used trichloroacetic acid (TCA), hydrochloric acid and sodium ethylenediaminetetraacetate (Na_2EDTA), for the extraction of oxytetracycline from catfish samples [19].

After the homogenization, the pretreatment procedure is similar to biological fluids pretreatment. However, the recoveries are usually lower because tissue samples are complex. For example, SPE using C_{18} cartriges was applied as sample pretreatment in the analysis of quinolones in pig kidney samples (recoveries over 80 %) [15] and in the analysis of tetracyclines in pig kidney and liver samples (recoveries between 69 and 80 %) [17].

For greasier tissues C_{18} sorbents tend to clog during SPE procedure and then repeatability and reproducibility may be poor. An example of this was the analysis of oxytetracycline in pig muscle samples, where the recoveries were around 20 % [17]. This was because muscle samples are fattier than other kinds of tissues such as liver or kidney samples. Polymeric sorbents are more recent and do not have these problems; they remain wet with water and retain a wide spectrum of both polar and nonpolar compounds. When these sorbents were

used to pretreat muscle samples in the analysis of oxytetracycline in muscle samples, recoveries were 76.4 % [17]. Other studies have used polymeric sorbents and also obtained good recoveries, e.g. the analysis of quinolones [12,13] and tetracyclines [18] in pig plasma samples by CE (recoveries over 90 %).

METHODS FOR ENHANCING SENSITIVITY

One of the main disadvantages of CE is that its concentration detection limit is poor. This is caused by the low sample introduction volume and the short optical pathlength for on-capillary detection. To avoid this problem often there are used highly sensitive detection systems such as laser-induced fluorescence (LIF), electrochemical and amperometric detectors. These systems can improve detection sensitivity by several orders. A CZE/LIF system was used to determine various quinolones in human plasma and microdialysates samples and obtained quantitation limits (LOQ) of 2.5 $mg \cdot kg^{-1}$ and 5 $mg \cdot kg^{-1}$, respectively [10]. Another example was the analysis of ciprofloxacin and its metabolite desethyleneciprofloxacin in human plasma samples, where the LOQ were 20 $\mu g \cdot kg^{-1}$ and 10 $\mu g \cdot kg^{-1}$, respectively [11].

Some of the metal complexes of tetracyclines (TCs) are very fluorescent and can be detected by LIF detection. Tetracycline, oxytetracycline and chlortetracycline were detected at 25 $\mu g \cdot kg^{-1}$ in milk and plasma samples by a CZE/LIF system [25]. The MRL for these compounds in milk for all producing species is 100 $\mu g \cdot kg^{-1}$, so this CZE/LIF method detected these compounds below the MRL levels.

CE with amperometric detection have also been used in the analysis of antibiotics in biological samples [43,44]. Ofloxacin and parimiazid were detected

in human urine at 8.5 $mg \cdot l^{-1}$ and 0.80 $mg \cdot l^{-1}$, respectively [44], and pipemidic acid was detected in human serum at 0.35 $mg \cdot l^{-1}$ by CE [43].
A simpler and more practical way to increase CE sensitivity is to use on-line (or on-capillary) sample stacking systems. Electrophoretic analyte-focusing systems are an elegant way to increase loadibility in CE [38]. Two stacking procedures have been recently used to analyse antibiotics in biological samples. These are field-amplified sample injection (FASI) and capillary isotachophoresis – zone electrophoresis (ITP – CZE). FASI/CZE system was used for analyzing amoxicillin in pig plasma samples, sensitivity increased 4-fold, and a detection limit (LOD) of 280 $\mu g \cdot l^{-1}$ was obtained [7]. This system was also used to analyse quinolones in pig plasma samples. In this case the sensitivity was 65 times higher than with conventional CZE and the detection limit was 25 $\mu g \cdot l^{-1}$ [12]. These studies show that the increment of the sensitivity is function of the sample and the compound.

Therefore, ITP-CZE was used for analyzing marbofloxacin in pig plasma samples. Fig. 6 [12] compares the analysis of a blank pig plasma sample with the analysis of a pig plasma sample spiked with 500 $\mu g \cdot l^{-1}$ of marbofloxacin by SPE/ITP/CZE system. The buffer system consisted of 10 mM sodium monohydrogen phosphate (pH 9.0) as the leading electrolyte and the BGE and 10 mM β-alanine (pH 9.0) as the terminating electrolyte. his system was 75 times more sensitive than conventional CE and the enhanced limit of detection (LOD) was 20 $\mu g \cdot Kg^{-1}$.

Other example is the use of an SPE/ITP/CZE system to analyse ciprofloxacin, enrofloxacin and flumequine in pig plasma samples. The buffer system consisted of 10 mM sodium monohydrogen phosphate and 5 mM NaCl (pH 9.0) as the leading and BGE and 10 mM β-alanine (pH 9.0) as the terminating electrolyte. In this way, the sensitivity was 40 times greater than with conventional CE. The LOD were therefore 70, 85 and 50 $\mu g \cdot l^{-1}$ for ciprofloxacin, enrofloxacin and flumequine, respectively [13].

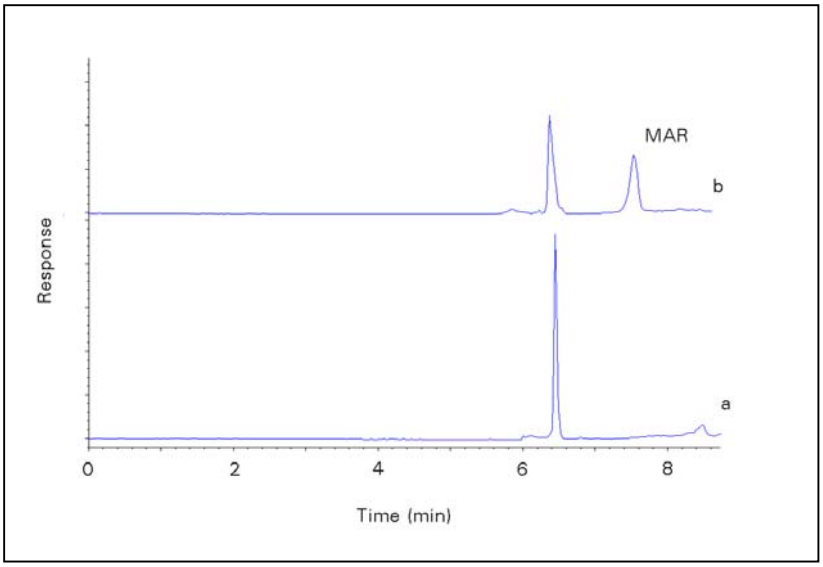

Fig. 6. Electropherogram of a blank pig plasma sample (a) and a spiked plasma sample (500 μg l^{-1} of marbofloxacin added to the original plasma sample) (b) after SPE pretreatment and analysed by ITP-CZE (from [12], reproduced with permission from Vieweg).

CONCLUSIONS

Several analytical methods for determining antibiotics by HPLC in biological samples have been published. However, CE is a good alternative because its advantages to over HPLC, such as the difficulties associated with peak tailing and low efficacy in HPLC. CE, both CZE and MEKC, is a suitable system to separate and determine several types of antibiotics in biological samples, and it is characterised for its simplicity and short analysis time. For this reason CE becomes a complementary tool to HPLC methods established until now to analyse antibiotics in biological samples.

Therefore, the main drawback of CE is that it is not enough sensitive in comparison with HPLC. To take full advantage of the separation power of CE for trace analysis of biological samples, there are considered several methods of improving determination limits. One of these is to increase detection sensitivity using highly sensitive detection systems (such as laser induced fluorescence detection and electrochemical detection). Another method is to increase the sample loadibility of CE system. Electrophorestic analyte-focusing techniques are an elegant way of increasing loadibility in CE (such as FASI and ITP). These systems could increase the sensitivity of CE system at least two orders of magnitude. By this way, we can consider CE as a real alternative to HPLC methods with a similar detection limits.

Because of the complexity of biological samples they usually need to be pretreated previous their injection onto CE instrument. In few cases the sample can be injected directly, only in the case of some biofluids such as urine or bile. Sample pretreatments used in CE are similar to sample pretreatment used in HPLC. However, in some cases, sample pretreatment in CE is simpler than in LC because its higher efficacy.

Some papers determined antibiotics by CE-MS [40,41]. However, as we know, there are no applications of their analysis in biological samples, possibly because this coupling provide unambiguous identification in complex samples.

We therefore conclude that CE can be used as an alternative to liquid chromatography and that it can be routinely used to analyse antibiotics in biomatrices.

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CAPÍTOL III. PART EXPERIMENTAL

III.1 Determinació d'amoxicil·lina en mostres de plasma per electroforesi capil·lar

La part experimental de la tesi doctoral es va iniciar amb el desenvolupament d'una metodologia analítica per a la determinació d'una penicil·lina, l'amoxicil·lina, per electroforesi capil·lar. Aquesta determinació es va realitzar en mostres de plasma de porc, essent el plasma una de les matrius biològiques més estudiades des del punt de vista analític. La importància de l'amoxicil·lina radica en què és un antibiòtic d'ampli ús com a bactericida tant en medicina humana com en veterinària.

Tal com s'ha descrit en el primer capítol, les penicil·lines contenen en la seva estructura un anell de tiazolidina unit a un anell β-lactama amb una cadena lateral (Figura 1). Aquesta família de compostos presenten tres grups funcionals en la seva estructura, un grup carboxílic, un grup hidroxil i un grup amino. En el cas de l'amoxicil·lina els valors de pKa per aquests grups són 2.4, 7.4 i 9.6, respectivament [1]. En aquest treball es va desenvolupar la determinació de l'amoxicil·lina per electroforesi capil·lar per zones (CZE), utilitzant tetraborat sòdic 20 mM a pH 9.0 com a tampó de separació. En aquestes condicions el compost està carregat negativament. Amb el mètode establert els límits de detecció (LOD) són de 200 $\mu g \cdot l^{-1}$.

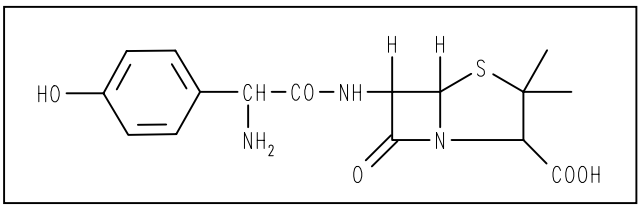

Fig. 1. Estructura de l'amoxicil·lina

En aquest treball, es va estudiar la possibilitat d'augmentar la sensibilitat del sistema. Per a tal objectiu s'han estudiat dos sistemes de preconcentració on-column, és a dir, sistemes que permeten preconcentrar la mostra dins el mateix

capil·lar. Aquests sistemes són: la injecció electrocinètica i la injecció de grans volums de mostra (FASI).

A la Figura 2 es mostra el procés de preconcentració de la mostra mitjançant l'ús de la injecció electrocinètica. En les condicions en què es troba la mostra l'amoxicil·lina està carregada negativament. Quan s'aplica un voltatge positiu el flux electroosmòtic es dirigeix cap al càtode mentre que el flux electroforètic es dirigeix cap a l'ànode. Com el flux electroosmòtic és més gran que l'electroforètic és d'esperar que la mostra s'introdueixi en el capil·lar per electroosmosi.

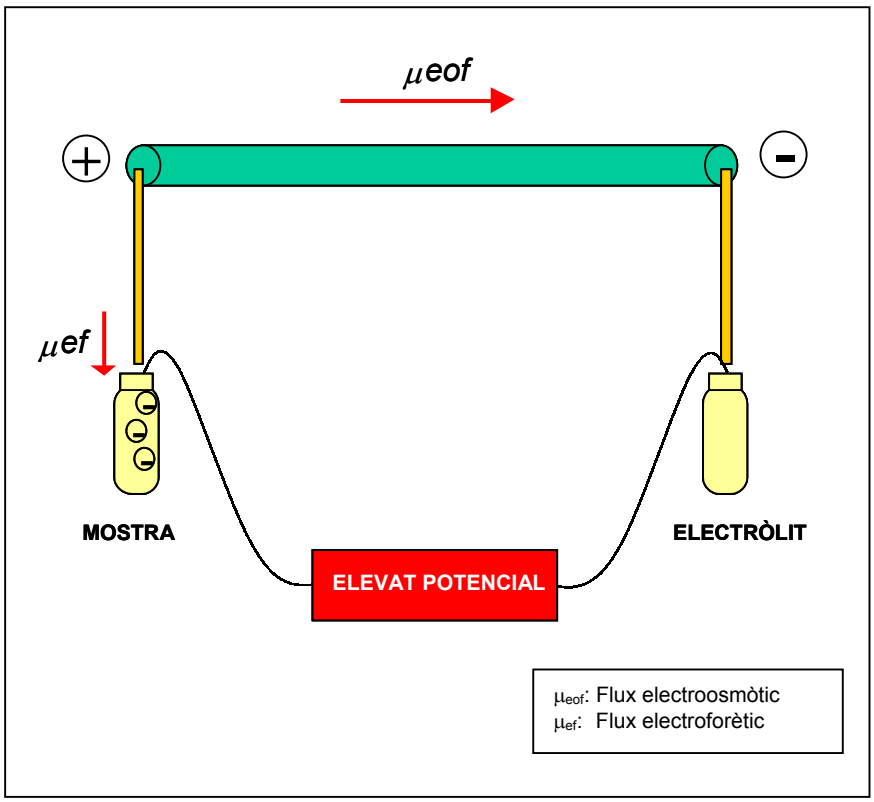

Fig. 2. Esquema de la injecció electrocinética de la mostra.

Malgrat això en aquestes condicions no es detecta. Això és degut a què la diferència entre els dos fluxos és petita. Per solucionar aquest problema addicional clorur sòdic 100 mm a la mostra. D'aquesta manera la conductància

equivalent de la mostra augmenta i l'amoxicil·lina és introduïda dins del capil·lar, ja que quan s'incrementa conductància de la mostra disminueix la mobilitat electroforètica de l'amoxicil·lina.

D´aquesta manera, el flux electroforètic es mou cap a l´ànode i no permetria la introducció de la mostra. Malgrat això, com el flux electroosmòtic del sistema es mou en sentit contrari i és superior al flux electroforètic, la mostra no és injectada. Per tal que la seva injecció sigui possible, s'afegeix una sal a la mostra (en aquest cas 100 mM de NaCl). D'aquesta manera augmenta la conductància de la mostra disminuint la seva mobilitat electroforètica. Mitjançant aquest sistema l'amoxicil·lina és introduïda en l'interior del capil·lar per electroosmosi. No obstant, el senyal que s'obté és comparable a la que s'obté quan la mostra s'injecta hidrodinàmicament en condicions normals, per tant no s'aconsegueix preconcentrar la mostra amb aquest sistema.

L'altre sistema de preconcentració utilitzat és FASI, sistema que s'ha esmentat en el capítol anterior. Aquest sistema permet injectar grans volums de mostra en el capil·lar hidrodinàmicament [2]. Un cop la mostra és introduïda dins el capil·lar mitjançant l'aplicació de voltatge s'aconsegueix preconcentrar la mostra dins el capil·lar a la vegada que s'elimina la matriu de la mostra, eliminant així possibles interferències. Es va estudiar aquest tipus d'*stacking* variant les dimensions del capil·lar, tant la longitud (entre 70 – 90 cm) com el diàmetre intern (entre 75 – 100 μm).

Utilitzant un capil·lar de 90 cm i 100 μm es va aconseguir augmentar el senyal fins a unes 35 vegades respecte la injecció hidrodinàmica convencional.

Un cop optimitzat el mètode mitjançant patrons estàndards, s'ha aplicat a l'anàlisi d'amoxicil·lina en mostres de plasma de porc. Quan s'injecta la mostra directament a l'instrument s'obté un electroferograma que presenta un gran nombre d'interferències i que fa impossible la determinació d'aquest compost.

Per tant, degut a la complexitat de la mostra ha estat necessari el seu pretractament.

S'han estudiat dos sistemes de pretractament: la desproteinització utilitzant àcid trifluoroacètic i l'extracció en fase sòlida (SPE) utilitzant cartutxos reblits de sorbent Sep Pak Plus $C_{!8}$.

En ambdós casos s'obté un electroferograma sense interferències si bé s'ha escollit treballar amb el sistema d'extracció en fase sòlida ja que s'obtenen majors recuperacions, de l'ordre del 90 %, mentre que mitjançant el sistema de desproteinització les recuperacions són del 75 %. La validació del sistema analític s'ha realitzat addicionant les mostres de plasma de porc amb diferents concentracions d'amoxicil·lina utilitzant la SPE com a sistema de pretractament i FASI com a sistema de preconcentració, obtenint un límit de detecció de 280 $\mu g \cdot l^{-1}$.

Els estudis que han estat realitzats en aquest treball han estat publicats a la revista Journal of Chromatography B, 731 (1999) 309-315, una còpia dels quals es mostra a continuació.

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DETERMINATION OF AMOXICILLIN IN PLASMA SAMPLES BY CAPILLARY ELECTROPHORESIS

ABSTRACT

We have developed a Capillary Zone Electrophoresis (CZE) method for determining amoxicillin in animal plasma samples. Sample clean-up involved solid-phase extraction onto Sep-Pak C_{18} cartridges followed by elution with water-methanol (85:15). This paper describes two different techniques to increase the sensitivity of the CZE method: field amplified sample injection (FASI) and electrokinetic injection. We have enhanced the detection limit (LOD) to 280 $\mu g \cdot l^{-1}$ by the FASI technique.

keywords: Solid-phase extraction; Injection methods; Amoxicillin; Antibiotics

INTRODUCTION

Many veterinary drugs are used to treat food-producing animals. Two commonly used types of antibiotics are sulphonamides and β-lactams. β-lactams antibiotics are two types of compounds: penicillins and cephalosporins. Amoxicillin is one penicillin widely used in clinical practice as a bactericidal against many gram-positive and gram-negative micro-organisms. One of the most difficult problems in pharmaceutical analysis is to separate and quantitatively determine penicillins, their degradation products and impurities. The iodometric method for determining amoxicillin in bulk drug substance is official in the Code of Federal Regulations [1]. Due to the lack of specificity of the official methods, others have been developed for determining amoxicillin in bulk drug substance and in biological fluids, as high-performance liquid chromatography (HPLC) [1-15] and capillary electrophoresis (CE) [16-29] techniques.

In the literature there have been several studies of amoxicillin by HPLC using ultraviolet detection. Some papers [1-3] have only been concerned with separating, identifying and quantifying its major impurities and decomposition products in these bulk drugs oral dosage forms. Others [4-10], however, was concerned with determining amoxicillin in biological fluids using different kinds of pre-treatment to obtain samples without interference and in a suitable medium.

Adamovics [11] suggested a method for injecting plasma samples directly. Unfortunately it was suitable only for plasma concentrations of between 20 and 100 $mg \cdot l^{-1}$, and this is too high for most applications. Tyczkoska et al. [12] injected plasma samples after filtration through a 30000 molecular mass cut-off filter to prevent the column from deteriorating. Since amoxicillin is insoluble in most organic solvents, currently available methods of measuring amoxicillin in biological fluids are usually based on protein precipitation by acid or organic solvents. These assays also tend to utilise UV detection [13], through post-column reactions and derivatization enhance assay sensitivity [9,10,14,15].

Solid-phase extraction (SPE) has gained popularity over the years in preparing samples for a wide range of analytes in complex matrices thanks to its better selectivity, simple operation, and lower consumption of solvents. However, there have been only a few reports of SPE methods of extracting amoxicillin by C_{18} cartridges from human plasma [4,6,7] and urine [5] or of systems which use solid-phase extraction for other β-lactams antibiotics [13,14]. Some authors have used tetrabutyl-ammonium bromide (TBA) to form an ion-pair with the acid group of amoxicillin. In these cases, the recovery of amoxicillin from plasma was greater than 80%, but other authors [6-8] have employed SPE without adding an ion-pair reagent. In these cases the recovery of amoxicillin changes from study to study, and values are between 60 and 90%.

When amoxicillin in plasma was analysed by HPLC and after on-line oxidation with an electrochemical detector [15], recoveries and sensitivity were higher than with the other methods. If we had an electrochemical detector we could use it to analyse the amoxicillin in our own body fluids using this type of detector.

This paper evaluates how effective capillary electrophoresis is when applied to biological fluids. Capillary electrophoresis, in the form of capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC), is a technique which has been used extensively to analyze pharmaceutical compounds [16-29], but has not been used to analyse amoxicillin in biological fluids.

Penicillin antibiotics, because of the pKa values of their different functional groups, were analysed by MECC [25-29]. Hows et al. [28] described the simultaneous separation of three types of compounds: sulphonamides, dihidrofolate reductase inhibitors and β-lactams antibiotics using MECC. This paper showed how factorial design is applied to optimise of these complex mixtures.

Although solid-phase extraction of amoxicillin has been used on biological samples [4-8], none of the authors reported the use of off-line solid-phase extraction followed by capillary electrophoresis analysis.

After evaluating two different types of sample pre-treatment, deproteinization with trifluoroacetic acid and solid-phase extraction using C_{18} cartridge, we developed a capillary zone electrophoresis method using a C_{18} cartridge extraction of amoxicillin and UV detection at 210 nm. We also studied different injection techniques (electrokinetic and field-amplified sample injection (FASI)) to enhance the sensitivity of the method [30-32]. This combination of extraction, injection and detection produces a method that is reliable and easy to perform.

EXPERIMENTAL

Instrumentation

Electrophoretic experiments were performed using a Prince CE System (Lauer Labs, Emmen, Holl,) with a UNICAM 4225 UV detector. Data were collected using the software provided with the HP3365 Series II Chemstation (Hewlett-Packard) which was operated under Windows 3.1 (Microsoft). The capillary was fused-silica (70 cm $\times$ 75 mm i.d.) supplied by Supelco (Bellefonte,USA). A detection window was prepared by burning off the polyimide coating 56cm from the capillary inlet.

Reagents and standards

Amoxicillin trihydrate was purchased from Sigma (Chemical Co). A standard stock solution of 1000 $mg \cdot l^{-1}$ was prepared in water which had been purified by

the use of a Milli-Q system (Millipore, Bedford, USA) and stored under refrigeration. Standard working solutions were prepared weekly or daily, depending on their concentration, by diluting the st,ard stock solution with Milli-Q quality water.

Trifluoroacetic acid (TFA) (Fluka Chemika, Switzerl,), acetonitrile (ACN) (Fisher Scientific UK, Leicestershire), methanol (Merck, Germany) and potassium dihydrogen phosphate (KH_2PO_4) (Fluka Chemika, Switzerl,) were used to pre-treat the sample.

Sodium tetraborate (Fluka, Buchs, Switzerl,), sodium dodecyl sulphate (SDS) (Sigma Chemical Co.) and HCl (Probus SA, Barcelona) were used to prepare the electrophoretic solution.

Electrophoretic conditions

The electrophoretic solution was prepared by adjusting the pH of 20 mM sodium tetraborate solution to 9 with 6 M HCl. Before use, the capillary was rinsed with 0.1M NaOH (Probus SA, Barcelona) (1000 mbar pressurised flow) for 15 min, then with H_2O MilliQ for 15 min and finally flushed with running buffer for 10 min. Before each analysis, the capillary was flushed with Milli-Q water for 2 min and the run buffer for 3 min successively. The detector was set at 210 nm. Injection was performed hydrodynamically at a pressure of 100 mbar for 1.8 seconds and the capillary temperature was 30ºC. The voltage was 15 kV.

Pre-treatment of the sample

Two types of pre-treatment were used:

Trifluoroacetic acid 3% in ACN

TFA 3% was prepared by dissolving 750 ml TFA 98 % in 25 ml of ACN. 400 ml of this solution and 100 ml of animal plasma were then mixed in a vortex and centrifuged at 7,000 rpm at 0ºC for 7 min. Finally, the clear supernatant was separated and filtered through a 0.45 mm nylon filter and stored at 0ºC until it was used.

SPE

Waters Sep-Pak cartridges plus (360 mg, C_{18}) were used to carry out SPE [7]. The cartridge was activated with 4 ml of methanol followed by 1 ml 0.02 M KH_2PO_4 at a flow rate of 1-2 ml/min using a water aspirator as a vacuum source connected to the cartridge pack. 1 ml of animal plasma sample was diluted with 1 ml of 0.02 M KH_2PO_4 and passed through the cartridge. The cartridge was washed with 1 ml of 0.02 M KH_2PO_4 and 0.5 ml of water. The amoxicillin was eluted from the cartridge with 2 ml aliquot of water-methanol (85:15).

Pig plasma was used in all experiments.

RESULTS AND DISCUSSION

To develop a method for determining amoxicillin, we first tried micellar electrokinetic capillary chromatography (MECC) because this technique is always used to analyse β-lactams [25-29], so we studied an electrolyte with a micellar agent as the SDS with a 20 mM borate buffer at pH 9 according to the literature [28]. Figure 1 shows electropherograms of a standard at 10 $mg \cdot l^{-1}$ with different concentrations of SDS: 50 mM SDS (Figure 1a), 75 mM SDS (Figure

1b) and 150 mM SDS (Figure 1c). The results show that analysis time increases and the peak shape was correct in all cases.

In amoxicillin the pKa values of the COOH, NH_2 and OH groups are 2.4, 7.4 and 9.6, respectively [4]. For this reason amoxicillin is negatively charged at the pH of the working electrolyte (pH 9), we could use capillary zone electrophoresis technique because of its simplicity. This mode of operation, however, has never been used to analyse a mixture of β-lactams antibiotics. We could use CZE because only amoxicillin was analysed. With the same borate buffer but without SDS, the migration time was still less and the peak shapes, sensitivity were similar. Figure 1d shows the electropherogram obtained in this case. Hence we have developed the study of amoxicillin using borate as running buffer without SDS.

After testing Ohm's law for this running buffer, the highest voltage we could apply was 15 KV since with higher voltages the analysis was not reproducible.

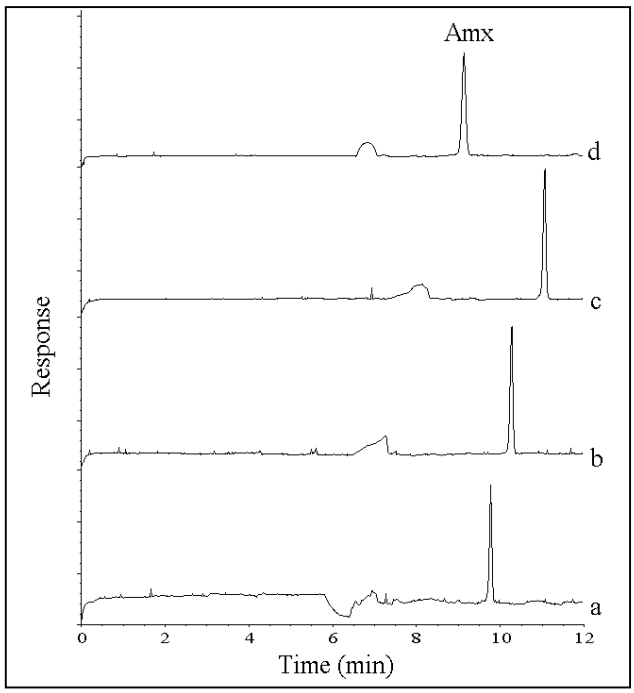

Fig. 1. Electropherograms of 10 $mg \cdot l^{-1}$ of amoxicillin from standard solution with 50 mM SDS (a), 75 mM SDS (b) and 150 mM SDS (c) in the running buffer, and without SDS (d). Running buffer: 20 mM sodium tetraborate, pH 9. Injection: 100 mbar for 1.8 min. Capillary temperature: 30ºC. Separation voltage: 15 kV.

To evaluate linearity we prepared and analysed different solutions of amoxicillin with concentrations ranging from 0.4 to 35 $mg \cdot l^{-1}$. These solutions were injected five times starting with the least concentrated and ending with the most. The area values obtained were successively analysed using ULC (Univariate Linear Calibration) software [33] to evaluate the correlation coefficient (r), relative standard deviation (R.S.D.) within solutions and limit of detection (LOD). Linearity was good, with a correlation coefficient of 0.994.

The detection limit (LOD) was calculated by the method of Widefordner and Long [34] using the ULC program with K equal to 6, the LOD was 0.2 mg $\cdot l^{-1}$. The R.S.D. of the areas was 1.3 % and the R.S.D. of the migration time was 0.9% for ten repeated injections of standard solutions of 10 $mg \cdot l^{-1}$. A fresh buffer was used after each sequence of three injections.

To improve detection limits, there are different injection modes for CE [30]. This paper studied electrokinetic injection [30], field-amplified sample injection [31,32]. In electrokinetic injection, the sample is introduced by applying a voltage across the capillary while one end is immersed in the sample solution, the other buffer.

The sample is drawn into the capillary by a combination of electrophoresis (EF) and electroosmotic (EOF) flows. When electrokinetic injection is performed by applying different voltages (5-15 kV) for different times (3-15 s), amoxicillin migrates to the cathode because of the EOF developed by the running buffer, but since the difference between EOF and electrophoretic mobility is little, no peak can be detected. On the other hand, the effect of adding salts to the

sample matrix was also examined. Addition of NaCl to the sample at a concentration of 100 mM increases the amount of amoxicillin injected. This improvement is due to the increase in the sample equivalent conductance. As a result, the electrophoretic mobility of the amoxicillin in the sample during the injection step decreases. In this case, amoxicillin will be introduced into the capillary not only depending on the individual equivalent conductance but also on the total conductance of the sample solution. In this case, the electrophoretic mobility of amoxicillin decreases when salt is added and consequently, a large quantity will be introduced by electroosmosis. Figure 2 shows the electropherograms when a standard solution of 10 $mg \cdot l^{-1}$ was injected electrokinetically at 15 kV for 0.25 min. without the addition of salt (Figure 2a) and with the addition of salt (Figure 2b). Results from this method are similar to those obtained from hydrodynamic injection. This shows that it was not possible to increase sensitivity in this way.

The other technique we studied to enhance detectability was Field-Amplified Sample Injection [31,32].

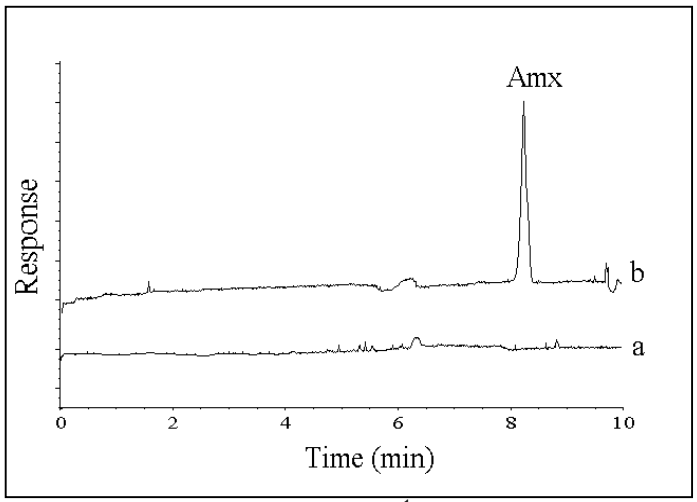

Fig. 2. Electropherograms of 10 $mg \cdot l^{-1}$ of amoxicillin from standard solution using electrokinetic injection: 15 KV for 0.25 min: (a) Without adding NaCl; (b) Adding 100 mM of NaCl to the sample. For other conditions see Fig. 1.

With this, sample stacking occurred when large volumes of sample were introduced into the capillary and sample matrix was removed by pumping it out of the column using the electroosmotic flow. The steps in this procedure are the following. First, a large sample plug is hydrodynamically injected into the column in a low-conductivity electrolyte. Second, the polarity is switched and the sample matrix is pushed out of the column. Third, when the sample matrix is almost completely out of the column (which can be determined by monitoring the electric current), the polarity is again reversed. Finally, the separation is performed. We have studied two parameters to increase the amount of amoxicillin injected and achieve our objective: length of capillary (70 and 90 cm) and its inner diameter (75 and 100 μm). When both parameters were increased there was an increase in the signal of amoxicillin. We used a standard solution of 10 $mg \cdot l^{-1}$. Results obtained could be observed in Table 1. Results were best for the 90 cm and 100 μm capillary (see Table 1). The sample was introduced hydrodynamically (200 mbar for 1 min), the sample vial was changed to buffer vial and a voltage with a reverse polarity (-10 kV outlet positive) was applied.

Table 1

The normalised results obtained using FASI method.

Hydrodynamic injection	Field-amplified sample injection (FASI)		
L_T: 70 cm I.D.: 75 μm	L_T: 70 cm I.D.: 75 μm	L_T: 90 cm I.D.: 75 μm	L_T: 90 cm I.D.: 100 μm
1	8.9	12.8	35.2

After 10 min the voltage was stopped, the polarity was switched back to its normal position for CZE (15 kV outlet negative) and CZE separation was began.

With this injection mode the LOD obtained was 15 $\mu g \cdot l^{-1}$ using a capillary of 90 cm of length and 100 μm of inner diameter.

When plasma was analysed by the HPLC method [8], the sample had to be pre-treated in order to decrease the high level of interference. To remove the protein content of plasma we therefore used TFA pre-treatment and it was analysed by the CZE method performed in this paper. Figure 3 shows electropherograms from the analysis of plasma with a standard addition of 50 $mg \cdot l^{-1}$, added to the original sample of plasma, without TFA sample pre-treatment (Figure 3a) and with TFA sample pre-treatment (Figure 3b). The sample therefore had to be pre-treated. TFA was used to obtain a selective CZE method. Recovery of amoxicillin from plasma was 75% using the trifluoroacetic treatment described in the experimental section and a standard addition of 50 $mg \cdot l^{-1}$ of amoxicillin in a plasma matrix.

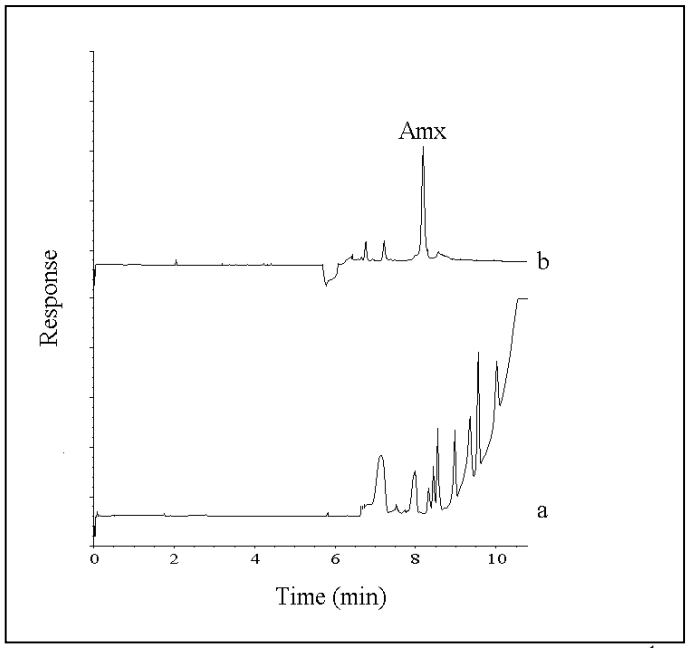

Fig. 3. Electropherogram of spiked plasma sample (50 $mg \cdot l^{-1}$ of amoxicillin added to the original sample of plasma) without (a) and with (b) sample TFA pre-treatment. For other conditions see Fig. 1.

After establishing pre-treatment, we studied the linear range and the LOD of amoxicillin in this kind of sample. To calculate them we have taken into account the dilution factor of samples, according to the type of pre-treatment used. Concentration ranged from 15 to 175 $mg \cdot l^{-1}$, and the LOD obtained was 7.5 $mg \cdot l^{-1}$ when the sample was injected hydrodynamically (100 mbar for 1.8 seconds). We therefore developed studies to enhance sensitivity in the CZE method.

To increase the sensitivity of the CZE method a FASI technique was applied but the sample was not preconcentrated and focused in the column. This studies were made with a capillary of 90 cm and 100 μm.

The problem may be due to an organic phase (ACN) in the injected sample following TFA pre-treatment. We removed this organic phase by heating the sample. As ACN is difficult to remove, we used cool acetone for pre-treatment. The organic phase was then removed, and FASI could be performed. The sample pre-treatment was not optimised by replacing acetonitrile with acetone, however, because this is out of the scope of this paper.

Another type of pre-treatment, solid phase extraction (SPE), was proposed for removing the matrix of the sample [7]. Applying solid-phase extraction to the analysis of β-lactam antibiotics in biological fluids is not very common. Many β-lactam antibiotics contain both amine and carboxylic acid groups, and are charged at any pH except at the isoelectric point. This would apparently make it difficult for them to be extracted efficiently by nonpolar C_{18} cartridges. The present application of a solid-phase extraction for amoxicillin demonstrates the potential usefulness of this approach in the extraction of other amphoteric β-lactam antibiotics from biological fluids by solid-phase cartridges. Figure 4a shows an electropherogram of a plasma sample after solid-phase extraction using a FASI technique to introduce the sample in the capillary. In this case the analysis time is longer because with the FASI optimised conditions the capillary

is 90 cm long and has an internal diameter of 100 μm. This is larger than when the analysis was performed without the FASI technique (70 cm x 75 μm). Electropherograms from ten different samples were similar and there was no evidence of interfering substances. Figure 4b shows an electropherogram of a spiked plasma sample after solid-phase extraction. The recovery of amoxicillin was determined by comparing the peak area of the extracted samples with the peak area of the same point on the calibration graph and was found to be 90 % ± 1.3 % (n=5).

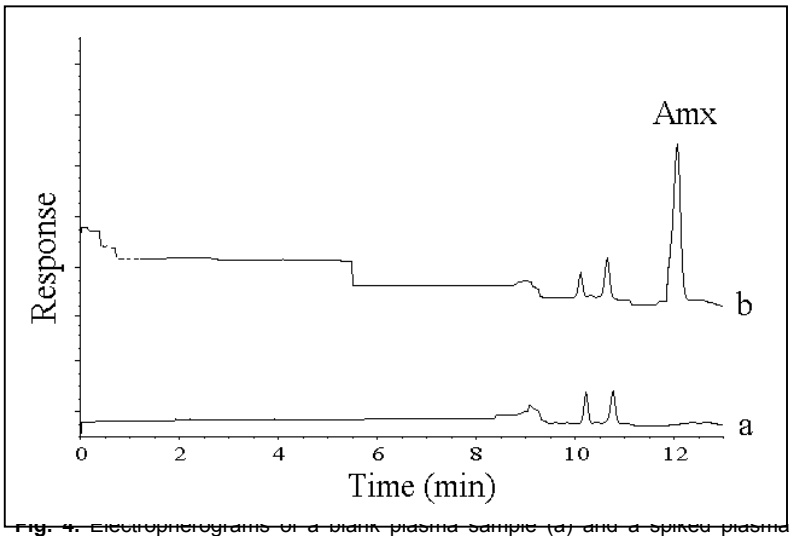

Fig. 4. Electropherograms of a blank plasma sample (*a*) and a spiked plasma sample (20 $mg \cdot l^{-1}$ of amoxicillin added to the original sample of plasma) (b) after solid-phase extraction. Capillary: 90 cm, 100 μm. Injection: FASI technique (conditions in the text). For other conditions see Fig. 1.

This recovery is higher than when TFA pre-treatment was used. The linear range of amoxicillin in plasma samples was from 10 to 70 $mg \cdot l^{-1}$. Pre-treating the sample in this way makes it possible to apply the FASI injection mode to enhance the sensitivity of the method, since no acetonitrile was used to elute the sample, instead of methanol was used. Using the same conditions for FASI injection previously optimised and with the same capillary dimensions, sensitivity increased 4-fold, which was significantly higher than with a TFA pre-treatment.

The LOD was 280 $\mu g \cdot l^{-1}$, higher than when FASI was applied to standard samples when the LOD was 15 $\mu g \cdot l^{-1}$. This difference probably arises because the relative standard deviation (RSD) values are higher than when the plasma samples were analysed. The RSD of the areas, calculated with a standard addition of 20 $mg \cdot l^{-1}$, was 14 % for ten repeated injections.

The detection limit was similar to that of a previous report [8] in which HPLC and SPE were used to monitor amoxicillin in plasma samples. This showed that CZE may be a valuable alternative technique to HPLC for analysing amoxicillin in plasma samples. The limit of detection was also similar to that obtained when different β-lactams were analysed in formulations by CZE [27] and MECC [26].

Other important reasons for the success of this assay are the clean background and high recovery with SPE and the fact that we can analyse the extract by CZE with FASI injection technique to enhance detectability. Another factor which contributes to the high degree of sensitivity when analysing plasma samples is the 210 nm UV light used to monitor amoxicillin.

CONCLUSIONS

This investigation has shown that analysing amoxicillin in animal plasma samples by capillary zone electrophoresis and introducing the sample into the capillary by FASI technique after solid-phase extraction with C_{18} cartridges, is an alternative to HPLC methods with a similar detection limit.

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III.2. Determinació d´oxitetraciclina en teixits de porc per electroforesi capil·lar

En aquest treball es mostra l´aplicació de l´electroforesi capil·lar en l´anàlisi de residus en teixits animals. El sistema que s´ha desenvolupat permet la determinació d´oxitetraciclina en ronyó, fetge i múscul de porc per electroforesi capil·lar.

Aquest compost pertany a la família de les tetraciclines, les quals es caracteritzen per la seva acció bacteriostàtica, inhibint la formació de proteïnes del bacteri. S´utilitzen tant en medicina humana com en veterinària. Tal com s´ha esmentat en el primer capítol de la tesi la seva estructura deriva d´un nucli d´hidronaftracè que conté 4 anells.

La concentració d´oxitetraciclina està regulada per la normativa europea en diferents teixits animals. Els valors de MRL fixats en els teixits de porc són de 600, 300 i 100 μg d´oxitetraciclina per kg de ronyó, fetge i múscul, respectivament.

En el sistema electroforètic s´ha utilitzat com a tampó carbonat sòdic 20 mM i etilendiaminotetracetat sòdic (Na_2EDTA) 1 mM a pH 11.2. En aquestes condicions l´oxitetraciclina està carregada negativament pel que es determina per electroforesi capil·lar per zones (CZE). El Na_2EDTA s´afegeix en el tampó de separació per tal d´evitar les interferències produïdes degut a la tendència de les tetraciclines de complexar-se donant lloc a complexos metàl·lics.

Degut a la complexitat de la mostra, per tal de determinar l´oxitetraciclina en els diferents teixits és necessari realitzar un tractament previ de la mostra, el qual consta de dues etapes, la primera consisteix en l´extracció de l´oxitetraciclina d´una mostra sòlida i la segona consisteix en l´eliminació d´interferències.
En la primera etapa s´homogeinitza la mostra mitjançant l'addició d'un tampó i triturant-la. El tampó que s'utilitza és el que es coneix com a tampó Na_2EDTA-McIlvaine, el qual conté àcid cítric, fosfat i Na_2EDTA. En aquest tampó l'oxitetraciclina és estable i soluble. Al llarg d'aquest procés s'elimina gran part

de les proteïnes presents a la mostra així com d'altres components. Aquest tampó ha estat prèviament usat per altres autors obtenint bones recuperacions [1-3]. La mostra s'homogeïnitza es centrifuga.

En la segona etapa, per tal d'eliminar les interferències presents en la matriu, es desenvolupa un sistema de SPE. Per du a terme aquest sistema s'utilitza el sorbent comercial Baker 10 C_{18}, ja que en altres estudis de tetraciclines s'havia utilitzat obtenint bones recuperacions [1,2,4]. En aquest estudi quan s'aplica aquesta metòdica en fetge i ronyó de porc s'obtenen recuperacions entre 56 – 79 %, en funció del tipus de teixit i la concentració, que són similars a les que havien obtingut altres autors.

Malgrat això, el mateix procés no s'ha pogut aplicar en múscul, degut a que aquest teixit és molt greixós i quan s'homogeinitza dóna lloc a una solució més complexa que en els altres dos teixits i el resultat és que en duu a terme la SPE el sorbent s'obtura i s'allarga molt el temps del pretractament, i per tant el temps total d'anàlisi. A més, les recuperacions que s'obtenen per aquest teixit són molt baixes, entre 15 – 20 %. Degut això es proposa l'ús d'un altre tipus de sorbent, l'Oasis HLB. Aquests sorbents es caracteritzen perque consisteixen en un copolímer, polidivinilbenzè-N-vinilpirrolidona. El monòmer divinilbenzè permet retenir els analits, mentre que la N-vinilpirrolidona li dóna al sorbent les suficients característiques hidrofíliques per prevenir els problemes que tenen els sorbents C_{18}, els quals no es poden assecar en cap moment ja que podria suposar que el procés no fos repetitiu i a més que el sorbent s'obturi. En aquestes condicions s'han obtingut recuperacions entre 59 – 76 %, en funció de la concentració d'oxitetraciclina en múscul.

Un altre problema que es planteja quan s'analitza múscul, és que el valor de MRL és més baix que pels altres teixits i mitjançant el sistema establert no es pot detectar aquest valor. Degut a això, s'han estudiat dos sistemes per augmentar la sensibilitat del mètode: FASI, sistema que ja s'ha aplicat en el

treball anterior i l'ús d'un capil·lar comercial de bombolla. Mitjançant el sistema FASI no s'obtenen bons resultats, possiblement degut a la complexitat de la matriu i s'opta per treballar amb el capil·lar de bombolla. Aquest tipus de capil·lar es caracteritza perque a la finestra de detecció el diàmetre intern del capil·lar és tres vegades més gran que en la resta del capil·lar i això permet augmentar el senyal uns tres cops en relació amb els capil·lars convencionals.

D'aquesta manera en mostres de ronyó i fetge mitjançant l'ús del sorbent Baker 10 C_{18} i usant capil·lars convencionals s'obtenen valors de LOD de 160 i 120 $\mu g \cdot Kg^{-1}$, respectivament. En el cas del múscul s'utilitza Oasis HLB com a sorbent i un capil·lar de bombolla, i s'obté un valor de LOD de 85 $\mu g \cdot Kg^{-1}$. Aquests valors són inferiors als valors límits (MRL) que estableix la normativa.

Per finalitzar l'estudi s'ha dut a terme un estudi farmacocinètic en ronyó de porc. Aquest estudi consisteix en administrar a un grup de porcs un medicament que contingui oxitetraciclina durant un cert període de temps. Després se sacrifica cada cert nombre de dies un número determinat de porcs. Finalment, s'analitza la concentració d'oxitetraciclina que resta en el ronyó dels animals als diferents dies de sacrifici. La normativa estableix que quan aquest valor està per sota dels MRL es considera que aquest teixit és apte pel consum. En aquest estudi es determina que el temps de sacrifici és de set dies.

Mitjançant aquest estudi s'arriba a la conclusió que la CE es pot utilitzar com a tècnica alternativa i complementària a la cromatografia de líquids per l'anàlisi d'oxitetraciclina en teixits de porc ja que permet obtenir uns valors de LOD comparables als d'aquesta tècnica, els quals són inferiors als MRL fixats per la normativa, i per tant el mètode desenvolupat és un mètode adequat per l'anàlisi de control de residus d'oxitetraciclina en teixits de porc.

Els estudis que han estat realitzats en aquest treball han estat publicats a la revista Chromatographia, 54 (2001) 355-359, una còpia dels quals es mostra a continuació.

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CAPILLARY ZONE ELECTROPHORESIS DETERMINATION OF OXYTETRACYCLINE IN PIG TISSUE SAMPLES AT MAXIMUM RESIDUE LIMITS

ABSTRACT

The determination of the antibiotic oxytetracycline (OTC), in pig tissues was investigated by capillary zone electrophoresis (CZE) with a prior solid-phase extraction (SPE) using alkyl-bonded silica and polymeric cartridges. The methodology developed allows determination of OTC in pig kidney, liver and muscle samples with detection limits below maximum residue limit (MRL) values, and the procedure to extract OTC and clean-up the matrix are simple and reliable. The limit of detection (LOD) for OTC was 160, 120 and 85 $\mu g\ Kg^{-1}$ for kidney, liver and muscle samples, respectively. The average recoveries from spiked samples (200 $\mu g\ Kg^{-1}$ and 1600 $\mu g\ Kg^{-1}$) were in excess of 63 % with coefficients of variation between 2.0 and 9.8 %. This method would be useful for routine monitoring of oxytetracycline residues in pig tissues.

keywords: Capillary zone electrophoresis; Solid-phase extraction; Oxytetracycline; Tissue samples

INTRODUCTION

Tetracyclines (TCs) are broad-spectrum antibacterial compounds commonly used in the prevention and treatment of diseases in livestock [1]. Therefore, monitoring of residual TCs is important from the viewpoint of veterinary food hygiene. The residues of the drugs may remain in tissues, milk, etc. intended for human consumption. To prevent health problems with consumers MRL of these compounds and other drugs in livestock products have been established. MRL are the maximum concentration of residue resulting from the use of a veterinary drug (expressed in mg Kg^{-1} or μg Kg^{-1} on a fresh weight basis) that the European Union (EU) permitted or recognized as acceptable in or on a food [2]. Oxytetracycline (OTC) is one tetracycline (TC) antibiotic most frequently used. MRL values are fixed for this compound in some tissues from pigs, poultries, sheep. MRL values for OTC in pig tissue samples are shown in Table 1. Consequently, one requirement of the analytical methods used to determine OTC residues in tissue samples is that they have detection limits below the MRL values.

A number of analytical methods for determining OTC by high-performance liquid chromatography technique (HPLC) in animal products have been published [3-16], e.g. the determination of OTC in milk samples [5,8,9], in plasma and / or urine samples [4] and in tissue samples [4,6,7,10-16]. There are few papers in which OTC is determined in biological samples by capillary electrophoresis (CE) [17-19]; e.g. the determination of OTC in catfish samples [17], and the analysis of different TCs in plasma [18,19] and milk samples [18].

CE has many advantages to analyze OTC over HPLC, such as the difficulties associated with peak tailing and low efficiency in HPLC due to interaction with the residual silanol groups on silica-base packing materials.

Table 1

MRL values of OTC fixed in tissue samples.

Pig samples	
Target tissue	**MRL values (μg OTC/kg tissue)**
Kidney	600
Liver	300
Muscle	100

CE has also the advantages of short analysis time and small consumption of sample and solvent volume in comparison with HPLC. OTC was efficiently separated by capillary zone electrophoresis (CZE) using different buffer systems, such as sodium phosphate – EDTA, sodium carbonate – EDTA and sodium borate – EDTA [20-23].

It is generally agreed that one of the first and most difficult steps required for TCs or any drug analysis is the extraction and clean-up of the drug from the biomatrix. The commonly techniques for the extraction and clean-up TCs from biomatrices involve some form of deproteinization agents to precipitate proteins presents in the sample matrix, liquid-liquid extraction (LLE) or solid-phase extraction (SPE). LLE was used to analyze TCs in plasma samples using an organic phase ethyl acetate [24] and ethyl acetate–2-propanol mixture [25]. Deproteinization agents used was chloride acid (HCl)-acetonitrile [4] or trichloroacetic acid (TCA) [5]. Recently, other possibility to pretreat this type of samples is the use of metal chelate affinity chromatography (MCAC) [3,6] which is based on the complex formation of cations with TCs. However, much of literature focuses on the use of solid-phase extraction (SPE) as a clean-up technique and in particular the use of silica-based cartridges to analyze TCs in tissue samples [12,26-28]. SPE is characterized because it is fast, reproducible, cleaner extracts are obtainable, emulsion creation is avoided and smaller samples are need. Moreover, it is cheap when labour costs of used solvents are

taken into consideration [29]. Extraction strategies needed for TCs depends upon the nature of the biomatrix.

Tissues matrices are by far the most complex matrices for drug extraction. The one common factor in all the methodologies to extract TCs from tissues samples is that the majority use aqueous solutions containing chelating agents to decrease the tendency of TCs to bind to cations in the matrix, for example EDTA, oxalic and citric acids which are the most commonly used chelating agents. Oka et al [12] investigated the suitability of various extractants for the analysis of TCs in pig liver (e.g. Na_2EDTA-McIlvaine´s buffer, phenol, metaphosphoric acid, trichloroacetic acid, hydrochloric acid and perchloric acid) and they concluded that the use of Na_2EDTA-McIlvaine´s buffer was the most suitable. Much of papers [12,27,30] developed the determination of TCs in biomatrices by HPLC using EDTA-McIlvaine's buffer combined with SPE using alkyl-bonded silica cartridges to clean-up the sample.

The objective of this paper is the determination of OTC in pig tissue samples by CZE method using SPE as a clean-up step, making standing out the importance of sample matrix.

To our knowledge, this is the first time CE has been applied to analyze oxytetracycline in tissue samples, and demonstrate that CE is a good alternative to HPLC because of its simplicity and because, in this paper, there is demonstrated the sensitivity of this technique is enough to determine OTC at MRL levels in pig tissues.

EXPERIMENTAL

Instrumentation

Electrophoretic experiments were performed using a HEWLETT PACKARD 3D CE (Waldbronn, Germany), with a DAD detector (Diode Array Detection). Data were collected with the software provided with the HP 3D Chemstation (Hewlett-Packard), which was operated under Windows NT Workstation (Microsoft). We used two different types of capillaries: a) fused-silica capillary (64.5 cm $\times$ 75 μm i.d.) and b) a commercial bubble-cell capillary fused-silica capillary (64.5 cm) with 50 μm i.d. and 150 μm diameter bubble area used for detection, supplied by Supelco (Bellefonte, USA). In the capillary (a) the detection window was prepared by burning off the polyimide coating 56 cm from the capillary inlet. In capillary (b) the detection window its ready-made.

Reagents and standards

OTC (oxytetracycline) was purchased from Across Organics (New Jersey, USA). Standard stock solution of 1000 mg L^{-1} of OTC which were prepared weekly, were dissolved in chloride acid (HCl) (Probus, Barcelona, Spain) 0.01 N and stored under refrigeration. Standard working solutions were prepared daily by diluting the standard stock solution with Milli-Q water.

Sodium carbonate (Prolabo, Bois, France) and sodium ethylenediaminetetraacetate (Na_2EDTA) (Probus, Barcelona, Spain) were used to prepare the electrophoretic solutions. Sodium hydroxide (Prolabo, Bois, France) was used to adjust the pH of the running buffer. Disodium hydrogen phosphate (Probus, Barcelona, Spain), citric acid monohydrate (J.T. Baker, Deventer, Holland) and Na_2EDTA were used to prepare Na_2EDTA-McIlvaine buffer [12,14]. Methanol (MeOH) (SDS, Peypin, France), ethyl acetate (Merck, Germany) and 2-propanol (Merck, Germany) were used during solid-phase extraction (SPE) method. Water was purified by a Milli-Q system, (Millipore, Bedford, USA). Chloride acid was used to dissolve standard solution.

Electrophoretic conditions

Running buffer was sodium carbonate 20 mM, EDTA 1 mM, adjusted to pH 11.2 (using NaOH 0.1 N). Before use, the capillary was rinsed with 1 M NaOH (Probus, Barcelona, Spain) (1000 mbar pressurized flow) for 15 min, then with H_2O Milli-Q for 15 min and with running buffer for 10 min. Before each analysis, the capillary was flushed with the running buffer for 3 min, and a fresh buffer was used after each sequence of three injections. The detector was set at 365 nm. Injection was performed hydrodynamically at a pressure of 50 mbar for 6 seconds of sample. Running buffer was then introduced to the capillary at 50 mbar for 6 seconds to decrease the dispersion of the sample during the analysis. The capillary temperature was 35 ºC and the separation voltage was 12 kV.

Extraction and clean-up procedure

A sample (5 g) fortified with oxytetracycline was placed in a centrifuge tube and 20 mL EDTA-McIlvaine´s buffer was added. The mixture was homogenized for 1 min using a Ultra-Turrax T-25 (Jankle & Kunkel, IKA-Labortechnik, Staufen, Germany) and then, the mixture was centrifuged at 15000 rpm during 5 min. After it, the pellet was blended and centrifuged two times more with 20 and 10 mL EDTA-McIlvaine´s buffer, respectively. Supernatants obtained were jointly centrifuged during 15 min, and, after filtration of the supernatant, the filtrate was applied on a solid-phase extraction cartridge.

Two different types of cartridges were used: 1) Baker 10 C_{18} cartridges (500 mg) and 2) Waters Oasis HLB cartridges (200 mg).

In the two cases SPE procedure was the same. The cartridge was activated with 5 mL of methanol followed by 5 mL Milli-Q water at a flow rate of 1-2 mL/min using a water aspirator as a vacuum source connected to the cartridge pack.

Supernatant obtained in the first step was passed through the cartridge. The cartridge was washed with 10 mL Milli-Q water. The oxytetracycline was eluted from the cartridge with 5 mL aliquot of ethyl acetate-methanol (50:50,v/v). The eluate was mixed with 5 mL of 2-propanol was added and then it was evaporated to dryness and the residue was redissolved in 1 mL of HCl 0.01 N.

Pig tissue samples were used in all experiments. Baker 10 C_{18} cartridges were used for kidney and liver samples and Waters Oasis HLB cartridges were used for muscle samples, which were filtered before passing through the cartridge.

RESULTS AND DISCUSSION

Capillary electrophoresis method

OTC is one TC antibiotic widely used to pretreat diseases in livestock. In this paper we have developed a CZE method to determine OTC in different pig tissue samples. Before analyzing OTC in these samples, it was necessary to establish a CZE method using standard solutions. In the bibliography, different buffer systems have been studied, like sodium phosphate – EDTA, sodium carbonate – EDTA and sodium borate – EDTA [20,21], and in this work we used sodium carbonate 20 mM at pH 11.2 adjusted with NaOH 0.1 N containing EDTA 1 mM to prevent well-known interference of tetracyclines with metal ions [20,21]. In these conditions, OTC is charged negatively because its pKa values [20]. In Figure 1, there is shown the electropherogram of 2000 μg Kg^{-1} OTC.

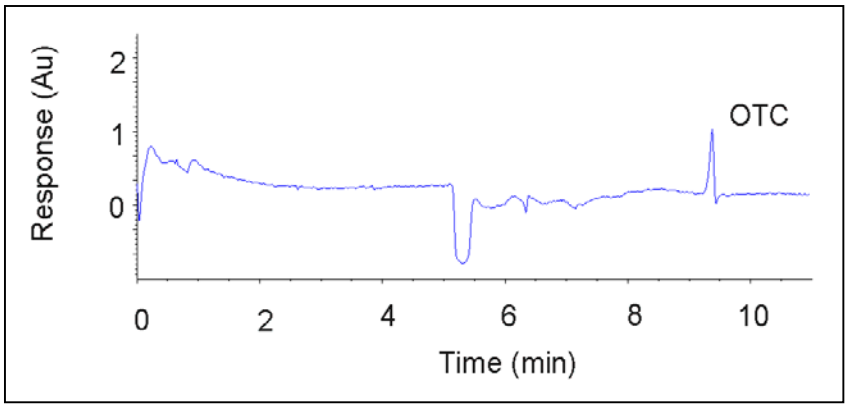

Fig. 1. Electropherogram of 2000 μg Kg^{-1} of OTC from standard solution. The electrolyte was sodium carbonate 20 mM at pH 11.2 adjusted with NaOH 0.1 N containing EDTA 1 mM. Injection: 50 mbar for 6 seconds. Capillary temperature: 35ºC. Separation voltage: 12 kV.

The linearity of OTC was made analyzing concentrations ranging from 200 to 2000 μg Kg^{-1}. These values are necessary to calculate later mean recoveries in each tissue. The area values obtained were successively analyzed using ULC (Univariate Linear Calibration) software [31] to evaluate the correlation

coefficient (r), relative standard deviation (R.S.D.) and limit of detection (LOD). Linearity was good, with a correlation coefficient of 0.9985. The detection limit (LOD) was calculated by the method of Widefordner and Long [32] using the ULC program with K equal to 3, and the LOD obtained was 100 μg Kg^{-1}. The % RSD of the areas was 6.8 % and the % RSD of the migration time was 2.3 % for ten repeated injections of standard solutions of 1600 μg Kg^{-1}. These values are shown in Table 2.

Table 2

Calibration data and precision studied with standard solutions.

Linearity (μg L^{-1})	Correlation coefficient (r^2)	LOD (μg Kg^{-1})	% RSD[c] (area)	% RSD[c] (time)
200-2000[a]	0.9985	100	6.8	2.3
200-2000 [b]	0.9978	60	8.0	0.5

[a]: Using a standard capillary

[b]: Using a bubble-cell capillary

[c]: Calculated for ten consecutive runs at 1600 μg L^{-1}

Kidney and liver samples

The objective of this paper is to determine OTC in pig kidney, liver and muscle samples at MRL levels through CZE method. The methodology to analyze this compound in tissue samples include and extraction of OTC from the matrix and a SPE as a clean-up step.

The extractant used was EDTA-McIlvaine´s buffer [12,27], and C_{18} cartridges were used to carry out the SPE procedure [12,27,28]. In this work we study different parameters of the SPE procedure to establish a method to be able to determine OTC by CZE in pig tissue samples without interferences and at MRL levels.

The parameters studied in the SPE procedure were the clean-up and the elution steps in order to minimize solvents used and also obtain a higher preconcentration factor. About the 50 mL obtained in the extraction of OTC from the kidney pig tissue with the EDTA-McIlvaine's buffer were introduced in the SPE cartridge after the cartridge was conditioned. The sample was passed through the cartridge, and different volumes of water were tested in order to obtain a clean sample with good recoveries of OTC, and the results showed that 10 mL of Milli-Q water was enough, while in other studies [12,27] were need higher volumes, which implied more time to develop clean-up. Then, we optimized the elution step. In order to find a suitable eluent and to simplify this step we studied different proportions of methanol – ethyl acetate because these two solvents are which gave higher recoveries in previous studies [27]. In this paper, we obtained the best conditions using methanol-ethyl acetate (50:50,v/v). Moreover, we investigated the use of different volumes of this mixture of solvents for elution of OTC from the C_{18} cartridge, and 5 mL of eluent were enough to carry out this step, obtaining good recoveries.

After SPE procedure the eluate was mixed with 5 mL of 2-propanol [27] and evaporated to dryness and the residue was dissolved in 1 mL of HCl 0.01 N, and analyzed by the CZE method.

In Figure 2, we compare the analysis of spiked kidney sample with 2000 μg Kg^{-1} (Figure 2b) with the analysis of a blank kidney sample (Figure 2a) in which there are shown that no interferences appeared at the migration time of OTC.

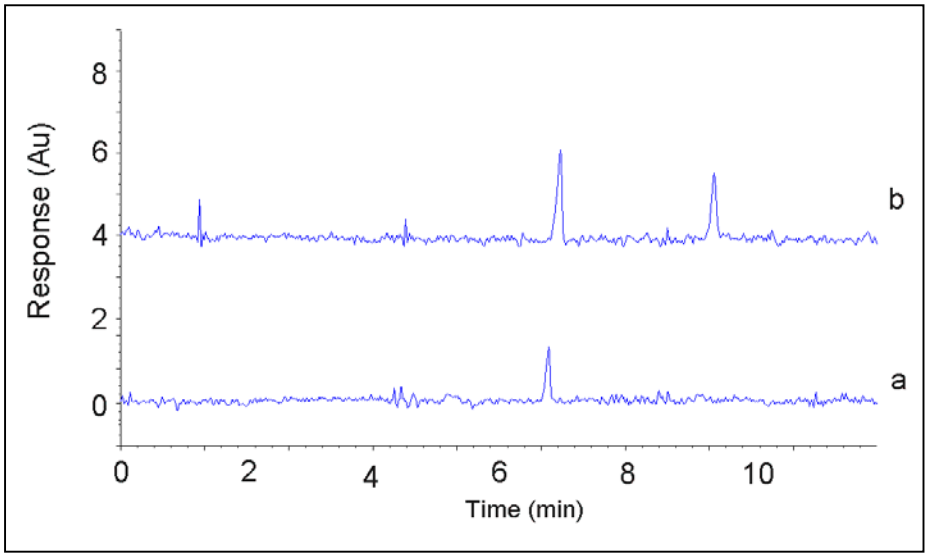

Fig. 2. Electropherogram of a blank kidney sample (a) and spiked kidney sample with 2000 μg Kg^{-1} of OTC (b). For other conditions see Figure 1.

The recovery values obtained were 70 %, for samples spiked with 200 μg Kg^{-1}, and 56 % for samples spiked with 1600 μg Kg^{-1} (see Table 3).

Calibration parameters were evaluated preparing spiked kidney samples with different amounts of OTC, between 200 to 2000 μg Kg^{-1}.

Data values as: linearity, the correlation coefficient, limit of detection (evaluated as before) and relative standard deviation are shown in Table 4. The detection limit obtained was 160 μg Kg^{-1}, and are below MRL level (600 μg Kg^{-1}).

Table 3

OTC recovery (% R) study and repeatability (% RSD) from pig tissue samples at different spiked level preconcentration.

	Spiked level preconcentration				
	200 μg Kg^{-1}		1600 μg Kg^{-1}		
Tissues	**% R**	**% RSD[a]**	**% R**	**% RSD[a]**	**Recovery (%)**
Kidney[b]	70	3.1	56	7.0	63

Liver[b]	79	3.9	77	9.8	78
Muscle[b]	20	4.2	16	5.7	18
Muscle[c]	76	2.0	59	3.3	68

[a] :Calculated for five consecutive runs at each concentration spiked with OTC standard.
[b]: Using Baker 10 C_{18} cartridges as SPE procedure
[c]: Using Waters Oasis HLB cartridges as SPE procedure

The same methodology could be applied to determine OTC in liver samples. In this case, the recovery obtained were higher: 79 %, for samples spiked with 200 μg Kg^{-1}, and 77 % for samples spiked with 1600 μg Kg^{-1} (see Table 3). Calibration parameters were obtained as before and are shown in Table 4. The detection limit was 120 μg Kg^{-1}. According to these results, by this method we could determine OTC in liver samples at MRL levels (300 μg Kg^{-1}).

Table 4

Calibration data and precision for oxytetracycline studied in different spiked tissue samples.

Samples	Linearity (μg Kg^{-1})	Correlation coefficient (r^2)	LOD (μg Kg^{-1})	% R.S.D.[a] (area)	% R.S.D.[a] (time)
Kidney[b]	200-2000	0.9941	160	7.0	0.7
Liver[b]	200-2000	0.9968	120	9.8	0.7
Muscle[c]	100-2000	0.9958	85	3.3	2.4

[a]: Calculated for five consecutive runs at 1600 μg L^{-1}
[b]: Using Baker 10 C_{18} cartridges as SPE procedure
[c]: Using Waters Oasis HLB cartridges as SPE procedure and a fused silica capillary with a bubble-cell

Muscle samples

In this case, we could not be applied the same SPE method as before because this type of tissue is fattier than the others and, in the extraction step we could not remove all pellet present in the sample and when we carried out SPE procedure all sample could not pass through Baker 10 C_{18} cartridges, which tended to clog, and the time needed to pass the sample through the cartridge increase dramatically, and the recovery obtained was very low (20 % for muscle samples spiked with 200 $\mu g\ Kg^{-1}$ and 16 % for muscle samples spiked with 1600 $\mu g\ Kg^{-1}$), and the % RSD was very high (about 13 %) compared to the other two tissues. See Table 3.

For this reason, we proposed the use of other type of cartridges to pretreat muscle samples: the use of Waters Oasis HLB cartridges, which contain a macroporous copolymer [poly(divinylbenzene-co-N-vinylpyrrolidone)] as sorbent that exhibits both hydrophilic and lipophilic retention characteristics. The lipophilic monomer is divinylbenzene, which provides the reversed-phase properties necessary for analyte retention. The hydrophilic monomer is N-vinyl pyrrolidone, which gives to the packing the necessary amount of hydrophilic property to prevent the wettability problems encountered with the C_{18} packings. These sorbents retain analytes even when the cartridges run dry. Moreover, while Baker 10 C_{18} cartridges used contain 500 mg of sorbent, Waters Oasis HLB cartridges only contain 200 mg of sorbent. However, Waters Oasis HLB cartridges not contain silanol group which simplifies the retention mechanism between sorbents and the analytes. This type of cartridges is the first time has been used to clean-up and preconcentrate OTC from animal tissues.

The optimum conditions obtained for the muscle tissue with the Oasis cartridges were the same as with C_{18} cartridges, and the recovery obtained with Oasis were higher compared to C_{18}, and the values were 76 % for muscle samples spiked with 200 $\mu g\ Kg^{-1}$, and 59 % for muscle samples spiked with 1600 $\mu g\ Kg^{-1}$, (see Table 3), and the % RSD values were significantly lower than the values obtained with this tissue treated with C_{18} cartridges.

In pig muscle samples, MRL values are lower than in the other tissues, for this reason we developed different types of stacking methods to increase the detection pathlength of CE method established. We studied two different sorts to increase the sensitivity of the method: 1) Field-Amplified Sample Injection (FASI) [33,34], and 2) using a commercial bubble-cell capillary. By FASI, we could usually increase the peak signal until 10-fold, depending on the compounds and the sample matrix. However, when we applied this procedure to tissue samples, we could not carry out FASI method. It could be due to the complex sample matrix. Other possibility was the use of a commercial bubble-cell capillary [34], which have a swelling in the detection area. This type of capillaries are characterized because of, in some cases, there could increase peak signal until nearly three-fold, depending on the type of compound to analyze and the sample matrix. In the capillary used the detection window has 150 μm bubble diameter, while the rest of capillary has 50 μm inner diameter. In this case, the peak signal was increased nearly three times compared with the standard capillary. The calibration parameters for standard solutions using bubble-cell capillary are shown in Table 2, jointly with data values obtained for standard solutions using standard fused-silica capillary.

Calibration parameters with pig muscle samples were obtained as before and are shown in Table 4. The detection limit was 85 μg Kg^{-1}. Thus, we could determine OTC in muscle samples, at the MRL levels, because its are fixed at 100 μg Kg^{-1} (see Table 1).

By this study we have developed a method which allows the determination of OTC in pig tissue samples by CZE method using SPE as clean-up step. In this paper we want to stand out the importance of sample matrix, because depending on it, SPE procedure carried out should be modified. We used two different types of sorbents: alkyl-bonded silica cartridges (Baker 10 C_{18} cartridges) to pretreat kidney and liver samples, and polymeric cartridges

(Waters Oasis HLB cartridges) to pretreat muscle samples. It is also possible to apply this method to the analysis of residual OTC in various animal tissues with similar recovery, accuracy and detection limits as in the case of pig tissues.

CE offers the advantage vs. liquid chromatography (LC) in terms of speed of the analysis and less interferences in the electropherogram. Figure 3 shows the chromatogram (a) and the electropherogram (b) of kidney tissue spiked at 200 μg Kg^{-1} of OTC.

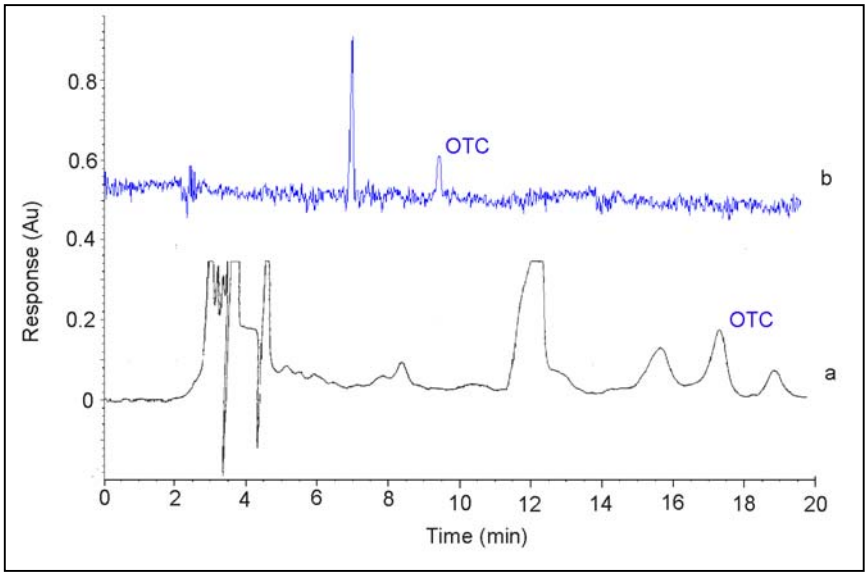

Fig. 3. Chromatogram (a) and electropherogram (b) of spiked kidney sample with 200 μg Kg^{-1} of OTC. Conditions in liquid chromatography were: instrument: HP 1050; column: Kromasil C_8, 5 μm 25 × 0.46 cm, Tecknokroma; mobile phase: oxalic acid / acetonitrile / methanol (85.5:12:2.5,v/v); 35 ºC; loop: 20 μl, λ: 350 nm. For conditions of capillary electrophoresis method see Figure 1.

The LC analysis takes 18 min compared to 10 min for CE, and in the electropherogram there are less interfering peaks, and also the peak shape are better. The CE method is less sensitive than existing liquid chromatographic methods but it may be used as a complementary tool in the control of residual OTC in pig tissues because the LOD is below the MRL values.

Pharmacokinetic study

After establishing CZE method we carried out a pharmacokinetic study in pig animals. It consisted to administrate during several days OTC together with the feed to various pigs. Then, we stopped to administrate it and start to slaughter some of them four, seven and nine days later. After it we carried out the analysis by CZE method determining OTC present in the different pig muscle samples.

In Table 5, there are shown the results obtained in these analysis. In this Table we can see that seven days later of the last dosage, OTC residues have been completely removed from muscle tissue, or unless they are in concentrations below LOD established by this method, and thus below MRL levels established by the legislation.

CONCLUSIONS

A CZE methodology was developed to analyze OTC in pig tissue samples below MRL values recommended by the European Union.

OTC and other drugs are often used in the prevention and treatment of diseases in livestock and the residues of these drugs may remain in biomatrices which are intended for human consumption. For this reason these are a legislation which regulate the presence of these residues. Thus, it is important to develop studies which allow to determine the presence of these drugs.

Table 5
OTC residues studied in a pharmacokinetic study from muscle tissue

Suppression Time	Animal	Mean Concentration
4 days	**M1**	< L.O.D.
	M2	112
	M3	120
7 days	**M1**	< L.O.D.
	M2	< L.O.D.
	M3	< L.O.D.
9 days	**M1**	< L.O.D.
	M2	< L.O.D.
	M3	< L.O.D.

CZE method established is characterized for its simplicity and sensitivity. For this reason, it becomes a complementary tool to HPLC methods established until now to analyze residual OTC in tissue samples.

ACKNOWLEDGEMENTS

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III.3. Determinació de kanamicina en serum per extracció en fase sòlida, derivatització *pre-column* i electroforesi capil·lar

En aquest treball s'ha desenvolupat la determinació de kanamicina per electroforesi capil·lar. Aquest compost pertany a la família dels aminoglicòsids. Aquests compostos actuen com a bactericides i s'utilitzen sobretot pel tractament d'infeccions urinàries i de l'abdomen. La kanamicina té un ampli ús terapèutic i s'ha utilitzat en el tractament de pacients amb tuberculosi.

En aquest treball s'ha desenvolupat un estudi per a determinar kanamicina per electroforesi capil·lar en mostres de sèrum humà. La presència de kanamicina o bé d'altres aminoglicòsids en l'home pot causar efectes secundaris com ototoxicitat i nefrotoxicitat. És per això que és important du a terme un control estricte quan s'utilitza aquest antibiòtic sobretot en teràpies de llarga duració. En la seva estructura contenen dos o més aminosucres units mitjançant un enllaç o-glicosídic a un nucli hexosa. L'objectiu d'aquest treball és determinar si les dosis administrades al pacient d'aquest antibiòtic estan en dosis terapèutiques o bé estan pròximes o per sobre del nivell tòxic.

Primer de tot s'ha desenvolupat un sistema per analitzar la kanamicina per electroforesi capil·lar. El tampó per a la separació consisteix en borat sòdic 30 mM (a pH 10.0) i 16 % metanol. La kanamicina és un compost que no absorbeix a la zona ultraviolada-visible. Per a la seva detecció hi ha varis sistemes proposats en la bibliografia, com la formació de complexos [1], mètodes indirectes [2] o bé la seva derivatització [3]. En aquest treball s'ha aplicat aquest últim sistema per a la determinació de kanamicina. Es tracta d'un sistema de derivatització *pre-column*, és a dir una derivatització que tingui lloc abans de la injecció en el capil·lar de separació. La derivatització s'ha dut a terme utilitzant o-ftadialdèhid (OPA) i àcid mercaptoacètic (MAA) en un medi de tampó borat amb un petit percentatge d´isopropanol. Perque es dugui a terme la reacció s'ajusta el pH a 11.5 i s'escalfa a 40 ºC durant 5 minuts. Ha estat necessari optimitzar les concentracions dels diferents reactius així com el volum de metanol per a que tingui lloc la derivatització.

A l'aplicar aquest mètode a l'anàlisi de kanamicina en sèrum humà ha estat necessari desenvolupar un sistema de *clean-up* previ a l'anàlisi degut a la complexitat de la mostra, per tal d'eliminar les interferències presents en aquesta. El sistema utilitzat ha estat un procés de SPE. En aquest cas, a diferència dels estudis anteriors, s'han utilitzat sorbents d'intercanvi catiònic feble (CBA). Això és degut a que els aminoglicòsids es caracteritzen per les seves propietats bàsiques i per tant és necessari l'ús d'aquests sorbents ja que en les condicions descrites aquests compostos estaran carregats negativament. En aquestes condicions les recuperacions han estat del 90 % aproximadament.

Mitjançant aquest procés de SPE-CZE descrit l'interval de linealitat és de 5 a 40 $mg \cdot l^{-1}$, obtenint-se un límit de detecció de 2 $mg \cdot l^{-1}$ i de quantificació de 5 $mg \cdot l^{-1}$. En dosis terapèutiques la concentració en què es pot trobar la kanamicina en sèrum humà és entre 5 i 35 $mg \cdot l^{-1}$. El sistema desenvolupat avarca tot aquest interval i per tant és adequat per aquest tipus d'estudi. Aquesta metodologia s'ha aplicat per a determinar la concentració màxima en sèrum de tres pacients. En cap dels tres casos es sobrepassen els 35 $mg \cdot l^{-1}$, per tant es troben dins dels nivells terapèutics.

Per altra banda la kanamicina s'elimina ràpidament del cos, de manera que al cap de poc més de 2 hores (entre 2.1 i 2.4 hores) la concentració ha de ser menor de 1 $mg \cdot l^{-1}$ [4]. Mitjançant aquest procés no es poden determinar aquestes concentracions, per tant és necessari augmentar la sensibilitat d'aquesta metodologia. Per aconseguir aquest objectiu s'utilitza el que es coneix com a injecció de grans volums de mostra (FASI). Mitjançant aquest sistema s'augmenta 20 cops la sensibilitat obtenint un límit de detecció de 100 $\mu g \cdot l^{-1}$ i un límit de quantificació de 200 $\mu g \cdot l^{-1}$. L'interval de linealitat en aquest cas va de 0.2 a 5 $mg \cdot l^{-1}$.

Aquest sistema ha estat aplicat a mostres de sèrum humà facilitades per un hospital de Bangladesh. Aquestes mostres corresponen a tres pacients que han

estat seleccionats per les diferents dosis rebudes, edat i estat físic. Aquestes mostres han estat recollides 2.5 hores després de l'última vegada que han estat medicats. Tal com s'ha descrit abans en aquestes condicions la concentració de kanamicina en el sèrum ha de ser mínima, menor a 1 $mg \cdot l^{-1}$. Quan s'analitza el sèrum mitjançant el sistema SPE/FASI/CE descrit prèviament es determina que en un dels tres pacients la concentració de kanamicina és superior a la que era d'esperar. La causa és que a diferència dels altres pacients aquest ja ha rebut 99 dosis de kanamicina mentre que els altres dos només n'han rebut 53 i 36, respectivament. Això indica que durant el tractament es produeix una certa acumulació de kanamicina. En els altres dos pacients s'obté una concentració mínima similar, menor de 0.05 $mg \cdot l^{-1}$. El pacient que ha rebut 53 dosis és més jove i més fort que el que n'ha rebut 35. Per tant, a partir d'aquests resultats és pot concloure que l'estat de forma i l'edat del pacient també influeix en l'eliminació d'aquest compost.

Per tant, l'electroforesi capil·lar és una tècnica adequada per anàlisis clíniques, ja que permet determinar d'una manera ràpida i senzilla compostos a nivells terapèutics.

Els diferents resultats d'aquest treball seran publicats properament a la revista Journal of Chromatography B (2002) i se n'adjunta la corresponent còpia a continuació.

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DETERMINATION OF KANAMYCIN IN SERUM BY SOLID-PHASE EXTRACTION, PRE-CAPILLARY DERIVATIZATION AND CAPILLARY ELECTROPHORESIS

ABSTRACT

Background: Kanamycin is an aminoglycoside antibiotic used as a second-line drug for tuberculosis (TB) patients. To ensure therapy efficacy and avoid potential ototoxicity and nephrotoxicity, careful monitoring of the drug level in the serum of patients receiving kanamycin is required, especially when therapy is of long duration.

Methods: Kanamycin in serum was pretreated by solid-phase extraction on a CBA weak cation-exchange cartridge. A mixture of 0.2 M borate (pH 10.5) – methanol (50:50, v/v) was used as eluting solvent. Capillary electrophoresis was employed for separation and quantitation after pre-capillary derivatization with o-phthalaldehyde (OPA)/ mercaptoacetic acid (MAA) reagent. For low level kanamycin, NH_4OH (25 %, m/v) -methanol (30:70, v/v) was used for analyte elution, after evaporation, residues re-dissolution, pre-capillary derivatization, the sample was analyzed by on-line field amplified sample injection (FASI) capillary electrophoresis

Results: A linear range of 4.0 μg/mL to 40 μg/mL was obtained with a LOD of 2.0 μg/mL and LOQ of 5.0 μg/mL by SPE/CE. Intra-day and inter-day RSD were 6.2% and 10.3%, respectively. Recoveries of around 90% were found. In SPE/FASI/CE, a linearity of 0.2 μg/mL to 5.0 μg/mL was obtained with a LOD of 0.1 μg/mL and LOQ of 0.4 μg/mL. Intra-day and inter-day RSD were 3.4% and 11.2%, respectively. Recoveries of around 60% were found.

Conclusions: Two methods were successfully applied to the analysis of kanamycin in sera of the TB patients in the concentrations covering peak level

and trough level. The results demonstrated the applicability for therapeutic drug monitoring and pharmacokinetic study.

Keywords: Capillary Zone Electrophoresis; Field-Amplified Sample Injection; Solid-Phase Extraction; Kanamycin

INTRODUCTION

Kanamycin is a broad spectrum aminoglycoside antibiotic produced by certain strains of Streptomyces kanamyceticus [1]. Due to its clinically useful activity against M. tuberculosis, kanamycin is widely used as a second-line anti-tuberculosis drug [2]. Like other aminoglycosides, the use of kanamycin can create potential side effects of ototoxicity and nephrotoxicity. Therefore, careful monitoring of the drug level in the serum of patients receiving kanamycin is required, especially when therapy is of long duration. The knowledge of the serum drug level is used to adjust the patient dose to ensure therapy efficacy and avoid possible risk of toxicity.

The existing methods for determination of kanamycin in blood sample include microbiological method [3], fluorescent immunoassay [4], fluorescent polarization immunoassay [5-6], fluorimetric determination [7] and chemiluminescence immunoassay [8]. However, these methods are no-specific and non-separative, the results are affected by the presence of other aminoglycoside antibiotics.

Methods for separation kanamycin from other aminoglycoside antibiotics have been described. TLC was first reported as a semiquantitative method for determination of kanamycin in serum and urine [9]. With the scanning denstitometry, TLC has been accepted as method for analysis of kanamycin by European Pharmacopoeias [10]. However, the sensitivity, resolution and accuracy of TLC are not high enough for the analysis of kanamycin in biological samples.

Combination of HPLC, post-column derivatization and fluorescence detection for the determination of kanamycin and other aminoglycosides in biomatrices have been reported [11-13]. Electrochemical detection was employed in kanamycin analysis by HPLC [14].

Due to its high resolution and low cost of operation, capillary electrophoresis has been increasingly viewed as a promising method for the analysis of drugs. Publications on the analysis of kanamycin by CE have been found [15-17]. Since kanamycin has no UV or fluorescence absorption, complexation formation [15], indirect method [16] and pre-capillary derivatization [17] were employed for detection. The sensitivity of the above CE methods is not a problem for the analysis of bulk pharmaceuticals and their formulations where the concentration of kanamycin in the range of 1-250 mg/mL. However, these methods can not be directly applied to clinical analysis, where kanamycin present in serum at a concentration range of 5 μg/mL to 35 μg/mL [18]. Because the complexity of sample matrix, the low injection volume and the very short optical path length for on-capillary UV detection will decrease the concentration sensitivity that can be achieved. Electrochemical detection methods have been reported in the determination of amimoglycoside antibiotics by CE [19-20] with the detection limits low enough to determine the amikacin and kanamycin in urine. However, electrochemical detection in CE requires special set-up and technique. It is not as convenient as UV detection, which is the most commonly used detection mode in capillary electrophoresis. No paper has been found in the literature on the analysis of kanamycin in serum by CE with UV detection and this initiates the present investigation.

In order to quantify low level of kanamycin in the presence of complex biological matrix, appropriate sample clean-up, matrix effects elimination and analytes enrichment procedures are required prior to CE separation and quantitation. Solid-phase extraction (SPE) can be used to simultaneously enrich the trace analytes and remove salts and protein. Due to its quick speed, higher reproducibility, cleaner extract and lower solvent consumption, it has gained prominence for biological sample handling in recent years [21]. Since aminoglycoside are polar and water soluble compounds, they are chemically ideal for aqueous extraction. Successful solid-phase extractions of

aminoglycoside antibiotics from biomatrices by using weak cation-exchanger [22-24] and high hydrophobic C-18 column [25] have been reported.

Field-amplified sample injection (FASI) is the simplest on-column concentration technique to increase CE sensitivity [26]. It is based on the principle that the velocity of an ion is dependent on its mobility and the electric field strength. If the conductivity of the sample zone is lower than that of the separation buffer, the ions in the sample region will experience a higher electric field and migrate rapidly, they will stacks up as a sharp band at the boundary between the sample plug and the separation buffer. With this technique, the concentration of analyte in the sample zone can increase from 10- to 1000-fold without any modification on the commercial instrument [26].

In the present study, a rapid and effective method based on solid-phase extraction on a carboxy propyl bonded phase (CBA) weak cation-exchange sorbent, pre-capillary derivatization with o-phthalaldehyde (OPA)/ mercaptoacetic acid (MAA) reagent and capillary electrophoresis for the determination of kanamycin in human serum was developed and validated. Optimization of the extraction parameters was studied. Field amplified sample injection was employed for the analysis of low level kanamycin. The applicability of the combination of SPE and CE was investigated. The advantages and limitation of the methods developed will be given and discussed.

EXPERIMENTAL

Instrumentation

An Agilent G1601 CE system equipped with a diode array detector was use for electrophoretic experiments. A HP 3D Chemstation (Rev. A. 08.03) was used

for data acquisition. An uncoated fused silica capillary of 50 μm i.d. × 42.5 cm (34 cm effective length) with an on-column detection window (Composite Metal Services, Hallow, UK) was used. An adjustable vacuum system was used for solid-phase extraction.

Reagents and standards

Sodium tetraborate decahydrate, 1,2-phthalic dicarboxaldehyde and mercaptoacetic acid were obtained from Acros Organic (Geel, Belgium), sodium dihydrogen phosphate from MERCK (Darmstadt, Germany), potassium hydroxide from Riedel de Haen (Seelze, Germany), sodium hydroxide and methanol (HPLC grade) from BDH (Poole, UK), boric acid from Merck Eurolab (Leuven, Belgium), picric acid from UCB (Brussels, Belgium). Commercial kanamycin sulfate was obtained from Fluka (Buchs, Switzerland). All solutions were prepared with ultrapure Milli-Q water (Millipore, Milford, MA, USA) and filtered with a 0.2-μm filter (Euroscientific Lint, Belgium). Solid-phase extraction cartridges (CBA, 100 mg/ 3 mL) were obtained from IST (Isolute, Sopachem, Brussels, Belgium).

Controlled human sera were obtained from Sigma (Poole, UK). Serum samples from tuberculosis patients receiving kanamycin were provided by the hospitals in Bangladesh. Peak sample was collected one hour after the injection, trough sample was collected just before the next injection. The samples were stored in plastic tubes and delivered to this laboratory by speed post. The serum samples were stored below 0 ^{o}C after arrival.

Derivatization reagent and separation buffer

The derivatization reagent consisted of 27 mg/mL OPA and 25 μL/mL MAA [17]. The reagent was freshly prepared by dissolving 540 mg of OPA in 2 mL of methanol before addition 15 mL of 30 mmol/L borate buffer (pH 11.5) and 500 μL of MAA. The resulting solution was adjusted to pH 11.5 with 8 M potassium hydroxide before making up to 20 mL with the 30 mmol/L borate buffer.

The separation buffer is composed of 30 mmol/L borax, pH 10.0, containing 16% (v/v) methanol [16]. The buffer was prepared by dissolving 1.144 g of sodium tetraborate decahydrate in 80 mL water, the pH was adjusted with 1 M sodium hydroxide. 16 mL methanol was added before making the solution volume up to 100 mL with water.

Capillary Zone Electrophoresis Conditions

CE separation conditions were adapted from the previously developed method in this laboratory [17]. The capillary was flushed very morning with 0.1 mol/L sodium hydroxide under the pressure of 100 kPa for 5 minutes, Milli Q water for 5 minute at 60 ^{o}C and running buffer for 5 minutes at 20 ^{o}C. When the capillary is new, 10 minutes flush of 1 mol/L sodium hydroxide was added to the beginning. The capillary temperature was set 20 ^{o}C during analysis with a separation voltage of 23.5 kV and UV detection of 335 nm. Derivatized sample was hydrodynamically injected at a pressure of 5 kPa for 8 seconds.

Solid-phase extraction procedure

Detailed procedures for the extraction kanamycin from serum with CBA-bonded phase sorbent were as follows: the SPE cartridge was conditioned with 1 mL methanol followed by 1 mL phosphate buffer (20 mmol/L, pH 7.4) at a flow rate

of 1-2 mL/min. The 4-time diluted serum sample was applied to the SPE cartridge at the flow rate of 1 mL/min. The cartridge was then washed with 2 mL phosphate buffer (20 mmol/L, pH 7.4) followed by 4 mL of borate buffer (20 mmol/L, pH 9.0). The cartridge was dried for 3 minutes before the trapped analytes were eluted into a 3-mL test tube with 1 mL of elution solvent (borate-methanol or NH_4OH-methanol) followed by a further volume of air.

Field-amplified sample injection

The procedure for on-line sample stacking in the present study involved following steps: (a) The capillary was filled with the sample by hydrodynamical injection at a pressure of 5 kPafor 300 seconds. (b) The capillary inlet was moved from sample vial to buffer vial, the polarity of the voltage was reversed and a voltage of 23.5 kV was applied. (c) The voltage was stopped when the observed current reached a value of approximately 95% of the actual current (current generated when the whole capillary filled with background electrolytes). (d) The polarity of the high voltage was switched back to normal configuration (positive at capillary inlet) and CE separation started.

RESULTS AND DISCUSSION

Optimatization and extraction conditions

Weak cation-exchanger containing carboxylpropyl bonded silica SPE cartridge was reported in successful extraction gentamicin from plasma and biological samples [22-23]. Due to the similar strongly basic nature of aminoglycosides, these existing extraction procedures, in principle, can be used for extraction kanamycin from serum. Necessary modifications were made in kanamycin

extraction in order to be compatible for subsequent derivatization and CE analysis

Since salts are present in serum at concentration around 0.15 mol/L, in order to facilitate maximum retention of the anlytes on CBA sorbent, serum sample was diluted 4 times with deionised water to reduce the viscosity and make ionic strength less than 0.05 mol/L.

SPE cartridge solvation, equilibration, sample loading and interference elution were performed as described in the existing work [22]. A solution containing 0.2 mol/L borate (pH 10.5)-methanol (50:50, v/v) was employed as elution solvent in the extraction of kanamycin from serum in this study. The use of an eluent containing 50% (v/v) methanol increased the efficiency of elution but also allowed the final extract compositions maintaining the optimum methanol concentration for the subsequent pre-capillary derivatization.

100 μL of OPA-MAA derivatization reagent and 10 μL picric acid (0.1 mg/mL) were added to the SPE extract (1 mL) and mixed well. After reaction at 40 ^{o}C for 5 minutes, the derivatized sample was injected directly into capillary for CE separation and quantitation. The addition of picric acid as internal standard can increase the precision of quantitation by accounting for variation on the final volume of the extract and for the variation in sample injection. A typical electropherogram of serum spiked with kanamycin was shown in Fig. 1. The purity of kanamycin peak was determined by comparison the UV spectra of different peak positions. The results showed the SPE clean-up procedure developed in this study yielded a very clean extract, no substance was found interfering with kanamycin in CE separation.

The recovery of kanamycin extracted from spiked human serum was calculated by corrected peak area comparison with the same concentration of kanamycin in elution solvent which was derivatized directly. Repeatability was based the three

analysis of the same concentration kanamycin spiked serum samples. With the procedure developed in this study, recoveries of kanamycin in the concentration range of 5 μg/mL to 40 μg/mL were found 90.8 % to 102 % with the relative standard deviations range from 8.6% to 14.7% (Table 1). Intra-day and inter-day precision were 6.2% and 10.3% RSD, respectively (30 μg/mL kanamycin-spiked serum, n=6).

Calibration and validation

A 5-point calibration was constructed for kanamycin in this study. Calibration standards spanned between 2.0 to 40 μg/L range were made up based on the known weight of kanamycin spiked into 1 mL serum. External standard calibration was used with analytical signal based on the corrected peak area obtained from the integration.

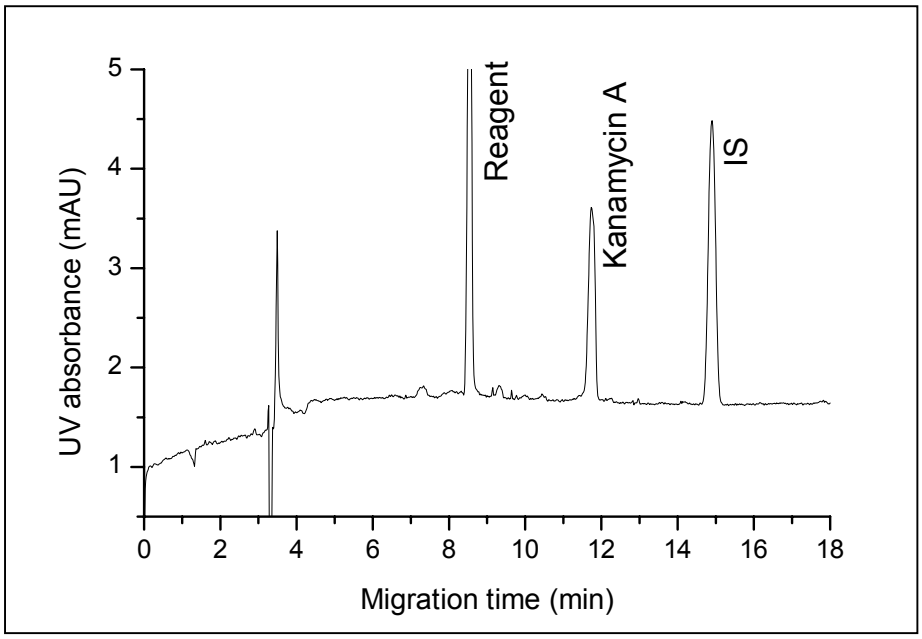


Fig. 1. Electropherogram of 30 mg/mL kanamycin-spiked serum by SPE/CE.

The linear range of the working curve was obtained by least square regression with correlation coefficient greater than 0.99. The lower end of the working range was taken as limit of quantitation (LOQ), which defined as ten times the background noise. The upper was taken 5 μg/mL greater than maximum peak kanamycin concentration in serum described in USDIP [18]. The limit of detection (LOD) was calculated based on S/N ratio = 3. A linear response over the concentration range of 5.0 μg/mL to 40 μg/mL was obtained with a LOD of 2.0 μg/mL and LOQ of 5.0 μg/mL ($y=0.222x+0.164$, $R^2=0.9991$, SD=0.0134) for the determination of kanamycin in serum using the SPE/CE method developed. The results showed the method cover the kanamycin blood concentration range described in USPDI [18]. Thus, this method can be applied directly to the analysis of kanamycin in real serum sample.

Table 1

Recovery and repeatability at different kanamycin level by SPE/CE

Concentration (μg/mL)	Recovery (%)	RSD (n=3)
5	92.9	9.5
20	95.8	10.3
40	94.4	14.7

Stability of kanamycin in serum

Because the serum samples had to be delivered from Bangladesh to Belgium, the stability of kanamycin in serum stored at room temperature was investigated. A 30 μg/mL kanamycin-spiked serum was divided into five batches. Serum aliquots were stored for 3, 4, 5 and 7 days before analysis. The stability was

tested by studying the recoveries of a kanamycin from the spiked serum over a given time interval. An average recovery of 91.67% was obtained with RSD of 10.29%. The results showed that the serum containing kanamycin could be stored at ambient temperature for one week without change in the concentration of kanamycin. Thus, the stability of kanamycin during delivery was generally well behaved.

Applicability of the SPE/CE method

To determine the kanamycin concentration in serum samples from TB patients. The serum was pretreated with the SPE procedure developed above, pre-capillary derivatization and CE separation and quantitation. A typical electropherogram of kanamycin in serum of a TB patient was shown in Fig. 2. The peak kanamycin serum levels of the three selected patients were found to be 32.97 μg/mL, 26.08 μg/mL and 33.59 μg/mL, respectively. The total analysis time for the whole procedure was less than 40 minutes.

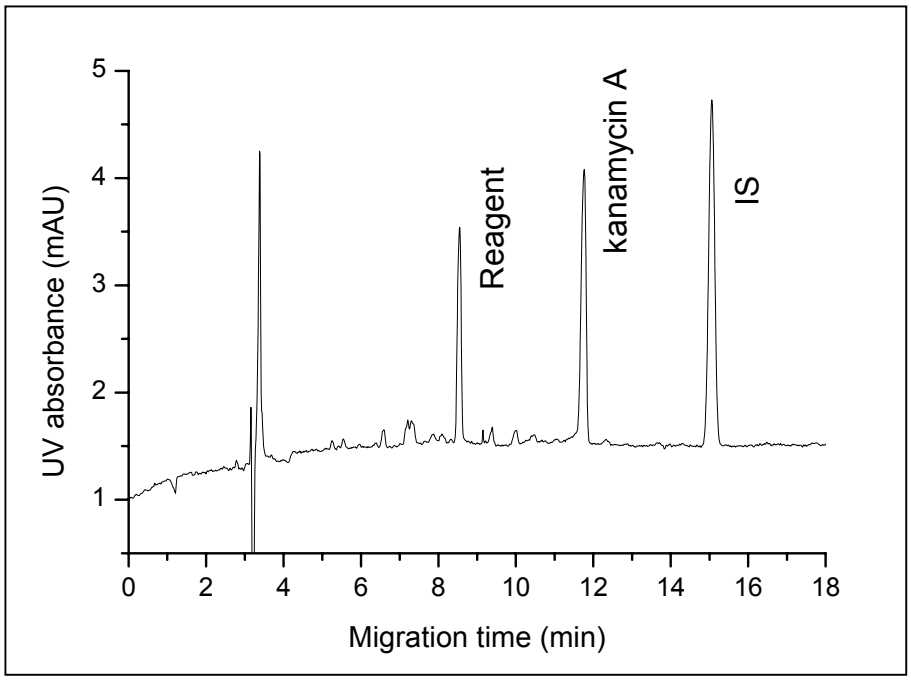


Fig. 2. A typical electropherogram of kanamycin in peak serum sample of a TB patient by SPE/CE

However, the results of all three trough samples were below the detection limit of the SPE/CE method described above. Safe trough concentrations in these patients could be reported according the USPDI guideline [18]. This is due to the fact that the patients were injected kanamycin at once-daily interval. The trough samples were collected just before the next injection. Since the half-live of the kanamycin is 2.1-2.4 hours [27, the trough concentration should be less than 1 μg/mL in an adult with normal renal function according to the pharmacokinetic study.

Optimization of field-amplified sample injection

In order to determine low level kanamycin in tough serum sample, an appropriate preconcentration method is needed to increase the sensitivity of the CE analysis. On-line field amplified sample injection is the simplest technique to solve this problem.

The volume of derivatization reagent

To meet the requirements of both derivatization and field amplified sample injection, kanamycin standard was prepared in Milli-Q water-methanol (50:50, v/v). However, salts were inevitably introduced into the sample solution by the addition of derivatization reagent and led to increase in conductivity of the sample buffer. Excess reagent not only introduced more salts to the sample buffer, but also caused the degradation of the kanamycin derivatives. Therefore, as less as possible reagent was added to the kanamycin sample while keeping

the necessary alkaline media for the derivatization. The minimum volume of derivatization reagent to keep a maximum reaction yield was found to be 15 μL.

pH of the derivatization reagent

The sensitivity of the CE method increased obviously after on-line sample stacking, but the peak of kanamycin A split into two peaks when using OPA-MAA reagent of pH 10.4 for derivatization. Fig. 3.

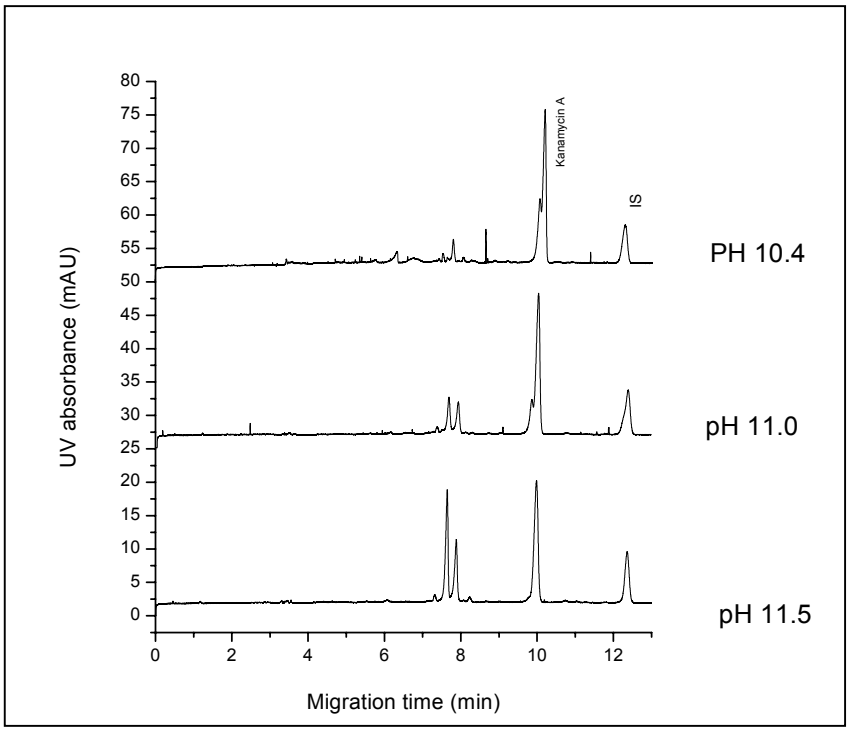


Fig. 3. The pH value of the derivatization reagent on the peak splitting of kanamycin by FASI/CE

However, the UV spectra of these two peaks were same, indicating these two compounds same or similar in structure. Increase the pH value of the derivatization reagent, the height of the smaller peak decreased. When the reagent pH was kept at 11.5, no peak splitting was observed. This maybe due to the fact that the carboxylic groups of kanamycin derivatives are fully ionized at higher pH, under the reversed electric field, they were stacked as a narrower band and resulted in sharper peak.

Buffer removal

After the polarity of high voltage was reversed, the positively charged ions were pumped out the capillary by the EOF, while the negative sample ions were stacked behind as a narrow zone. It was found that switching the polarity back when the observed current around the 95~ 98% of the actual current could result in better peak shape. The can be explained that more sample buffer removed, narrower sample band obtained.

Capillary loading length

The length of the capillary with sample load was measured as follow: hydrodynamically injecting the sample into the capillary under a pressure of 5 kPa mbar, the time needed to fill the effective length was obtained by monitoring the UV absorbance. By repeating the above experiment, the filling rate could be calculated. It took 3.25 minutes to fill the effective length in the present study. Although, increase the sample loading, the peak height increases non-linearly, in order to obtain as high as possible sensitivity, the whole capillary was loaded with sample at 5 kPa with an injection time of 300 seconds in this study.
With field amplified sample injection followed by CE separation, the concentration of kanamycin and picric acid were found to increase 28-fold and

36-fold, respectively. This is due to the fact that smaller picric acid ion moved faster than kanamycin derivatives in the sample zone and resulted in better stacking.

Optimization of SPE for FASI/CE

Sine field amplified sample injection required the sample prepared in low conductivity buffer (at least 10-time lower than the separation buffer), it cannot be used for the direct injection of biological samples because of the high concentration of salts and the presence of proteins. Thus, a sample pretreatment procedure for salt and proteins removal is necessary. Although, the SPE procedure described above could remove the proteins and the salts existing naturally in serum, it introduced a high concentration of borate at the analyte elution step. The borate salt in the final extract resulted in too high conductivity of the sample buffer to perform field amplified sample injection.

There are two methods to get a low conductivity sample buffer. One is using a low conductivity solution as analyte elution solvent, and another is desalting the SPE extract. Although the mechanisms indicates a buffer adjusted to two pH units above the pKa of the analyte or a buffer with pH < 2.8 can be used for elution the analytes from CBA sorbent. Only 16.4 % recovery was found when using 10 mmol/L sodium hydroxide – methanol (10:90, v/v) as elution solvent, no kanamycin was recovered with 30 mmol/L HCl –methanol (10:90, v/v) solution. Thus, desalting had become the only way to get a low conductivity sample buffer in this study. The Milipore drop dialysis method was used to desalt sample with high ionic strength of sodium chloride [28]. However, the large size of borate salt is not easily dialyzed as the small ions. Using a concentrated NH_4OH in methanol for elution gentamicin from CBA cartridge was reported by Cherlet et al [23]. The ammonium was later removed by evaporation due to its volatile

property. Thus, in principle, a solution of NH_4OH-methanol sounded to be ideal for kanamycin elution and subsequent desalting.

Since salts were retained on the CBA sorbent and the wall of the tubing in the SPE system after interference elution step. A diluted borate buffer (20 mmol/L, pH 9.0) was used following phosphate buffer as interference elution solvent. To void these non-volatile salts being eluted to the final extract, a small volume of water was applied to the cartridge before drying for analyte elution.

Optimization of NH_4OH content in elution solvent

Solutions containing different volume ratios of NH_4OH (25%, m/v) and methanol were used to elute kanamycin from CBA cartridge. The dependence of recovery on NH_4OH content in elution solvent was shown in Fig. 4. The results show kanamycin recovery increased significantly from 27% to 61% with increasing content of NH_4OH in methanol from 10% to 30%. Further increase in NH_4OH content was found to give a slightly higher recovery. Since the higher content of NH_4OH solution in the extract, the longer the evaporation time is needed. As a compromise of sample preparation time and kanamycin recovery, A mixture of NH_4OH (25%, m/v) – methanol (30:70, v/v) was chosen for analyte elution in the present study. The elution volume was optimized by comparison the recoveries from different volume of elution solvent. No significant difference was found between the recoveries from 1 mL and 2 mL elution solvent. To short the sample preparation time, 1 mL of the elution was used in this study.

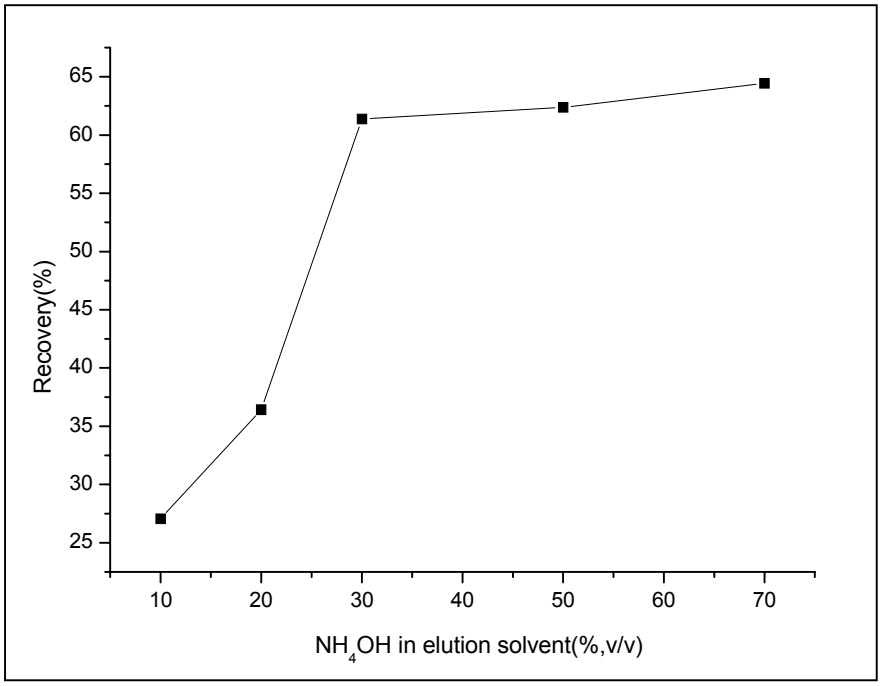


Fig. 4. Effect of NH_4OH on the recovery of kanamycin from serum

Extract

In order to eliminate the interfering effect of ammonium in the derivatization procedure and obtain a low-conductive sample buffer for sample stacking, ammonium removal is required. Thus, the SPE extract which containing NH4OH and methanol was evaporated to dryness at the temperature of 100 ^{o}C in a water bath. The dry residue was re-dissolved in 1 mL of methanol-water (50:50, v/v) before derivatization.

Analytical parameters of SPE/FASI/CE

With the SPE procedure developed, the extract of kanamycin free serum was very clear and showed no peaks that would interfere with the determination of

kanamycin by CE. The electrophoregrams of kanamycin-free serum and kanamycin-spiked serum were shown in Fig. 5 and Fig. 6.

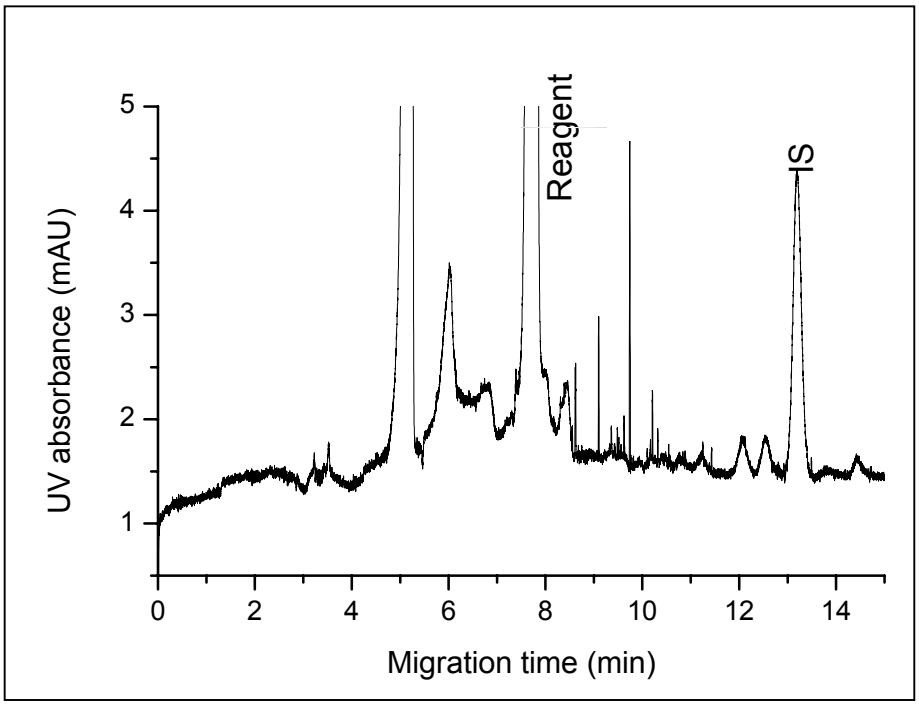
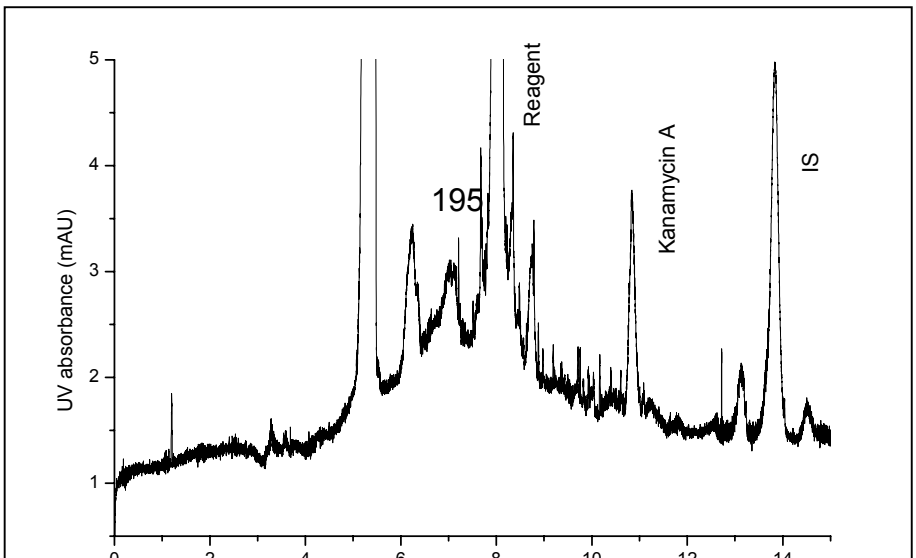


Fig. 5. Electropherogram of kanamycin-free serum by SPE/FASI/CE

was obtained (y=0.820x-0.035, R^2=0.9995, n=5, SD=0.0569), with a LOD of 0.1 μg/mL and LOQ of 0.4 μg/mL. Intra-day and inter-day precision for whole analysis procedure including solid-phase extraction, evaporation, pre-capillary derivatization and on-line sample stacking capillary electrophoresis were 3.4% and 11.2%, respectively (1 μg/mL kanamycin-spiked serum).



The recoveries of different concentration levels from solid-phase extraction and evaporation were ranged from 60.42% to 69.54% (Table 2). The total analysis time for the whole procedure was less than two hours.

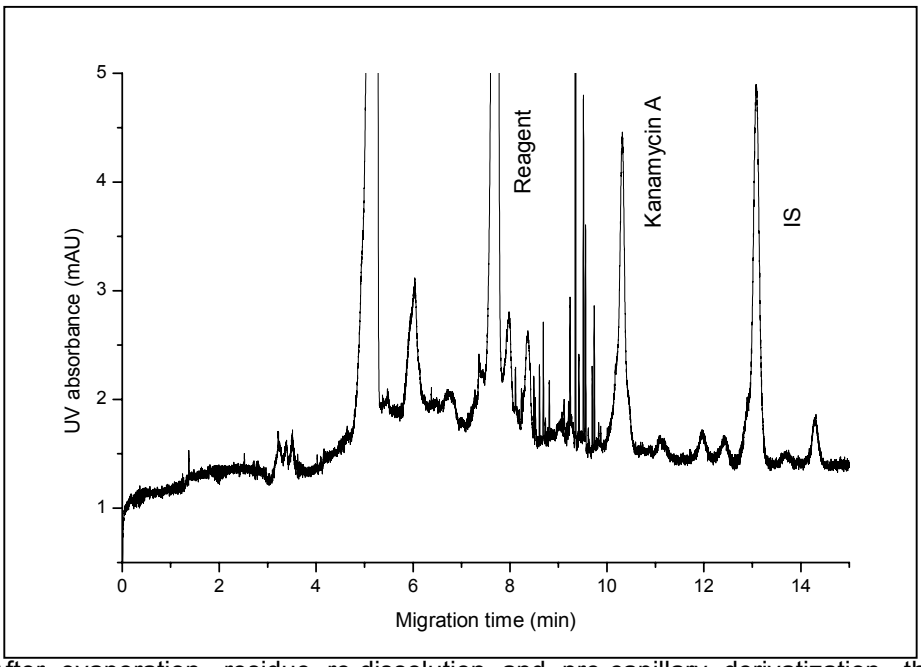
Table 2

Recovery and repeatability at different kanamycin level by SPE/FASI/CE

Concentration (μg/mL)	Recovery (%)	RSD (n=3)
0.5	68.7	8.2
1.0	62.4	3.8
5.0	61.9	11.2

APPLICATION OF SPE/FASS/CE

Tough serum samples were pretreated with the modified SPE procedure, a solution containing NH_4OH (25%, m/v) - methanol (30:70, v/v) was used as elution solvent.



After evaporation, residue re-dissolution and pre-capillary derivatization, the ka CE se

Electropherogram of kanamycin in trough serum sample of a TB patient by

A typical electropherogram of trough serum sample from a TB patient on kanamycin was shown in Fig. 7. The kanamycin concentrations in the tough serum samples from the three selected patients were found to be 0.87 μg/mL, 1.93 μg/mL and 0.80 μg/mL, respectively.

Interpretation of the kanamycin levels in serum of patients

According to the patient information provided by the hospital, serum samples from three patients were selected based on different doses, age and clinical

status for the analysis of kanamycin by the methods developed in the present study. The results are shown in Table 3.

Table 3

Kanamycin level in serum of three selected patients

Patient	Peak concentration* (μg/mL)	Trough concentration** (μg/mL)
T-66	32.97	0.47
SSK-1	26.08	1.28
MDR-4	33.59	0.48

* Analysis by SPE/CE
** Analysis by SPE/FASI/CE

It was found only one patient's peak serum kanamycin concentration in the recommended therapeutic range, the other two's were higher but not exceeded the toxic value. Results showed the trough serum kanamycin concentrations of all patients below than the recommended maximum value of 5 μg/mL. The reasons for the occurrence of low kanamycin level in trough serum sample are due to the short kanamycin half-life and once-daily drug injection remedy.

All trough concentrations in the selected patients in this study were higher than the theoretical value of 0.05 μg/mL (calculate from peak concentration, kanamycin half-life and sampling time), this indicated the accumulation of kanamycin in patient serum due to long-term of therapy. The higher trough concentration found in patient SSK-1 can be explained by the fact that this patient received 90 doses of kanamycin by the time of sampling, while patient T-66 and MDR-4 received 53 doses and 35 doses, respectively. The similar tough drug levels were found in patient T-66 and MDR-4, although patient T-66 received more doses and higher dose of kanamycin. This may due to the fact

that patient T-66 was younger and heavier than MDR-4, resulted in better kanamycin excretion.

CONCLUSIONS

Two hyphenate techniques based on solid-phase extraction and capillary electrophoresis were developed in this study. A SPE procedure with borate (0.2 mol/L, pH 10.4)-methanol (50:50,v/v) as elution solvent followed by pre-capillary derivatization and capillary electrophoresis was developed and has been successfully applied to analysis of kanamycin in peak serum sample from the selected patients. Results showed this SPE/CE method could be used directly in clinical analysis.

Table 4
Analytical parameters of the two methods developed in this study

	Linear range (μg/mL)	Linear equation	Correlation Coefficient	LOQ (μg/mL)	LOD (μg/mL)	Recovery (%)	Intra-day RSD (%) n= 5	Inter-day RSD (%) n= 5
PE/CE	5.0-40	y= 0.222X+0.164	0.9991	5.0	2.0	90	6.2	10.3
PE/FASI/CE	0.4-5.0	y= 0.820X-0.035	0.9995	0.4	0.1	60	3.4	11.2

Due to its simplicity, short analysis time and high recovery, this method is recommended for solving the suspect of treatment failure and toxicity.

In SPE/FASI/CE method, NH_4OH (25 %, m/v)-methanol (30:70, v/v) was used to elute the trapped kanamycin from the SPE cartridge. After evaporation, residues re-dissolution, pre-capillary derivatization, the kanamycin sample was analyzed by on-line field amplified sample injection capillary electrophoresis. This method had been successfully used for the analysis of kanamycin in trough serum sample. Due to its low LOD and LOQ, this method is recommended for pharmacokinetic study.

The analytical parameters of the two methods developed for determination of kanamycin in serum were shown in Table 4. The results indicate the two methods developed in the present study cover the concentration range of kanamycin present in serum at both peak level and trough level and demonstrate the applicability for therapeutic kanamycin monitoring and pharmacokinetic study.

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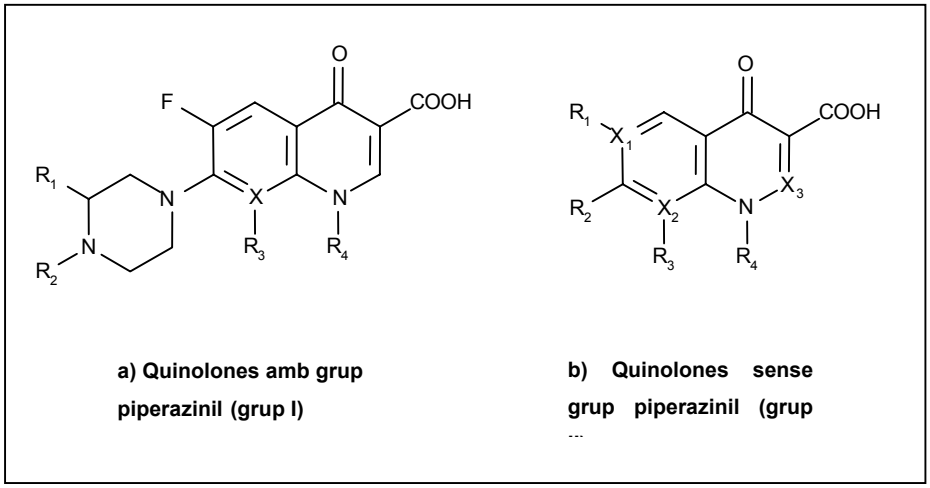
III.4. Determinació de quinolones en plasma per electroforesi capil·lar emprant l´extracció en fase sòlida

Una altra família d'antibiòtics estudiada són les quinolones. Formen part d'un important grup d'antibiòtics sintètics amb acció bactericida. Actuen de manera selectiva inhibint la síntesi de DNA dels bacteris. Totes elles tenen en la seva estructura un grup carboxílic en la posició 4 que fa que siguin actives contra varis microorganismes grampositius i gramnegatius. Algunes quinolones contenen un grup fluoro en la posició 6 i un grup piperazinil en la posició 7, i es coneixen com a fluoroquinolones. Es caracteritzen perque tenen major activitat antibacteriana i són actives contra gran varietat d'enfermetats infeccioses. A la Figura 1 es mostra l'estructura general d'aquests dos grups de quinolones.



a) Quinolones amb grup piperazinil (grup I)

b) Quinolones sense grup piperazinil (grup

Fig. 1. Estructura de les quinolones amb grup piperazinil, grup I (a) i les que no el contenen, grup II (b)

En aquest estudi s'ha desenvolupat un sistema d'electroforesi capil·lar per a separar deu quinolones, cinc de les quals (lomefloxacina, enoxacina, norfloxacina, àcid pipemídic i ofloxacin) contenen el grup piperazinil (i per tant pertanyen al grup de les fluoroquinolones), grup I, i les altres que no el contenen (cinoxacina, flumequina, àcid nalidíxic, àcid piromídic i àcid oxolínic), pertanyen al grup II. També s´ha realitzat l'aplicació del mètode establert a l'anàlisi de plasma de porc.

L'electròlit que s'utilitza per iniciar aquest estudi és dihidrogenfosfat sòdic 18 mM ajustat a pH 7.3 amb tetraborat sòdic 50 mM. En aquestes condicions les quinolones del grup I són neutres i les del grup II estan carregades negativament. Això és degut als valors de pKa dels seus grups funcionals. El valor de pKa pel grup carboxílic és entre 5.5 i 6.0, mentre que el valor de pKa del grup amino és entre 7.5 i 8.5 [1]. Degut a això s'obtè a l'electroferograma un grup de cinc compostos molt mal resolts que corresponen a les quinolones del grup I i un segon grup més ben resolt corresponent a la resta de quinolones.

Per tal d'aconseguir separar les quinolones del grup I es va du a terme un sistema MEKC afegint un surfactant a l'electròlit, el dodecil sulfat sòdic (SDS) [2]. En aquestes condicions es comença a millorar la separació, però malgrat això vàries quinolones estan solapades.

Per tal de millorar la separació s'afegeixen diferents modificadors a l'electròlit, tals com octanosulfonat sòdic i tetrabutilamoni (TBA), per formar parells iònics amb les quinolones carregades negativament, i variar així la selectivitat del sistema. Malgrat això tampoc s'aconsegueix millorar la separació de forma considerable.

Ja que la MEKC no dóna els resultats esperats, es va realitzar l'optimització de la separació per CZE en funció del pH. S'estudia un interval de pH entre 8.1 – 9.2. D'aquesta manera s'aconsegueix ionitzar tots els compostos, almenys en part. A pH 8.1 s'aconsegueixen separar els deu compostos i afegint un 10 % de metanol (MeOH) a l'electròlit s'aconsegueix una bona resolució.

Per tant, les condicions òptimes han estat l'ús de la CZE emprant com a electròlit tetraborat sòdic 40 mM a pH 8.1, contenint un 10 % (v/v) MeOH.

Aquest sistema ha estat aplicat a la determinació d'aquests compostos en plasma de porc. El tractament de mostra s'ha realitzat mitjançant és el

tractament per SPE utilitzant el sorbent comercial Sep Pak Plus C_{18}. Es va escollir aquest sorbent perque ja s'havia utilitzat en l'extracció d'altres quinolones en fluids biològics obtenint-se bones recuperacions [3]. Per dur a terme la SPE amb el sorbent esmentat anteriorment és necessari que totes les quinolones siguin neutres. Degut a l'estructura que presenten aquests compostos es va escollir treballar a un pH de la mostra entre 3 i 4 i afegir octanosulfonat sòdic, i així les quinolones del grup I tenen la seva càrrega positiva neutralitzada per l'octanosulfonat sòdic i les quinolones del grup II estan neutres. Amb aquest sistema s'obtenen recuperacions entre 90 – 129 % per tots els compostos, excepte per un d'ells, l'àcid piromídic.

Per tant, en aquest treball es mostra la capacitat de la CZE per a la separació d'un gran nombre de compostos i la seva aplicació a l'anàlisi de mostres reals. Malgrat això el mètode no és molt sensible ja que els valors dels LOD són entre 1.1 – 2.4 $mg \cdot l^{-1}$.

A continuació s'inclou una còpia de l'article publicat en la revista Journal of Chromatography B, 742 (2000) 255-265 sorgit arran dels estudis realitzats en aquest treball.

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DETERMINATION OF QUINOLONES IN PLASMA SAMPLES BY CAPILLARY ELECTROPHORESIS USING SOLID-PHASE EXTRACTION

ABSTRACT

The potential of capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) have been investigated for the separation and quantitative determination of ten quinolone antibiotics. The influence of different conditions, such as the buffer and pH of the electrolyte, the surfactant and the ion-pairing agents added to the electrolyte and the organic modifier were studied. A buffer consisting of 40 mM sodium tetraborate at pH 8.1 containing 10% (v/v) methanol was found to be a highly efficient electrophoretic system for separating lomefloxacin, enoxacin, norfloxacin, pipemidic acid, ofloxacin, piromidic acid, flumequine, oxolinic acid, cinoxacin and nalidixic acid. A solid-phase extraction method to remove the sample matrix (pig plasma samples) was developed on a C18 cartridge using a mixture of methanol-water (70:30, v/v). The method is specific and reproducible and mean recoveries were in the range 94.0 ± 4.2 % and 123.3 ± 4.1 % for pig plasma samples over the range used. A linear relationship between concentration and peak area for each compound in pig plasma samples was obtained in the concentration range 5 - 20 mg l^{-1} and detection limits were between 1.1 - 2.4 mg l^{-1}.

Keywords: Quinolones

INTRODUCTION

Quinolones are an important group of synthetic antibiotics with bactericidal action. The antibacterial activity of these compounds was shown to result from selective inhibition of bacterial DNA synthesis [1,2]. They all have a carboxylic acid group in position “4”, so are often referred to as 4-quinolones. These carboxylic acid groups are active against many gram-positive and gram-negative bacteria [3]. Numerous structurally related quinolones have been synthesised and several are in routine clinical use throughout the world. Their antibacterial activity is greatly increased by the addition of 6-fluoro and 7-piperazinyl groups to the molecule. The more recent introduction of fluorinated quinolones represents a particularly important therapeutic advance, since these agents have broad antibacterial activity and are an effective oral for the treatment for a wide variety of infectious diseases [4]. They are also widely used to treat and prevent of veterinary diseases in food-producing animals [3].

The need to identify quinolones in various biological tissues and fluids is obvious. Numerous techniques have been developed for their analysis in biological fluids and pharmaceutical preparations. Most of the analytical methods published are based on high-performance liquid chromatography (HPLC) [3,5-9], and some have been reported to determine them in various biological matrices [3,6-8]. Carlucci [8] wrote a review which included a different HPLC assay which was designed to determine different fluoroquinolones in biological fluids.

Other studies have concentrated on the structure and pKa values of quinolones. For instance, Barbosa et al. [9-12] studied the variation of their dissociation constants and those of other types of compounds like diuretics and peptides in several acetonitrile-water mixed solvents. Different gradient elution HPLC was used to determine impurities and degradation products of some quinolones because of their large differences in the chromatographic behaviour [5]. Reverse-phase liquid chromatography (RPLC) was used to quantify structure-

retention relationships of different quinolones [10]. All of these studies provide information about the behaviour of these compounds.

Capillary electrophoresis (CE) is a highly efficient method of separation which is generally used to determine charged components because it combines high resolution and easy automation with modest sample requirements and low solvent consumption [13-15]. Little attention has been paid, however, to separate quinolone antibiotics.

It should be possible to perform capillary electrophoresis (CE) analysis [16-20] on quinolones because they have carboxylic acid function in their structures (Fig.1), and because CE is an attractive resolution technique because of its high efficiency and short analysis times and because it requires smaller volumes of analytes and running solution additives than HPLC. Both capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) were used.

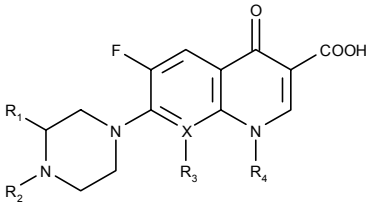
CE has been used to determine ciprofloxacin in pharmaceutical formulations [16] and for the enantioselective separation of ofloxacin and DU-6859 using either vancomycin [21] or the γ-cyclodextrin-zinc(II)-o-phenylalanine mixture [18] as a chiral selector. Sun and Chen [17] performed a capillary electrophoretic separation of fourteen antibacterial quinolones using standard solutions. To optimize the separation they employed a triangular overlapping mapping scheme in which three factors relevant to the running buffer composition were chosen: the concentrations of sodium cholate (the micelle-forming surfactant), sodium heptanesulfonate (ion-pairing agent) and the percentage volume of acetonitrile.

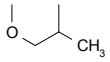
Although several reports have shown that CE is suitable for pharmaceutical analysis, only a few have been applied to biological samples [19,20]. They were applied predominantly to human body fluids; the sample was pretreated by

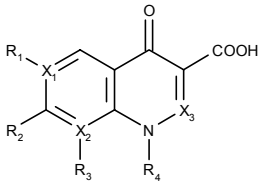
deproteinization adding different reagents to the sample. Möller et al. [19] developed a CE method with laser-induced fluorescence to determine moxifloxacin in plasma and microdialysate and the results obtained were cross-validated with an established HPLC-method. Pérez-Ruiz et al. [20] determined six quinolone antibiotics simultaneously by CZE in serum and urine samples.

Solid-phase extraction (SPE) has gained popularity over the years in the preparation of samples for a wide range of analytes in complex matrices because of its better selectivity, its simpler operation, and the lower consumption of solvents. However, few reports have employed SPE for extracting antibiotics from plasma samples [22-25]. Manceau et al. [26] used SPE as a pretreatment in the analysis of enrofloxacin and ciprofloxacin in biological fluids by HPLC.

This paper studied the optimization of the separation of ten antibacterial quinolones (Fig.1) using CZE and MEKC modes. For this purpose the pH and composition of the electrophoretic buffer and type and proportion of the organic modifier were optimized. The animal plasma was pretreated using a C_{18} solid-phase extraction and different parameters were optimized. The optimum CZE method is rapid and is useful for determining of these 10 antibiotics in animal plasma samples.



	R1	R2	R3	R4	X
Enoxacin (1)	H	H	---	Et	N
Lomefloxacin (2)	CH_3	H	F	Et	C
Norfloxacin (3)	H	H	H	Et	CH
Ofloxacin (4)	H	CH_3			C
Ciprofloxacin (5)	H	H	H		CH



	R1	R2	R3	R4	X1	X2	X3
Cinoxacin (6)			H	Et	C	C	N
Flumequine (7)	F	H			C	C	CH
Nalidixic acid (8)	H	CH_3	---	Et	C	N	CH
Piromidic acid (9)	---		---	Et	N	N	CH
Oxolinic acid (10)			---	Et	C	N	CH

Fig.1. Structures of the quinolone antibiotics studied

EXPERIMENTAL

Instrumentation

Electrophoretic experiments were performed using a Hewlett Packard 3D CE (Waldbronn, Germany) equipped with a diode array detector. Data were collected using the software provided with the HP Chemstation version A.03.01 chromatographic data system. The capillary was uncoated fused-silica (64.5 cm $\times$ 75 μm i.d.) supplied by Supelco (Bellefonte,USA). A detection window was prepared by burning off the polyimide coating 56 cm from the capillary inlet.

Reagents and standards

Enoxacin, lomefloxacin, norfloxacin, ofloxacin, pipemidic acid, cinoxacin, flumequine, nalidixic acid, piromidic acid and oxolinic acid were purchased from Sigma (Sant Louis, USA). Standard stock solutions of 1000 mg l-1 were prepared in NaOH 0.1 N and stored under refrigeration. Standard working solutions were prepared weekly or daily, depending on their concentration, by diluting the standard stock solution with water which had been purified by a Milli-Q system (Millipore, Bedford, USA).

Methanol (MeOH) (Merck, Germany), acetic acid (Probus SA, Barcelona), sodium acetate (Prolabo, Bois) and sodium octanesulfonate (OCN) (Sigma, Sant Louis, USA) were used to pretreat the sample.

Sodium tetraborate (Fluka, Buchs, Switzerland), potassium dihydrogen phosphate (KH_2PO_4) (Fluka Chemika, Switzerland), sodium dodecyl sulphate (SDS) (Sigma, Sant Louis, USA), tetrabutyl-ammonium bromide (TBA) (Sigma, Sant Louis, USA), OCN, acetonitrile (ACN) (Merck, Germany), HCl (Probus SA, Barcelona) and MeOH were used to prepare the run buffer solution.

Electrophoretic conditions

The electrophoretic solution was a stock solution of 40 mM sodium tetraborate containing 10% of MeOH. This running buffer was adjusted to pH 8.1 with HCl 6 N. Before use, the capillary was rinsed with 1 M NaOH (1000 mbar pressurised flow) for 5 min, then with MilliQ water for 10 min and finally flushed with running buffer for 3 min successively. The detector was set at 260 nm. The injection was hydrodynamic at a pressure of 50 mbar for 4.8 seconds. Capillary temperature and separation voltage were 30 ºC and 30 kV, respectively.

Solid-phase extraction as pretreatment of the sample

Waters Sep-Pak cartridges plus (360 mg, C18) were used to pretreat the sample. The cartridge was activated with 6 ml of MeOH followed by 1 ml of acetic acid 0.7 M/sodium acetate 0.04 M buffer (pH 3-4) and 1 ml of OCN 5 mM, at a flow rate of 1-2 ml/min using water aspirator as a vacuum source connected to the cartridge pack. 2 ml of sample which contains OCN 5 mM was passed through the cartridge. The cartridge was washed with 1 ml extraction buffer and 0.5 ml MilliQ water. The compounds of the sample were eluted from the cartridge with 2 ml of MeOH/water (70:30,v/v).

RESULTS AND DISCUSSION

Optimization of capillary electrophoresis separation

The aim of this paper is to develop a simple and rapid method of separating and identifying ten quinolones. These compounds are characterised by their structure. We can divided them into two groups: compounds with piperazinyl substituent (group I) and compounds without (group II). Group I comprises: lomefloxacin (1), enoxacin (2), norfloxacin (3), pipemidic acid (4) and ofloxacin (5); group II comprises: piromidic acid (6), oxolinic acid (7), flumequine (8), cinoxacin (9) and nalidixic acid (10). The pKa values of the compounds of group I range from 7.5 to 8.5 for the ammonium form and from 5.5 to 6.0 for the carboxylic function. The pKa value of the compounds of group II is due to carboxylic function [27].

Because of these pKa values it is reasonable to start with a buffer system with a pH range of 6.5 - 7.5 using the CZE mode, since at this pH quinolones of group I are neutral because they are in zwitterionic or unionized form at their isoelectric points (between pH 6.5 - 7.5), whereas quinolones of group II are negatively charged because they are purely acid and their pKa values are due to carboxylic function. The optimization of the separation was carried out using standard solutions of 25 mg l^{-1} of these ten quinolones. The running buffer used was sodium dihydrogen phosphate 18 mM (adjusting pH to 7.3 with sodium tetraborate 50 mM). Under these conditions the ten quinolones could not be separated, and the electropherogram (Fig.2) showed that the five piperazinyl-carrying quinolones migrated as a group ahead of the five piperazinyl non-carrying quinolones, which formed another group of somewhat better-separated peaks.

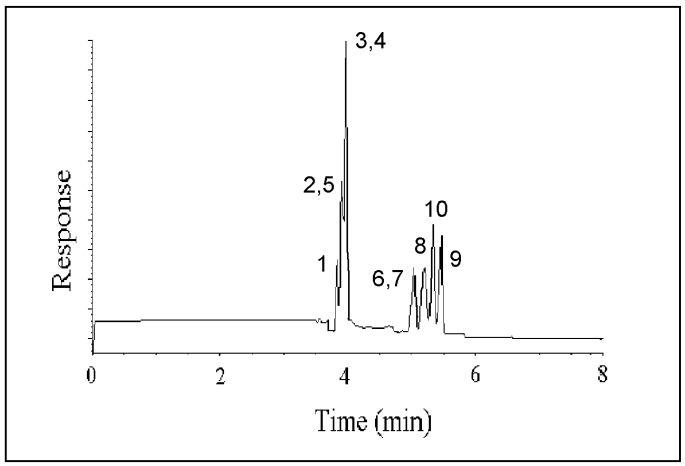


Fig.2. CZE electropherogram of 25 mg l^{-1} of quinolone antibiotics from standard solution. Electrophoretic conditions: 18 mM sodium dihydrogenphosphate adjusted to pH 7.3 with 50 mM sodium tetraborate; injection, 50 mbar for 4.8 s; separation voltage, 30 kV; capillary temperature, 30ºC; UV detection, 260 nm. Compound identities as shown in Fig.1.

MEKC is generally employed when the simple CZE mode cannot separate all compounds. First of all we studied the addition of a surfactant to the running buffer (the same running buffer as before). Sun and Chen [17] studied different bile salts to separate a group of quinolone antibiotics and the results were good. However, in this paper we used SDS because this surfactant is widely used in MEKC and also because of economic considerations. The working range was set at 8-25 mM of SDS. With greater concentrations of this surfactant separation was worse. Under these conditions results were best when SDS 20 mM was used, and there were seven peaks (Fig.3a). However, without adding any modifiers, several peaks were still partially merged. Under such circumstances, the ion-pairing reagent sodium octanesulfonate 5 mM was added (Fig.3b) to improve selectivity and peaks shapes according to the literature [17], but there was only a slight improvement. We had to improve the resolution and efficiency of the separation, so we studied the addition of another ion-pairing reagent,

TBA. Results were best when 5 mM of TBA was added to the running buffer (Fig.3c) but separation still had to be improved. We also studied a different gradient voltages, however it did not increase the resolution.

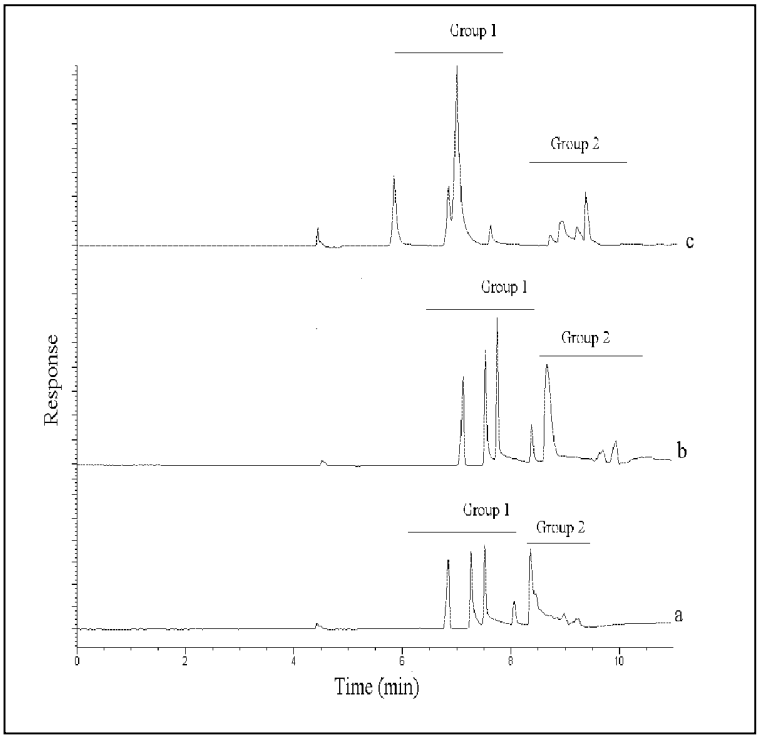


Fig.3. MEKC electropherograms of 25 mg l^{-1} of quinolone antibiotics from standard solution with: (a) 20 mM SDS, (b) 20 mM SDS and 5 mM OCN and (c) 20 mM SDS and 5 mM TBA in the running buffer. For other conditions see Fig.2.

After all these analysis, separation had only improved a little. We therefore decided to change the pH of the running buffer to ionize, at least partly, all the quinolones in this study and so simplify the method. In this case the running buffer was sodium tetraborate 40 mM and the pH was studied between 9.2 and 8.1 adjusted with diluted HCl. Figure 4 shows that the best result was obtained at pH 8.1. Under these conditions we can see ten peaks.

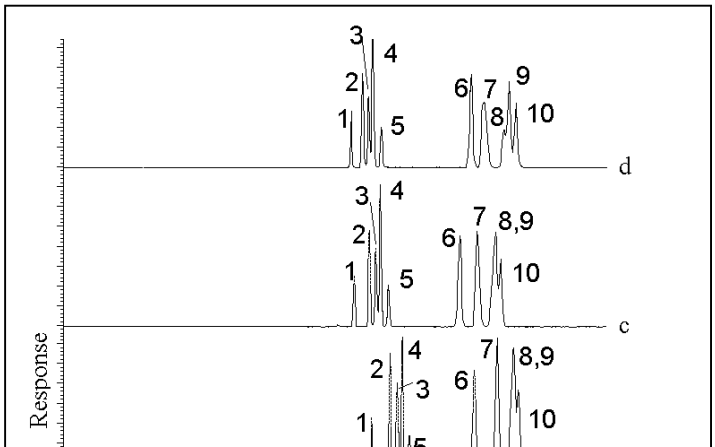


Fig.4. CZE electropherograms of 25 mg l^{-1} of quinolone antibiotics from standard solution with a running buffer of 40 mM sodium tetraborate at (a) pH 9.2, (b) pH 8.5, (c) pH 8.3 and (d) pH 8.1 adjusted with diluted HCl. For other conditions see Fig.2.

From here onwards it is only necessary to improve the resolution of the peaks. We also considered adding organic solvents to the electrophoretic buffer because they affect several variables, including viscosity, dielectric constant and zeta potential. The presence of acetonitrile or methanol increased the migration time of all the compounds. Increasing the concentrations of the organic solvents up to approximately 10% (v/v) improved the separation of the ten quinolones. The best results were obtained when MeOH 10% was added (Fig.5a). As we can see, the quinolones in group I were eluted first, followed by those in group II.

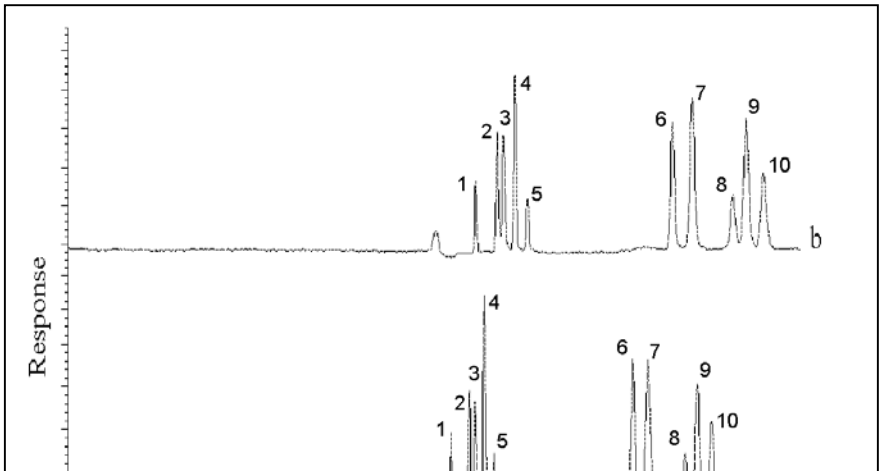


Fig.5. CZE electropherograms of: (a) 5 mg l^{-1} of quinolone antibiotics from standard solution and (b) pig plasma sample with standard addition of 5 mg l^{-1} of quinolone antibiotics after SPE. Running buffer of 40 mM sodium tetraborate at pH 8.1 and methanol 10% (v/v). For other conditions see Fig.2.

This was because the compounds in group I are bulkier and because all quinolones of group II are charged negatively and quinolones of group I are in the unionized form or negatively charged depending of the value of aminogroup pKa. If its pKa is lower than 8.1 these quinolones are negatively charged, but if it is higher then they are in the unionized form.

Optimization of solid-phase extraction method in biological samples

To analyse quinolones in plasma samples by CE it is necessary to remove interferences which are in the background of the sample. This paper proposes using SPE to pretreat the sample. The present application of SPE demonstrates the potential usefulness of nonpolar C_{18} cartridges for a group of quinolone antibiotics. This pretreatment was carried out at a pH of between 3 - 4 since at this pH quinolones in group I are charged positively and those in group II are in

the unionized form because of their pKa values. To carry out SPE using C_{18} cartridges OCN must be added to the sample to neutralize the positive charge of quinolones of group I.

With the SPE method we had to optimize a number of factors: the volume of elution, the percentage of MeOH in the MeOH/H_2O mixture for elution and the sample volume to pass through the cartridge. All of these studies were performed using 5 ml of a standard solution of 25 mg l^{-1} of ten quinolones. First we studied the elution volume (1 and 2 ml of a mixture of MeOH/H_2O (70/30)), and the results were best with 2 ml. We also studied different mixtures of 60/40 and 70/30 (MeOH:H_2O, v/v) to make a recovery study. With a 75/25 mixture we have a few problems with the current in the electrophoresis system and it is impossible to make the analysis. Table 1 shows the recovery values (% R) of the different quinolones over the percentage of MeOH and their relative standard deviation (% R.S.D.). In all cases the recovery for piromidic acid (6) is very low, which could be due to its structure. The best results were obtained using a mixture of 70/30 with recoveries nearly to 100 % and reproducibility as % R.S.D. in measured areas was between 2.3 and 15.1 %. We then studied the volume of sample to pass through the cartridge. These were 2, 5, 10 and 15 ml.

Table 1

Recovery values (%R) and Relative Standard Deviation (R.S.D.) of quinolones using SPE with different percentages of MeOH/H_2O (%, v/v).

Compound	60/40		70/30	
	%R	% R.S.D. (area)	%R	% R.S.D. (area)
1	27	4.0	103	3.3
2	26	7.1	127	4.4
3	29	8.2	111	3.1
4	45	5.7	114	3.1
5	35	3.6	128	12.0
6	2	14.1	39	3.3
7	16	17.1	93	2.3

8	51	7.7	125	15.1
9	25	8.2	103	3.9
10	59	6.0	131	10.0

*Studies performed using 5 ml of sample volume of a standard solution of 25 mg l^{-1} and 2 ml of elution volume

Recovery values and their R.S.D. are shown in Table 2. The recoveries obtained up to 5 ml of sample are good for all compounds except for piromidic acid (6), as we verified earlier. When we used sample volumes upper to 5 ml the recoveries were lower.

Table 2

Recovery values (% R) obtained with different sample volumes.

Compounds	%R (2ml)	%R (5ml)	%R (10ml)	%R (15ml)
1	112	103	90	65
2	112	127	121	92
3	109	111	89	70
4	111	114	109	88
5	89	136	121	96
6	27	39	24	---
7	94	139	142	96
8	90	93	71	57
9	93	103	81	65
10	125	131	89	74

To evaluate the calibration graph for each compound, a linear regression was performed with concentrations of calibration standards against measured peak areas using the optimum conditions.

All solutions were prepared in 70/30 (MeOH:H_2O, v/v) as this is the solvent composition introduced to the capillary electrophoresis system after solid-phase treatment. The results are shown in Table 3. These solutions were injected five times starting with the least concentrated and ending with the most. The area values obtained were successively analysed using ULC (Univariate Linear Calibration) software [28] to evaluate the correlation coefficient (r^2), relative standard deviation (R.S.D.) and limit of detection (LOD).

Table 3

Calibration data and precision for the ten quinolone antibiotics studied with standard solutions.

Compounds	Linearity (mg l^{-1})	Correlation coefficient (r^2)	LOD (mg l^{-1})	% R.S.D.[a] (area)	% R.S.D.[a] (time)
1	0.8 – 45	0.9978	0.4	5.3	2.9
2	1.0 – 45	0.9957	0.3	4.3	3.1
3	0.8 – 45	0.9954	0.2	6.2	3.2
4	0.8 – 45	0.9973	0.2	5.1	3.3
5	0.8 – 45	0.9971	0.4	5.9	3.3
6	0.8 – 60	0.9944	0.3	6.2	2.1
7	0.8 – 35	0.9973	0.4	7.6	3.6
8	0.8 – 60	0.9986	0.1	6.7	3.4
9	0.8 – 35	0.9998	0.3	13.0	3.7
10	1.0 – 35	0.9991	0.2	15.7	4.0

[a]: Calculated for ten consecutive runs at 25 mg l^{-1}

As we can see in Table 3, the linearities and correlation coefficients of the different compounds were good. The detection limit (LOD) was calculated by Widefordner and Long´s method [29] using the ULC program with K equal to 3. The LOD values for quinolones were between 0.1 and 0.4 mg l^{-1}. The R.S.D. of the areas and of the migration time were calculated for ten repeated injections of standard solutions of 25 mg l^{-1} . A fresh buffer was used after each sequence of three injections. The R.S.D. of migration time are between 2.1 and 4 %, while the R.S.D. of areas are between 4.3 and 15.7 %.

To demonstrate how useful the CE method is for determining quinolones, pig plasma samples were analysed. Samples were spiked with different quantities of the compounds up to a concentration range of between 5 and 20 mg l^{-1} before SPE treatment and 2 ml of sample was passed through the cartridge. The correlation coefficients are as good as in standard solutions. The LOD of these quinolones are between 1.1 and 2.4 mg l^{-1} with the method set out in this paper. The results are summarised in Table 4.

Table 4

Calibration data and precision for the ten quinolone antibiotics studied with pig plasma sample after SPE with standard addition.

Compounds	Linearity (mg l^{-1})	Correlation coefficient (r^2)	LOD (mg l^{-1})	% R.S.D[a] (area)	% R.S.D[a] (time)
1	5 – 20	0.9998	1.2	15.3	4.5
2	5 – 20	0.9990	2.0	6.7	2.5
3	5 – 20	0.9950	1.8	9.3	3.5
4	5 – 20	0.9970	2.2	8.2	2.8
5	5 – 20	0.9998	2.2	9.6	2.6
6	5 – 20	0.9918	1.2	19.0	3.8
7	5 – 20	0.9994	1.6	14.4	4.9
8	5 – 20	0.9964	2.3	17.4	4.0
9	5 – 15	0.9942	1.1	15.9	5.0
10	5 – 20	0.9970	2.4	16.0	3.4

[a]: Calculated for ten consecutive runs at 5 mg l^{-1} spiked with a standard of ten quinolones.

The limit of quantification (LOQ) in pig plasma samples is the lowest concentration from which it is possible to quantify the analyte with reasonable statistical certainty. The LOQ was calculated by Widefordner and Long´s method [29] using the ULC program [28] with K equal to 6. The LOQ for pig plasma estimated by measuring the response of 10 drug-free pig plasma sample was determined to be in the range 2.2 mg l^{-1} and 4.8 mg l^{-1}. The LOQ estimated by the smallest validated concentration within the limits of precision and accuracy set for the method was determined to be 5 mg l^{-1} for all quinolones. Repeatability of the method did not exceed 19.0 % measured in peak are and 5.0 % measured in migration time.

The extraction efficiency (recovery) was determined by comparity peak areas from drug-free samples spiked with known amounts of drugs (in the range of concentrations of the calibration curves) and standard solutions injected directly in the electrophoresis system. Each sample was determined in triplicate.

The recoveries obtained for pig plasma (Table 5) were stable over the range used (5-20 mg l^{-1}). They varied from 90.0 % to 129.3 %, except for piromidic

acid. The mean recovery varied from 94.0 ± 4.2 % to 123.3 ± 4.1 % for the three tested concentrations representing the entire range of the calibration curve (low, medium and high concentrations).

Fig.5b shows the separation of these quinolones from a pig plasma sample spiked with a standard solution of 5 mg l^{-1}.

CONCLUSIONS

Our results suggest that 40 mM of borate buffer at pH 8.1 containing 10% (v/v) methanol is a very efficient running buffer for separating quinolone antibiotics. This method in which SPE treatment was used as a clean-up step for obtaining a background free of interferences in the electropherograms of real samples, successfully separated and determined the ten quinolones in pig plasma samples.

Table 5

Recovery (%R) study and repeatability (%RSD) from pig plasma samples at different spiked level preconcentration.

	Low concentrations		Medium concentrations		High concentrations		
	5 ppm		10 ppm		20 ppm		
Compounds	**Recovery (%R)**	**R.S.D.**	**Recovery (%R)**	**% RSD**	**Recovery (%R)**	**% RSD**	**Mean recovery (%)**
1	104.3	4.1	120.5	2.2	106.7	5.2	110.5 ± 8.7
2	99.9	0.1	119.6	3.9	106.2	1.8	108.6 ± 10.1
3	119.9	4.2	122.3	2.8	127.8	2.5	123.3 ± 4.1
4	98.0	9.6	110.8	5.2	119.3	2.2	109.4 ± 10.7
5	97.7	13.8	115.1	8.2	91.7	7.1	101.5 ± 12.2
6	68.0	8.7	63.4	15.6	73.0	17.0	68.1 ± 4.8
7	90.2	7.5	98.5	14.7	93.4	9.0	94.0 ± 4.2
8	107.7	8.4	126.0	6.4	128.7	2.5	120.8 ± 11.4
9	100.9	8.2	102.5	12.8	107.5	13.6	103.6 ± 3.4

10	106.7	15.6	123.9	7.6	124.7	8.3	118.4 ± 10.2

[a] :Calculated for ten consecutive runs at each concentration spiked with a standard of ten quinolones.

This investigation has shown the method developed is an alternative to HPLC methods in terms to short analysis time (13 min) and simple clean-up system, and the LODs obtained are in accordance to other methods developed to analyse quinolones with a capillary electrophoresis system using UV-visible detection but a little higher when quinolones are analysed by HPLC.

We will continue working to decrease detection limits and we will study different types of SPE cartridges. We will also try to increase the sensitivity of the CE method with different preconcentration techniques.
Testing of the suitability of the method for the analysis the same molecules in plasma of other species or in the other tissues is in progress in our laboratory. The method could be successfully applied to monitor structurally related novel fluoroquinolone antibiotics in plasma.

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III.5. Determinació de tetraciclines en mostres biològiques per electroforesi capil·lar

En aquest treball es mostra el potencial de separació de la tècnica de CE en medi no aquós, el que es coneix com a NACE (*nonaqueous capillary electrophoresis*), en l'anàlisi de tetraciclines.

Des dels primers estudis per CE, l'aigua sempre ha estat el solvent més utilitzat en els sistemes electroforètics, això és degut a les seves característiques, com són el baix cost, baixa viscositat, no volàtil, seguretat i disponibilitat. A més les característiques àcid-base són perfectament conegudes, és compatible amb diversos tipus de detectors, solubilitza gran part d'electròlits,... Malgrat això l'aplicació de CE en medi aquós està limitat a compostos amb alguna característica polar [1]. Per altra banda, els solvents orgànics es caracteritzen per la seva versatilitat, àmplia varietat de propietats físico-químiques (com diferents constants dielèctriques, polaritats, densitats, viscositats i propietats àcid-base [1-3]). A la Taula 1 es mostren algunes de les propietats físico-químiques de diferents solvents orgànics utilitzats en CE [1,3]. La gran versatilitat d'aquests solvents permet augmentar la selectivitat dels sistemes electroforètics.

En el present treball s'ha fet un estudi comparatiu en el qual es compara la selectivitat aconseguida a l'analitzar quatre tetraciclines: oxitetraciclina, tetraciclina, 4-epioxitetraciclina i 4-epitetraciclina, en un sistema electrolític en un medi aquós i en un medi no aquós. Aquest estudi mostra que mentre la separació d'aquests compostos no ha estat possible en medi aquós, quan es treballa amb NACE s´augmenta la selectivitat del sistema de tal manera que s'aconsegueix una completa separació de tots els compostos.

Taula 1.

Propietats físiques i químiques d'alguns solvents utilitzats en CE a 25 ºC

Solvents	ε^a	ζ^a (10^{-2} Nm^{-1})	η^a (mPa s)	$T_{eb}{}^a$ (ºC)	pKa
Aigua	78.39	7.181	0.890	100.0	14.00
Metanol (MeOH)	32.70	2.212	0.544	64.6	17.20
Acetonitril (ACN)	17.51	2.760	0.369	81.6	≥33.3
Formamida	111.0	5.791	3.343	210.5	16.8[b]
N-Metilformamida (NMF)	182.4	3.87	1.678	182.0	10.74
N,N-Dimetilformamida (DMF)	36.71	3.52	0.794	153.0	29.4
Dimetilsulfòxid (DMSO)	46.68	4.286	1.987	189.0	33.3
1-propanol	20.33	2.330	1.945	97.2	19.43
2-propanol	19.92	2.124	2.038	82.3	20.80
1-butanol	17.51	2.416	2.544	117.7	21.56

[a] ε (permititivitat relativa), ζ (tensió superficial), η(viscositat dinàmica), T_{eb} (temperatura d'ebullició)
[b] A 20 ºC

Primer es desenvolupa un sistema en medi aquós utilitzant com a tampó carbonat sòdic 20 mM i EDTA 1 mM a pH 11.25, sistema que ja havia estat utilitzat prèviament en altres estudis per a la determinació de tetraciclines [4]. En aquestes condicions els quatre compostos estan carregats negativament, però malgrat això no es poden separar. Per tal de millorar la separació s'afegeixen solvents orgànics (metanol, acetonitril i isopropanol) en l'electròlit en proporcions d'entre el 5 i 20 %. En les millors condicions (quan s'afegeix un 10 % d'acetonitril), en l'electroferograma s'obtenen 3 pics, ja que dos dels quatre compostos (la 4-epitetraciclina i la 4-epioxitetraciclina) coelueixen.

Com no s'aconsegueix la completa separació dels quatre compostos s'opta per augmentar la selectivitat del sistema mitjançant la utilització d'un sistema electroforètic en medi no aquós.

En aquest sistema s'han optimitzat diversos paràmetres: diferents tampons per dur a terme la separació (acetat amònic, acetat sòdic i formiat amònic), diferents àcids per acidificar el tampó (àcid metanosulfònic, àcid clorhídric, àcid acètic, àcid fòrmic i àcid cítric) i diferents solvents orgànics per dissoldre el tampó (acetonitril, metanol, N-metilformamide, N,N-dimetilformamida, dimetilsulfòxid).

Els tampons triats es caracteritzen perque tots ells són solubles en medi no aquós i normalment donen una bona resolució en la separació de diferents tetraciclines. El medi àcid s'utiliza per acidificar el medi i que totes les tetraciclines estiguin carregades negativament. Els àcids triats permeten una bona resolució en el procés de separació. El valor de pH només té un sentit directe en els medis aquosos. En els medis no aquosos el valor de pH es defineix com a pH aparent o pH*. Aquest valor serveix per mesurar les propietats àcides i bàsiques en aquests medis.

En aquest treball s'han estudiat diverses mescles de solvents orgànics per a determinar quines ofereixen una major selectivitat en la separació dels compostos esmentats. El medi que s'ha escollit ha consistit en metanol/acetonitril (MeOH/ACN) (50:50,v/v). El MeOH, generalment, augmenta la solubilitat dels compostos, i a més, millora la detectabilitat del mètode. Per altra banda, l'ACN disminueix la viscositat del medi. De vegades s'aconsella la utilització de tres solvents, MeOH – ACN – altre solvent, per tal de millorar la selectivitat del mètode. En aquest estudi s'ha estudiat l'addició d'aquest tercer solvent, la N-metilformamida (NMF), la dimetilformamida (DMF) i el dimetilsulfòxid (DMSO). En cap dels casos s'ha aconseguit millorar la separació. A més, aquests solvents absorbeixen a la zona ultraviolada, de manera que fan disminuïr la sensibilitat del mètode.

El mètode establert ha estat aplicat a l'anàlisi de les quatre tetraciclines esmentades en plasma de porc. Com en el cas de l'amoxicil·lina, ha estat necessari el desenvolupament d'un sistema de pretractament de la mostra, el qual s'ha realitzat mitjançant la tècnica de SPE.

S'han estudiat diferents sorbents comercials, Sep Pak Plus C_{18}, LiChrolut EN i Oasis HLB. El primer es tracta d'un sorbent C_{18}, el qual es caracteritza perque les interaccions que tenen lloc són principalment interaccions hidrofòbiques amb grups alquílics hidrofòbics. Els altres dos sorbents (LiChrolut EN i Oasis HLB) són sorbents polimèrics, els quals es caracteritzen perque les interaccions que tenen lloc són π-π.

Per altra banda, s'ha estudiat com varien les recuperacions en funció del pH de la mostra (pH 3, 5 i 8). S'observa un augment creixent de les recuperacions en tots els sorbents a mida que disminueix el pH de la mostra. Això pot ser degut a que aquests compostos contenen diversos grups hidroxil en la seva estructura, i a pH bàsics aquests no són molt estables i pot disminuir la seva afinitat pels sorbents.

En el cas dels sorbents Sep Pak Plus C_{18} les recuperacions no han estat molt altes, entre el 60 – 70 %. Això pot ser degut als grups silanol residuals presents en el sorbent poden produir l'adsorció de les tetraciclines. En el cas dels sorbents polimèrics no hi ha aquest problema degut a l'absència d'aquests grups.

Els millors resultats s'han obtingut amb l'ús dels sorbents Oasis HLB amb recuperacions de l'ordre del 90 – 100 %, excepte per la 4-epioxitetraciclina, on les recuperacions són de l'ordre del 74 %. Els sorbents LiChrolut En donen unes recuperacions similars als d'Oasis HLB, però una mica més baixes. Això pot ser degut al diferent empaquetat i d'altres característiques del sorbent.

El mètode establert permet eliminar les interferències que conté la matriu de la mostra i permet una completa separació i determinació de tots els compostos.

En aquest treball s'ha demostrat el potencial que presenta l'ús de NACE, i que és un bon sistema per a la separació i determinació de les quatre tetraciclines

objecte d'estudi. A més, també s'ha demostrat l'aplicabilitat d'aquest mètode en la determinació de les esmentades tetraciclines en mostres de plasma de porc.

Els estudis que han estat realitzats en aquest treball han estat publicats a la revista Chromatographia, 52 (2000) 279-284, una còpia dels quals es mostra a continuació.

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DETERMINATION OF OXYTETRACYCLINE AND SOME OF ITS IMPURITIES IN PLASMA SAMPLES BY NONAQUEOUS CAPILLARY ELECTROPHORESIS USING SOLID-PHASE EXTRACTION AS A CLEAN-UP STEP

ABSTRACT

The potential of a nonaqueous capillary electrophoresis (NACE) system for separating oxytetracycline from three of its impurities — tetracycline, 4-epioxytetracycline and 4-epitetracycline — using UV detection has been studied. The running buffer was: 25 mM sodium acetate, 1 mM EDTA, methanesulfonic acid, pH* 4, dissolved in MeOH/ACN (50:50, v/v). The method was also used to determine these compounds in pig plasma. A solid-phase extraction (SPE) procedure as a clean-up step has also developed. For this we tested Sep Pak C_{18}, LiChrolut EN and OASIS cartridges. OASIS cartridges were best. Recoveries were 90 - 100 % for all compounds except EOTC which had a recovery of 74 %.

keywords: Capillary zone electrophoresis; Nonaqueous medium; Solid-phase extraction; Tetracyclines; Plasma samples

INTRODUCTION

Tetracyclines (TCs) are a group of clinically important natural products and semi-synthetic derivatives characterised by broad spectrum activity against pathogenic micro-organisms. These therapeutic compounds are used to control bacterial infections in humans and animals. They have also found applications in the preservation of harvested fruits and vegetables, the extermination of insect pests, and as an animal feed supplement [1,2]. The chemical structures of this group of antibiotics are closely related and are derived from a common hydronaphthacene nucleus containing four fused rings [2]. The structures of the tetracyclines we studied are given in Figure 1.

Oxytetracycline (OTC) is a broad-spectrum antibiotic of the TC family. It is produced by fermentation by *Streptomyces Rimosus*. During its synthesis some side products, such as 2-acetyl-2-decarboxaamidooxytetracycline (ADOTC) and tetracycline (TC), can be formed. Contact with acid can convert OTC to 4-epioxytetracycline (EOTC) through an equilibrium reaction and to anhydrooxytetracycline (AOTC) by irreversible dehydration. Contact with acid can also convert TC to 4-epitetracycline (ETC). The pK values relevant to acid-base equilibrium in aqueous solution of OTC are 3.27, 7.32, 9.11 and 10.7 [3] and of TC are 3.30, 7.68, 9.69 and 10.7 [4].

TCs are commonly used in prevention and treatment of diseases in livestock. They are widely used in pigs, cattle, sheep and poultry. The residues of the drugs may remain in the animal products intended for human consumption. To prevent health problems for consumers maximum residues levels (MRLs) of these compounds and other drugs in liver stock products have been established [5, 6]. Consequently, one requirement of the analytical methods used to determine of tetracycline residues in tissues samples and other types of samples is that they have detection limits below the MRLs. For plasma samples MRLs are not fixed.

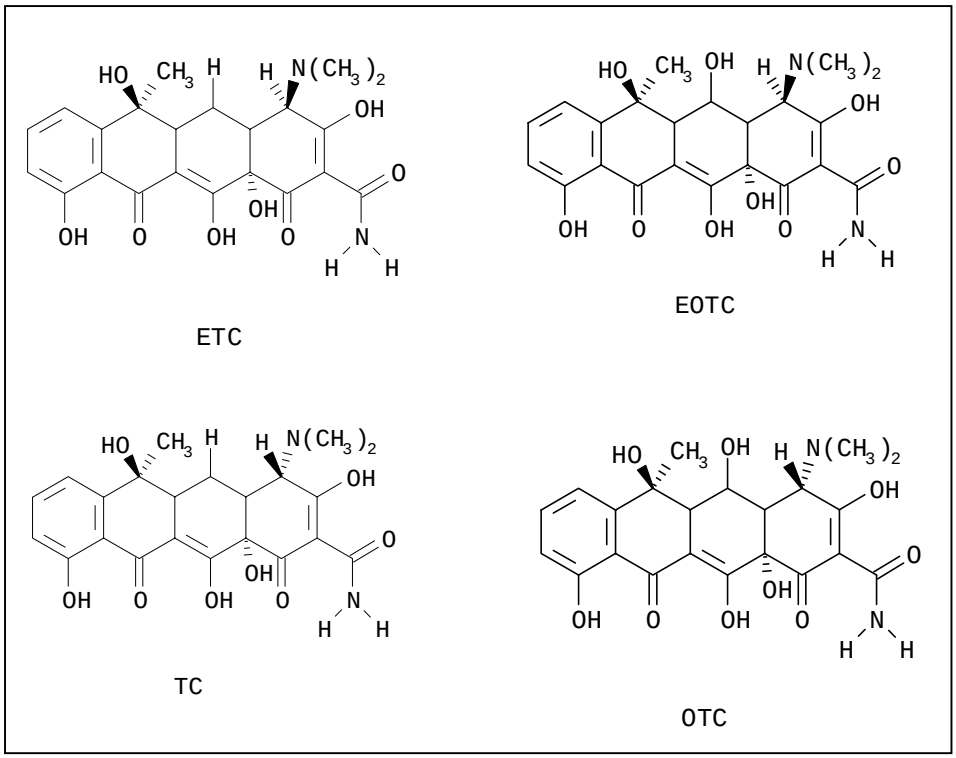

Fig. 1. Structures of four tetracyclines

In the literature there are several liquid chromatographic (HPLC) methods to separate, identify and quantify TCs and their major impurities and degradation products using standard solutions [7, 8]. Some of these studies have been applied to determine these compounds in veterinary products like oral medications or injectable solutions [9]. HPLC methods have also been used to determine TCs in biological samples like plasma [10, 11], milk [12–15] and tissue samples [5,14–22].

Recently there have been several reports on the separation of TCs by aqueous capillary electrophoresis (CE) [1–4,23–25]. Tavares et al. [2] studied the separation of OTC, TC and five other TCs by capillary zone electrophoresis (CZE) using phosphate buffer. Sodium carbonate was used as basic run buffer in the separation of different mixtures of TCs by CZE [3, 4, 25]. EDTA, 1 mM.

was added to the running buffer to prevent metal-ion complexation. Some authors have investigated using MECC to improve the selectivity of the TC analysis [1,23,24]. These papers studied the addition of different non-ionic surfactants like Triton X-100, Brij-35, Tween 20 and Tween 80 to the running buffer.

In CE the running buffer is generally dissolved in water. This is because of its unique characteristics, i.e. low cost, low viscosity, involatility, safety and availability. Moreover, water has a well-known acid-base chemistry, it is compatible with various detection schemes and can solvate a variety of electrolytes. However, the application of aqueous CE is limited to compounds that are somewhat polar. To analyse nonpolar solutes modifiers, such as micelles, urea and organic cosolvents must be added. These modifiers alter the bulk properties of the solvent and influence the solvation of analytes, thus affecting the susceptibility of analytes to electrophoretic migration. To maximise the advantages of using an organic cosolvent a totally nonaqueous medium is prepared [26–29]. This is the case of non-aqueous capillary electrophoresis (NACE). Recent applications of NACE in the determination of TC antibiotics have been successful [30,33]. Selectivities which were very difficult to obtain in aqueous buffers even when using MECC or complexing agents were easily obtained when using nonaqueous systems. In the present paper, a simple method was optimized by NACE using UV detection to determine OTC and three of its impurities: tetracycline (TC), 4-epioxytetracycline (EOTC) and 4-epitetracycline (ETC).

On account of their complexity biological samples cannot generally be injected directly to the analytical instrument. Sample pretreatment is necessary to obtain them without any interferences and in a suitable medium. A number of LC methods have been described for determining residues of TC antibiotics in plasma samples [14,15,31,32], e.g., using trifluoroacetic acid to precipitate plasma proteins with recoveries of around 70 % [31], or using ethyl acetate to extract TCs from plasma [14,15]. These methods are both sensitive and precise

and have high recoveries, but they are also time-consuming. Another type of extraction proposed was to use an ion-pair such as tetrabutyl-ammonium [32], in this case the recovery was low, between 60 – 65%. A large number of papers focus on solid-phase extraction (SPE) [5, 14–17,32,37] as a clean-up technique and in particular on silica-based cartridges. However, the recoveries are sometimes low. Although CE has been successfully used in the analysis of commercially available pharmaceutical formulations [2, 4], few applications have been reported for biological samples until now e.g. for milk samples [33] and cooked channel catfish [34], in which an SPE procedure with a C_{18} cartridge was used for pretreatment.

This paper compares different types of SPE cartridge: Sep Pak C_{18}, LiChrolut EN and OASIS cartridges, to clean-up pig plasma samples before their analysis by non-aqueous CE.

EXPERIMENTAL

Instrumentation

Electrophoretic experiments were performed using a HEWLETT PACKARD 3D CE (Waldbronn, Germany), with a DAD detector (Diode Array Detection). Data were collected with the software provided with the HP 3D Chemstation (Hewlett-Packard), which was operated under Windows 95 (Microsoft). The capillary was fused-silica (64.5 cm $\times$ 75 μm i.d.) from Supelco (Bellefonte, USA). A detection window was prepared by burning off the polyimide coating 56 cm from the capillary inlet.

Reagents and standards

OTC, TC, ETC and EOTC were from Acros Organics (New Jersey, USA). Standard stock solutions of 1000 mg L^{-1} of OTC, EOTC, TC and ETC, which were prepared weekly, were dissolved in methanol-acetonitrile (50:50, v/v) and stored refrigerated. Standard working solutions were prepared daily by diluting the standard stock solution with methanol-acetonitrile (50:50, v/v).

Trifluoroacetic acid (TFA) (Fluka Chemika, Buchs, Switzerland), acetonitrile (ACN) (Fisher Scientific UK, Leicestershire) and methanol (MeOH) (Merck, Germany) were used to pretreat the sample.

Sodium tetraborate (Fluka), sodium carbonate (Prolabo, Bois, France), sodium acetate (Aldrich Chemical Co. Inc., USA), ammonium acetate (Aldrich) and ammonium formate (Aldrich) were used to prepare the electrophoretic solutions. Water purified by a Milli-Q system, (Millipore, Bedford, USA). Methanesulfonic acid (Fluka), hydrochloric acid, acetic acid, formic acid and citric acid (all Probus) were used to acidify the running buffer. Acetonitrile (ACN) (Fisher Scientific UK, Leicestershire), methanol (MeOH) (Merck, Germany), N-methylformamide (NMF) (Fluka), N,N-dimethylformamide (DMF) (Merck) and dimethylsulfoxide (DMSO) (Merck) were used to dissolve the electrolyte. Sodium ethylenediaminetetraacetate (EDTA) (Probus,) was used to prevent metal-ion complexation.

Electrophoretic conditions

The running buffer was 25 mM sodium acetate, 1 mM EDTA, 26 mM methanesulfonic acid with a mixture of MeOH-ACN (50:50,v/v) as a solvent. The apparent pH (pH*) of this nonaqueous electrolyte was 4. Before use, the capillary was rinsed with 1 M NaOH (Probus) (1000 mbar pressurised flow) for 15 min, then with H_2O Milli-Q for 15 min, MeOH for 15 min and finally flushed with running buffer for 10 min. Before each analysis, the capillary was flushed with MeOH for 3 min and with the running buffer for 3 min. Detection was at 265 nm. Injection was hydrodynamic at 50 mbar for 3 s of sample. Running buffer was then introduced to the capillary at 50 mbar for 6 s to diminish the dispersion

of sample during analysis. The capillary temperature was 25 ºC and the separation voltage was 30 kV.

Pretreatment of the Sample

Solid-phase Extraction (SPE)

We used three different types of cartridges for SPE: 1) Sep Pak C_{18} cartridges (360 mg), 2) LiChrolut EN cartridges (200 mg) and 3) Waters Oasis HLB cartridges (60 mg).

In all cases the procedure was the same: the cartridge was activated with 5 mL MeOH followed by 5 mL H_2O Milli-Q at a flow rate of 1 – 2 mL min^{-1} using a water aspirator as vacuum source connected to the cartridge pack. 2 mL of sample was passed through the cartridge. The cartridge was washed with 2 mL H_2O MilliQ. Compounds were eluted from the cartridge with 2 mL aliquots of MeOH-ACN (50:50,v/v).

Pig plasma samples was used in all experiments.

RESULTS AND DISCUSSION

Aqueous Capillary Electrophoresis

This paper describes the determination of oxytetracycline (OTC) and three of its impurities: tetracycline (TC), 4-epioxytetracycline (EOTC) and 4-epitetracycline (ETC) in pig plasma samples. First, we optimized a CE method to analyse these TCs in standard solutions. We used sodium carbonate-EDTA system as the electrolyte because it obtained good resolutions in the analyses of other TCs [3,4,24]. The running buffer was sodium carbonate 20 mM, EDTA 1 mM,

adjusted to pH 11.25 (using NaOH 0.1 N), with water as a solvent. In these conditions all compounds are charged negatively, because their pK values [3,4]. Under these conditions only one peak appears. None of the compounds could be separated. We considered the possibility of using sodium borate as electrolyte but the results were worse. So, with sodium carbonate as electrolyte we added various organic solvents to the running buffer to improve separation. Solvents studied were: MeOH, ACN and 2-propanol in proportions of 5, 10 and 20 %. In all cases the analysis time increased because of the viscosity of the organic solvents. When MeOH or 2-propanol was added, only one peak was obtained. However, when ACN was added, two peaks appeared in the electropherogram, except when the proportion was 10%, when three peaks appeared. In this case, the elution order was TC, ETC – EOTC (coeluted) and OTC. From these results, however, separation needs to be improved.

Nonaqueous Capillary Electrophoresis

On account of the wide variety of physical and chemical properties characterising organic solvents we can optimize a number of operating parameters in CE to enhance the advantages of using an organic cosolvent, e.g., we can use a completely nonaqueous medium [29]. Selectivity can be manipulated by varying the types and composition of organic solvents, changing ionic strength, alterating pH* values and adding cyclodextrins soluble in organic solvents. This produces a wide variety of applications. The pH value is only defined in dilute aqueous solutions and has no direct significance in nonaqueous solvents. When we use a non-aqueous medium we define pH as apparent pH or pH*. However, acidic or basic electrolytes still exhibit a major influence on separation selectivity. The pH* value is used to measure acidic and basic properties of non-aqueous media [35, 36]. Methanol-acetonitrile (50:50, v/v) is usually the mixture used to dissolve the running buffer in nonaqueous medium. This is because the MeOH content enhances solubility of analytes and improves detectability. Adding up to 50 % ACN shortened the analysis time (as expected from ACN´s lower viscosity) but maintained full resolution of all components [37].

Several authors have developed the determination of a number of TC antibiotics by NACE systems [30,33,38]. Separation was complete and resolution good because of the nature of the organic solvents and because the electrolytes greatly influence selectivity and efficiency in the electrophoretic system. Moreover, NACE systems make possible direct injection of samples which are dissolved in organic solvents e.g. those obtained with SPE or protein precipitation pretreatment. For these reasons and because it is very difficult to separate all compounds by CE in aqueous media we have developed NACE method to separate and identify them. As the bibliography shows [28,35,37,38] to operate NACE system the electrolyte must be optimized, i.e. the acid for acidifying the running buffer and the organic solvents for dissolving the running buffer. Here we have optimised several parameters: a) electrolytes (ammonium acetate, ammonium formate and sodium acetate), b) different acids to acidify the running buffer (methanesulfonic acid, chloride acid, acetic acid, formic acid and citric acid) and c) organic solvents to dissolve the running buffer (ACN, MeOH, NMF, DMF and DMSO). The electrolytes chosen for this study were those soluble in most of the usual organic solvents and produced better resolution of different TCs in other studies in non-aqueous media. An acidic medium was used to keep the TCs as cations. Acids chosen for acidification were those producing a better resolution of peaks in the separation of TCs in previous papers [38].

The electrolyte system first used was 25 mM ammonium acetate, 1 mM EDTA and different acids for acidification. The running buffer and standard solutions were diluted with MeOH-ACN (50:50, v/v). The analysis was performed at pH* 3. Best results were with methanesulfonic acid so that the electrolyte had a pH* of 3. In this case three peaks appeared in the electropherogram (Figure 2a) and the elution order was the same as for the best conditions with an aqueous medium (adding 10 % ACN to running buffer). However, analysis time is less in non-aqueous medium [28, 35]. Results are similar when methanesulfonic and

formic acids are used. However, methanesulphonic acid produced slightly better resolution.

We then used ammonium formate as the electrolyte and methanesulfonic and formic acids to acidify the running buffer. The concentration of the running buffer, pH* and conditions of the analysis instrument were the same as before. With formic acid we obtained three peaks. With methanesulfonic acid resolution increased slightly. The electropherogram has four peaks (Figure 2b) but two of them are slightly merged. Another electrolyte studied to improve separation was sodium acetate with the addition of methanesulfonic, formic and acetic acids. With acetic acid there were problems in stabilising the baseline. As seen in Figure 2c, the best result was with methanesulfonic acid as almost complete separation of the four compounds was achieved. This figure shows the results obtained with methanesulfonic acid and the three different electrolytes.

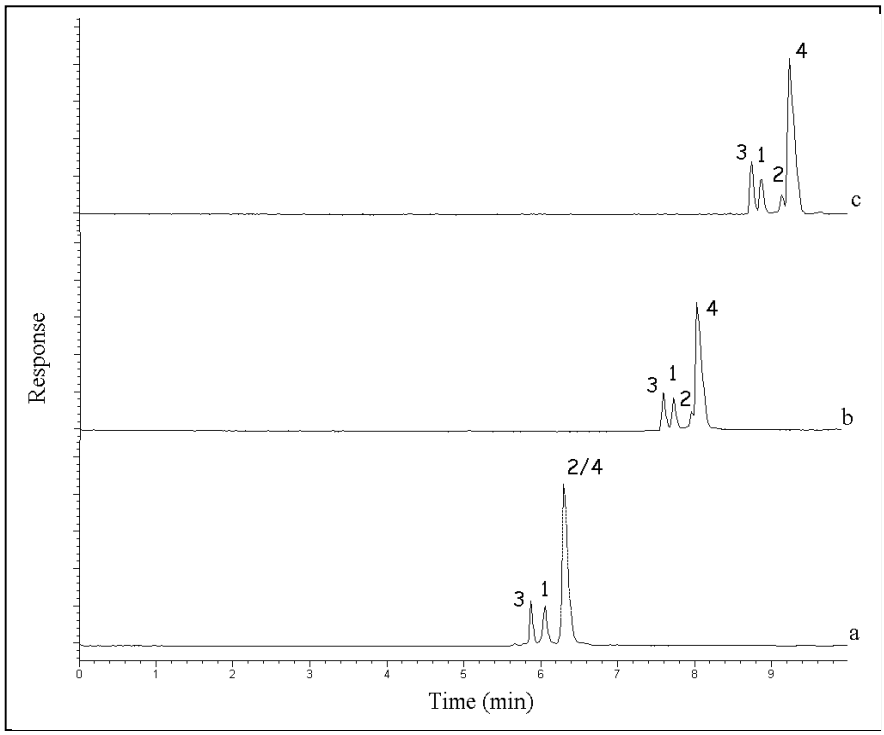

Fig. 2. Electropherogram of 100 mg L^{-1} of OTC and 20 mg L^{-1} TC, ETC and EOTC from standard solution. The electrolyte was ammonium acetate (a), ammonium formate (b) and sodium acetate (c). Running buffer: 25mM electrolyte, 1mM EDTA, pH* 3 (methanesulfonic acid). Injection: 50 mbar for 0.3 min of sample and 50 mbar for 0.6 min of running buffer. Capillary temperature: 25ºC. Separation voltage: 30 kV. For peak assignments see Figure 1.

Up to this point, all running buffers were dissolved in a mixture of MeOH-ACN (50:50, v/v). To improve resolution different mixtures of three organic solvents were studied: MeOH-ACN-another organic solvent (50:45:5, v/v). Solvents added were NMF, DMF and DMSO which have a greater viscosity than ACN and MeOH.

A high viscosity leads to a decrease in electroosmotic flow (EOF) and therefore to a reduction in efficiency unless this is compensated by an increase in the dielectric constant and an improvement in the resolution of the analysis [38]. The running buffer for this study was 25 mM sodium acetate, 1 mM EDTA, methanesulfonic acid, pH* 3. Figure 3 shows the electropherograms obtained when the following were used to dissolve the running buffer, MeOH-ACN-NMF (50:45:5, v/v) (Figure 3a), MeOH-ACN-DMF (50:45:5, v/v) (Figure 3b) and MeOH-ACN-DMSO (50:45:5, v/v) (Figure 3c) and MeOH-ACN (50:50, v/v) (Figure 3d). In all cases four peaks appeared in the electropherogram but no analysis improved on the resolution with a mixture of three organic solvents and, moreover, the signal decreased. This is because organic solvents like NMF, DMF and DMSO have high UV background absorbance at shorter wavelengths and would reduce sensitivity [37].

These solvent mixtures were also used with ammonium acetate and ammonium formate, but the results were not so good. The best results were obtained when the running buffer was: 25 mM sodium acetate, 1 mM EDTA, methanesulfonic acid, pH* 3 and MeOH-ACN (50:50,v/v) as the solvent (Figure 2c). Other possibilities for improving separation could have been altering MeOH-ACN ratios in the running buffer, but were not studied here.

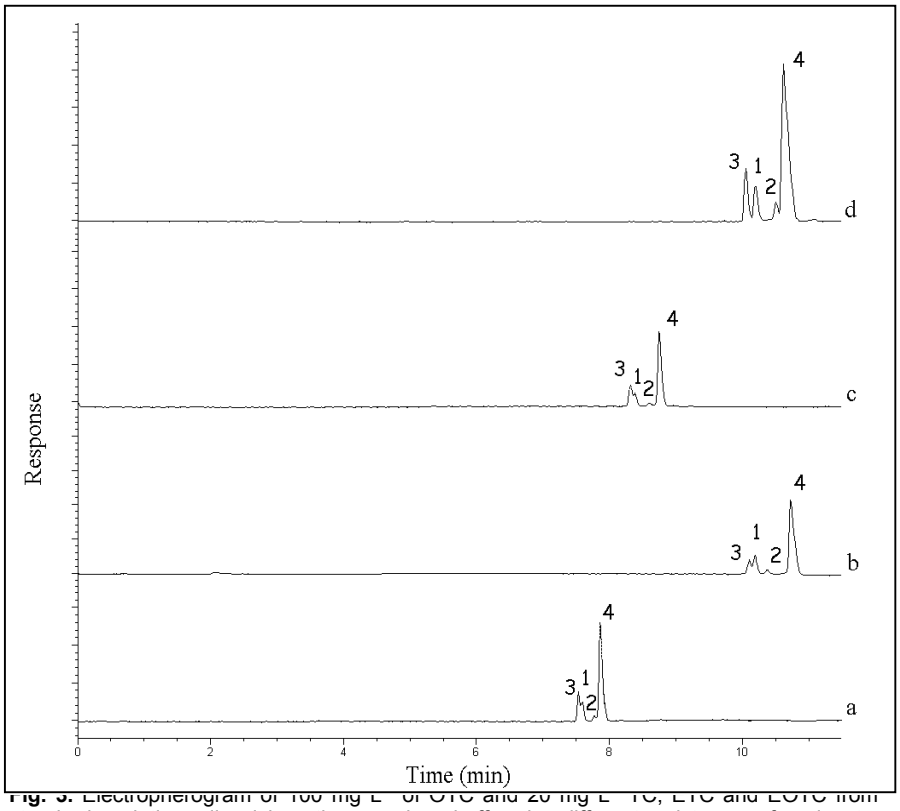

Fig. 3. Electropherogram of 100 mg L^{-1} of OTC and 20 mg L^{-1} TC, CTC and EOTC from standard solution dissolving the running buffer in different mixtures of solvents: MeOH/ACN/NMF (50:45:5,v/v) (a), MeOH/ACN/DMF (50:45:5,v/v) (b), MeOH/ACN/DMSO (50:45:5,v/v) (c) and MeOH/ACN (50:50, v/v) (d). Running buffer: 25 mM sodium acetate, 1 mM EDTA, pH* 3. For other conditions see Figure 2.

To improve separation the pH* value in the running buffer was increased to change the number of ionic species in the analysis medium. At a pH* of 4 the four compounds were separated with good resolution and efficacy (Figure 4).

To evaluate the calibration graph for each compound, a linear regression was performed with concentrations of calibration standards against measured peak areas under optimum conditions. All solutions were prepared in MeOH-ACN

(50:50, v/v). They were injected five times, starting with the least concentrated solution and ending with the most concentrated one. Areas obtained were successively analysed with ULC (Univariate Linear Calibration) software [39] to evaluate the correlation coefficient (r^2), relative standard deviation (R.S.D.) and limit of detection (LOD).

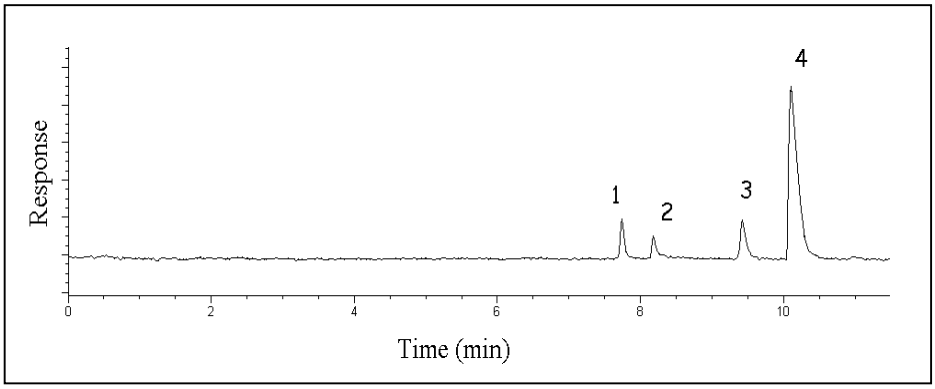

Fig. 4. Electropherogram of 100 mg L^{-1} of OTC and 20 mg L^{-1} TC, ETC and EOTC from standard. Running buffer: 25 mM sodium acetate, 1 mM EDTA, pH* 4. For other conditions see Figure 2.

These values are shown in Table 1, which shows that the linearities and correlation coefficients of the different compounds were good. The LOD was calculated by Widefordner and Long´s method [40] using the ULC program with K = 6. LOD´s for tetracyclines were 0.2 - 0.3 mg L^{-1}. R.S.D.s of the areas were calculated for ten repeated injections of standard solutions of 20 mg L^{-1}. A fresh buffer was used after each sequence of three injections. R.S.D.s of areas are 2.1 - 6.9 %.

Table 1

Calibration data and precision for four tetracycline antibiotics with standard solutions.

Compounds	Linearity (mg L^{-1})	Correlation coefficient (r^2)	LOD	R.S.D.[a] (area)
ETC	1-100	0.9995	0.3	2.5
EOTC	1-100	0.9985	0.3	6.9
TC	1-100	0.9976	0.2	2.1
OTC	1-100	0.9987	0.2	6.8

[a]: Calculated for ten consecutive runs at 20 mg L^{-1}

SPE as Pretreatment of Plasma Samples

SPE using C_{18} cartridges as a clean-up step is the most often used method for analysing TCs in tissue samples by HPLC [5,15-17,20-22,34]. Here we analysed OTC and three of its impurities in pig plasma samples. SPE was selected for its advantages over other clean-up methods and tested on one type of C_{18} cartridge (Sep Pak C_{18}) as well as other types of cartridges which can retain these compounds. LiChrolut EN and Oasis HLB cartridges were selected for the polar characteristics and bulk of the compounds. LiChrolut EN cartridges contain a polymer sorbent material based on ethylvinylbenzene-copolymer with a particle size distribution 40 – 120 μm, while Oasis HLB contain a macroporous copolymer [poly(divinylbenzene-co-N-vinylpyrrolidone)] as a sorbent that exhibits both hydrophilic and lipophilic retention characteristics.

We have determined which kind of cartridge produced highest recoveries, using standard solutions of 10 mg L^{-1} OTC, TC, ETC and EOTC dissolved in Milli-Q water and at different pH: 3, 5 and 8. The SPE procedure was the same for all three cartridges: 5 mL MeOH and 5 mL Milli-Q water to activate the cartridge. 2 mL sample was then passed through the cartridge, which was then washed with 2 mL Milli-Q water. Compounds were eluted with 2 mL MeOH-ACN (50:50, v/v). Recoveries (% R) and relative standard deviations (% R.S.D.) of the areas were calculated for ten repeated injections of standard solutions of 10 mg L^{-1} and are shown in Table 2. Recoveries were highest with OASIS cartridges. Table 2

shows that recoveries decrease when the pH increases with Sep Pak C_{18} and LiChrolut En cartridges.

Table 2.

Comparison of recovery (% R) and repeatability (% R.S.D.) from analysis of standard solutions (10 mg L^{-1} of OTC, TC, ETC and EOTC) after carrying out SPE procedure with different cartridges at different pH of sample.

		OASIS		SepPak		LiChr.	
pH	**Comp**	**%R**	**R.S.D. (%)**	**%R**	**R.S.D. (%)**	**%R**	**R.S.D. (%)**
3.0	ETC	108.2	11.2	78.2	4.9	94.5	12.9
	EOTC	102.5	2.7	78.7	5.1	78.7	10.1
	TC	101.4	4.5	63.6	2.0	96.3	12.0
	OTC	108.5	6.2	62.3	4.3	110.4	10.9
5.0	ETC	104.0	15.1	36.1	6.7	52.4	11.6
	EOTC	87.1	13.7	30.9	12.9	49.2	5.6
	TC	91.9	1.9	41.6	4.3	67.4	1.9
	OTC	94.3	2.8	62.3	4.3	56.6	9.4
8.0	ETC	103.1	2.4	24.1	10.1	50.7	7.5
	EOTC	92.7	4.3	22.5	8.8	33.7	11.8
	TC	99.5	1.8	31.5	5.7	55.4	9.6
	OTC	92.5	2.9	41.5	4.5	49.1	10.9

On C_{18} sorbents the main retention mechanisms are the hydrophobic interactions with hydrophobic alkyl groups, while with polymeric sorbents they are the π-π binding interactions. The fact that recoveries are lower when the pH increases could be because hydroxyl groups are more unstable and reduce the affinity between hydroxyl groups with these sorbents.

On the other hand, Oasis cartridges contain a macroporous copolymer as sorbent that confers both hydrophilic and liphophilic retention characteristics which could mean that recoveries do not change much when the pH changes. Moreover, the lower recoveries obtained with C_{18} sorbents are due to their

residual silanols which could lead to their significant adsorption of TC drugs [22,41]. Other cartridges (LiChrolut EN and OASIS) do not have this problem because they have not silanol groups [42]. For these reasons, recoveries were highest when OASIS cartridges were used.

OASIS cartridges were then used in a clean up procedure with pig plasma samples, at pH 8 and as, at this pH and with this kind of cartridge recoveries were high, we determined OTC and its impurities in these conditions. Recoveries were high, around 90 – 100 %, except for EOTC which had a lower recovery (74 %), possibly due to its structural characteristics. The recoveries and R.S.D. are shown in Table 3.

Table 3

Recovery (% R) and repeatability (% R.S.D.) from analysis of spiked pig plasma samples (10 mg L^{-1} of OTC, TC, ETC and EOTC) after carrying out the SPE procedure with OASIS cartridges.

Compounds	% R	% R.S.D.
ETC	99	7
EOTC	74	5
TC	93	9
OTC	94	13

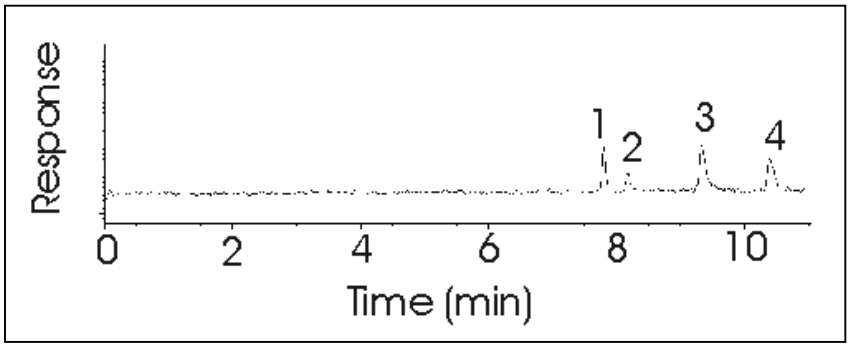

Fig. 5. Electropherogram of spiked plasma sample (10 mg L^{-1} of OTC, TC, ETC and EOTC added to the original sample of plasma) after carrying out the SPE procedure. For other conditions see Figure 2.

CONCLUSIONS

This paper reports the analysis of OTC and three of its impurities (ETC, EOTC and TC) in pig plasma samples by NACE method and OASIS HLB cartridges as a clean-up step. The method achieved a satisfactory separation of TCs and the SPE clean-up in plasma samples produced good recoveries. NACE therefore has great potential as an alternative tool for analysing oxytetracycline in plasma samples. Other biological matrices will studied in the future.

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III.6. Ús de CE en medi no aquós per a determinar un conjunt de quinolones en mostres de ronyó de porc

En aquest estudi s'ha desenvolupat una altra aplicació de NACE, la determinació de diferents quinolones en ronyó de porc.

S'analitzen 7 quinolones: ciprofloxacina, enrofloxacina, danofloxacina, difloxacina, marbofloxacina, flumequina i àcid oxolínic. Totes elles es caracteritzen pel seu ampli ús en veterinària i perque la seva concentració màxima en teixits animals destinats al consum humà (MRLs) està regulada per la normativa europea. Els MRL per aquests compostos en ronyó de porc es mostren a la Taula 1.

Taula 1.
Valors de MRL de diferents quinolones en ronyó de porc.

Substància farmacològicament activa	**Residu**	**MRL ($\mu g \cdot Kg^{-1}$)**
Enrofloxacina	Enrofloxacina+ciprofloxacina	300
Marbofloxacina	Marbofloxacina	150
Flumequina	Flumequina	1500
Difloxacina	Difloxacina	800
Àcid Oxolínic	Àcid Oxolínic	150
Danofloxacina	Danofloxacina	200

Tal com s'ha demostrat en el treball anterior el NACE és un sistema que permet millorar la selectivitat en la separació de diferents compostos degut a l'àmplia varietat de propietats físiques i químiques que presenten els diferents solvents orgànics.

S'ha escollit com a electròlit acetat amònic 20 mM dissolt en MeOH/ACN. Tal com s'ha estudiat en el treball anterior hi ha algunes quinolones que només tenen un grup ionitzable, un grup carboxílic, com són en aquest cas la flumequina i l'àcid oxolínic, mentre que d'altres en presenten dos, un grup

carboxílic i un grup piperazinil, com són la resta de quinolones d'aquest estudi. En aquest treball s'ha optimitzat el pH* del tampó de separació, així com la proporció de MeOH/ACN més adequada per dissoldre el tampó.

En aquest treball s'ha estudiat quin és el pH* òptim de treball. Per les quinolones, el pKa del grup carboxílic és entre 5.5 i 6.6, i el del grup piperazinil és de 7.5 i 8.6 [1]. En medi orgànic aquests valors poden variar una mica. És per això que es va fer un estudi previ per veure en quines condicions s'aconsegueix una millor separació. S'ha estudiat un interval de pH* entre 5 – 11, aconseguint separar tots els compostos a un pH* de 5.4 i 10. El pH* òptim de la separació és pH* 10.

Posteriorment es va estudiar el medi orgànic en què es dissol la solució tampó. Després d'estudiar diverses mescles de MeOH/ACN, la millor separació s'obté treballant amb una proporció de 50:50, v/v. Aquests dos solvents són els més utilitzats en NACE, perquè d'altres solvents com N-metilformamida, N,N-dimetilformamida, dimetilsulfòxid, etc... es caracteritzen per la seva absorció en la zona ultraviolada-visible [2], el que faria disminuir la sensibilitat del mètode.

En les condicions esmentades la separació no és completa, pel que és necessària l'addició d'algun modificador en el tampó, com acetat sòdic i àcid acètic per tal de modificar una mica les característiques àcid-base del medi [3]. Malgrat això, no s'aconsegueix millorar la separació.

Per tal de millorar la separació dels compostos el que es fa és afegir un surfactant, el policatió de bromur d'hexadimetrina (HDB), en el tampó de separació. Aquest producte inverteix la direcció del flux electroosmòtic. Quan no s'afegeix aquest HDB, a l'aplicar voltatge el flux electroosmòtic es desplaça cap al càtode i els analits, degut al pH* del tampó, estan carregats negativament i tenen tendència a desplaçar-se cap a l'ànode degut al flux electroforètic. En aquest cas com el flux electroosmòtic és més gran que l'electroforètic tot el

sistema tendeix a desplaçar-se cap al càtode. Quan s'afegeix HDB en el medi s'inverteix el sentit del flux electroosmòtic. Per tant, a l'invertir el voltatge (s'aplica voltatge negatiu), el flux electroosmòtic i l'electroforètic es desplacen cap al mateix sentit, cap a l'ànode. En aquestes condicions la separació no és completa, però els pics presenten una eficàcia més gran.

A l'addicionar un 4 % d'àcid acètic en el tampó s'aconsegueix una gran millora en la separació i només hi ha dos compostos que coelueixen, els dos que no contenen el grup piperazinil en la seva estructura, la flumequina i l'àcid oxolínic. Això és degut a que amb l'addició de l'àcid acètic el pH* del tampó passa a ser de 5.4. En aquestes condicions és d'esperar que totes les quinolones estiguin carregades positivament excepte aquestes dues que estaran neutres, sense ésser possible la seva separació en aquest sistema.

En aquestes condicions, i sense fer cap tipus de sistema de preconcentració, els límits de detecció per tots els compostos és de 120 $\mu g \cdot l^{-1}$, per tant inferior als valors de MRL fixats per la normativa europea. Així doncs es tracta d'un sistema molt sensible i aplicable a l'anàlisi de residus.

L'aplicabilitat del mètode es mostra analitzant aquests compostos en ronyó de porc. Com en l'estudi de tetraciclines en diferents teixits de porc, primer s'ha d'homogeneïtzar la mostra per tal d'extreure aquests compostos de la matriu sòlida i després s'utilitza un sistema de SPE per tal d'eliminar les interferències de la matriu.
Per homogeneïtzar la mostra s'utilitza àcid clorhídric 0.1 N. Un cop extretes les quinolones del teixit es realitza un clean-up a través d'un sistema de SPE. En aquest cas s'utilitza un sorbent C_{18} com en el treball anterior, en el qual s'analitzaven diferents quinolones en plasma de porc. En aquest cas les recuperacions han estat de l'ordre del 80 %.

Amb aquest sistema de clean-up s'aconsegueix eliminar la gran majoria de components de la matriu, eliminant la gran majoria d'interferències del sistema i s'aconsegueix un mètode adequat per a la determinació d'aquests compostos.

En aquest treball es demostra l'efectivitat de NACE per a la separació de diferents quinolones i a més es demostra la seva aplicabilitat en l'anàlisi d'aquestes en mostres biològiques, concretament en ronyó de porc.

Els estudis que han estat realitzats en aquest treball han estat publicats a la revista Electrophoresis, 23 (2002) 506-511, una còpia dels quals es mostra a continuació.

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USING NONAQUEOUS CAPILLARY ELECTROPHORESIS TO ANALYZE SEVERAL QUINOLONES IN PIG KIDNEY SAMPLES

ABSTRACT

This paper shows the potential of nonaqueous capillary electrophoresis (NACE) for analyzing enrofloxacin (ENR), ciprofloxacin (CPR), danofloxacin (DAN), difloxacin (DIF), marbofloxacin (MAR), flumequine (FLU) and oxolinic acid (OXA) in pig kidney samples. Organic solvents have widely variety of physical and chemical properties, so it has possible to improve separation selectivity with respect aqueous medium. In this paper we have studied the effects of parameters such as the composition of the organic media, the choice of electrolyte, the pH* of the background electrolyte (BGE), the addition of modifiers and the reversal of electroosmotic flow. Separation was good with 20 mM ammonium acetate, 0.004 % polycation hexadimethrine bromide (HDB) and 4 % acetic acid (pH* 5.4) in MeOH/ACN (50:50,v/v) medium. We used a quick and simple sample-preparation method, hydrochloric acid as an extractant and solid-phase extraction (SPE) with Baker C_{18} cartridges as the clean-up step. Recoveries for all quinolones were over 80 %.

keywords: Nonaqueous capillary electrophoresis; Quinolones; Tissue samples; Solid-phase extraction

INTRODUCTION

Quinolones (Qs) are a group of synthetic antimicrobial agents that are widely used in human and veterinary medicine. They are derived from nalidixic acid, which is a naphthyridine derivative used to treat urinary-tract infections [1]. Their common skeleton is termed 4-oxo-1,4-dihydroquinoline. Therefore, they have a carboxylic acid group in position “4” and are often referred to as 4-quinolones. This group makes these compounds active against many gram-positive and gram-negative bacteria. Their antibacterial action is greatly increased by adding 6-fluoro and 7-piperazinyl groups to their structure [2].

Qs are extensively used to treat and prevent veterinary diseases in food-producing animals. This is potentially hazardous because their residues may remain in edible animal tissues [3]. Governments in many countries have set up programs for monitor antibiotics in food. In most European countries the legislation and use of veterinary drugs is regulated on a national and European Union level (EU). The EU included a number of quinolones in Council Regulation (EC) No. 2377/90 [4], which establishes the maximum residue limits (MRLs) of veterinary products in foodstuffs of animal origin [5,6]. Analytical methods are therefore needed to monitor the levels of drug residues in animal products.

In the literature the most common methods for analyzing these agents are based on liquid chromatography (LC). These studies have analyzed Qs in standard solutions [7] as well as biological samples [2,8-14]. However, CE has proved to be a good alternative to LC for its characteristics. This technique combines high resolution and easy automation with modest sample requirements and low solvent consumption. CE is a highly efficient way of separating a wide variety of ionic species because it is highly selective and efficient, and its analysis time is short [15,16]. Some studies have determined quinolones by capillary electrophoresis (CE) [1,2,15-20]. The analysis of Qs by CE is usually studied to separate and determine them in standard solutions [17] or in pharmaceutical

formulations [1]. Few studies have determined Qs in biological samples [2,19,20].

Generally, in CE, the background electrolyte (BGE) is dissolved in aqueous medium. This is because the separation mechanism is based on the difference in the migration rate of charged species in an electrical field. Water has a well-known acid-based chemistry, and moreover it is compatible with several detection schemes and can dissolve numerous BGE. For many years, therefore, organic solvents have been an alternative to aqueous media in conventional electrophoresis and have often been used as modifiers in CE. However, their potential as background electrolyte solutions in CE has only recently begun to attract close attention. The low currents in nonaqueous capillary electrophoresis mean that higher electrolyte salt concentrations and electric field strengths can be used, and also that the sample load can be scaled-up by using capillaries with a wider inside diameter.

One of the most attractive features of organic solvents is that their physical and chemical properties are very different, both from each other and from water. Accordingly, the separation selectivity is improved if we use neat organic solvents, and this selectivity can easily be altered if we change the nature of the organic solvent or use mixtures of organic solvents. Thus we have achieved separations that are impossible in aqueous mediums. Most organic solvents are capable of dissolving electrolytes at least to some extent, and acids and their ammonium salts are the most often used electrolytes. Electrolyte cation or anion also has a clear effect on the capillary electrophoretic selectivity in nonaqueous media [21].

To choose an organic solvent or solvent mixture we take into account parameters such as viscosity, the dielectric constant, the solubility of the electrolytes and UV transparency. The parameters with the greatest effects are those which optimize the separation in CE (e.g. analysis time, selectivity, efficiency and detectability) [15,21,22].

Some organic solvents, like methanol or acetonitrile, are highly volatile. This could be a problem if the BGE vials or sample vials are not well sealed, because the loss of solvent through evaporation may change the concentrations. This could gradually increase ionic strength and produce results with poor reproducibility. The advantage of their volatility, however, is that they can be used in CE-MS experiments [15,22].

Another advantage of only nonaqueous media for CE is that they make sample preparation easier because extracts obtained with organic solvents can be injected directly into the nonaqueous system. For example, the eluate from the solid-phase extraction cartridge, when MeOH or ACN is the eluent, may be used for CE without further treatment.

Barbosa et al. [17,18] determined several quinolones with CE by adding organic solvents to water. This affected their susceptibility to electrophoretic migration. However, to maximize the advantages of organic solvents, we suggest the use of an organic cosolvent to prepare a totally organic medium. In this paper we establish a simple and easy method for determining seven quinolones – enrofloxacin (ENR), ciprofloxacin (CPR), danofloxacin (DAN), difloxacin (DIF), marbofloxacin (MAR), flumequine (FLU) and oxolinic acid (OXA) – by nonaqueous capillary electrophoresis (NACE). We studied several parameters such as the choice and composition of the electrolyte, the composition of the organic medium and the pH* of the background electrolyte to find a suitable separation with a short analysis time.

This method is applied for determining Qs in pig kidney samples. We have developed a rapid and specific sample preparation using hydrochloric acid as an extractant and SPE with C_{18} cartridges to purify the sample.

EXPERIMENTAL

Instrumentation

Electrophoretic experiments were performed using a Hewlett Packard 3D CE (Waldbronn, Germany), with a DAD detector (Diode Array Detection). Data were collected with the software provided with the HP 3D Chemstation (Hewlett-Packard), which was operated under Windows NT (Microsoft). The capillary was fused-silica (64.5 cm $\times$ 75 μm i.d.) supplied by Beckman Coulter, Inc. (Fullerton, U.S.A.). A detection window was prepared by burning off the polyamide coating 56 cm from the capillary inlet.

Reagents and standards

Marbofloxacin (MAR) was kindly supplied by Vétoquinol (Lure, France), ciprofloxacin (CPR) and enrofloxacin (ENR) by Cenavisa (Reus, Spain), danofloxacin (DAN) by Pfizer (Sandwich, NJ, USA), flumequine (FLU) and oxolinic acid (OXA) were purchased from Sigma (Sant Louis, USA), and difloxacin from Abbott (North Chicago, IL, USA). Standard stock solutions of 1000 mg/L were prepared in NaOH (Prolabo, Bois, France) 0.1 N and stored under refrigeration at 4 ºC. Standard working solutions were prepared weekly or daily, depending on their concentration, by diluting the standard stock solution with methanol/acetonitrile (MeOH/ACN) (50:50,v/v).

Methanol (MeOH) (SDS, Peypin, France), water (water purified by a Milli-Q system) (Millipore, Bedford, USA) and hydrochloric acid (HCl) (Prolabo, Bois, France), Milli-Q were used to pretreat the sample.

Ammonium acetate (Sigma, Sant Louis, USA), sodium acetate (Aldrich Chemical Co. Inc., USA) and hexadimethrine bromide (HDB) (Sigma, Sant

Louis, USA) were used to prepare the electrophoretic solutions. Acetic acid (Prolabo, Bois, France) was used to acidify the running buffer. Sodium hydroxide was used to obtain a basic medium. Acetonitrile (ACN) (SDS, Peypin, France) and MeOH were used to dissolve the electrolyte. Methanol (MeOH) (SDS, Peypin, France) was used during solid-phase extraction.

Capillary Zone Electrophoresis Conditions

Background electrolyte (BGE) was 20 mM ammonium acetate, 0.004 % (w/v) HDB and 4 % (v/v) acetic acid with a mixture of MeOH/ACN (50:50,v/v) as a solvent. Before use, the capillary was rinsed with 1 M NaOH (dissolved in aqueous medium) (Probus SA, Barcelona) (1000 mbar pressurised flow) for 15 min, then with H_2O Milli-Q for 15 min, MeOH for 15 min. Finally, it was flushed with running buffer for 15 min. Before each analysis, the capillary was flushed with the running buffer for 3 min. The detector was set at 290 nm. Injection was performed hydrodynamically at a pressure of 50 mbar for 3 seconds of sample. Running buffer was then introduced to the capillary at 50 mbar for 6 seconds to diminish the dispersion of the sample during the analysis. The capillary temperature was 25 ºC and the separation voltage was 30 kV.

Extraction and clean-up

A sample (0.5 g) fortified with Qs was placed in a centrifuge tube and 5 mL HCl 0.1 N was added. The mixture was homogenized for 1 min using an Ultra-Turrax T-18 (Jankle & Kunkel, IKA-Labortechnik, Staufen, Germany) and then centrifuged at 12000 rpm for 15 min. The pellet was then blended and centrifuged one more time 5 mL HCl 0.1 N. The supernatants obtained were jointly centrifuged for 15 min and then filtered. The filtrate was placed on a solid-phase extraction (SPE) cartridge.

Baker C_{18} cartridges (500 mg) were used to develop SPE. The cartridge was conditioned with 5 mL of methanol followed by 5 mL Milli-Q water at a flow rate of 1-2 mL/min using a water aspirator as a vacuum source connected to the cartridge pack. The supernatant previously obtained was passed through the cartridge. The cartridge was washed with 1 mL Milli-Q water and the compounds were eluted from the cartridge with a 2 mL aliquot of MeOH (2 % HCl). The collected eluate was evaporated to dryness at 40 ºC under a stream of nitrogen, the residue was redissolved in 1 mL of MeOH, and an aliquot was analyzed by capillary electrophoresis.

RESULTS AND DISCUSSION

Nonaqueous Capillary Electrophoresis

This paper describes the determination of several quinolones – ENR, CPR, DAN, DIF, MAR, FLU and OXA – in pig tissue samples by nonaqueous capillary electrophoresis (NACE). This is a CE mode in which BGE as well as sample solutions are dissolved in organic solvents which are characterized because their physical and chemical properties differ widely, both from each other and from water. Thus, selectivity manipulation in nonaqueous CE can be achieved simply by changing the organic solvent or varying the proportions of two solvents [21-23].

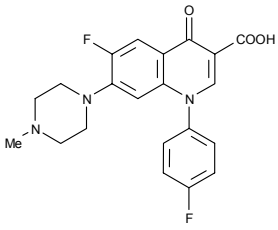
Important parameters to consider are the acid-base properties of the solutes, which are expressed by their pKa values. Two of the compounds studied (FLU and OXA) have only one relevant ionizable functional group (carboxylic group), while the others have two ionizable groups (the ammonium and carboxylic groups). Fig. 1 shows their structures. In aqueous medium the pKa values of these compounds range from 5.5 to 6.6 for the carboxylic group (pK_1) and from 7.5 to 8.6 for the ammonium group (pK_2). These values may change by many

orders of magnitude according to the differentiating effect of many organic solvents [22]. This means that the degree of dissociation depends on the protolytic behavior in the different organic solvents leading to variations of the degree of ionization in different organic solvents.

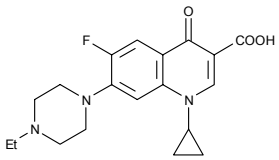
In nonaqueous systems, the pH is generally defined as apparent pH or pH*. The pH is only defined in aqueous systems and has no direct significance in nonaqueous systems. Although serious problems may arise, the pH is often measured directly in the organic medium with the glass electrode using aqueous calibration buffers and neglecting liquid junction potentials [24].

Choice of electrolyte and organic solvent

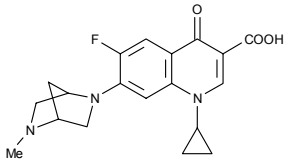
The most polar solvents like MeOH, formamide, N-methylformamide (NMF), N,N-dimethylformamide (DMF) and dimethylsulfoxide (DMSO) posses a good solvating power towards the electrolytes commonly used in NACE, but NMF, DMF and DMSO would have reduced sensitivity because they have high background UV absorbance at a shorter wavelength [25,26].



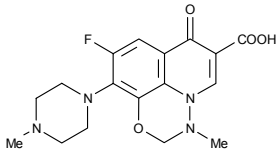
1. Difloxacin (DIF)



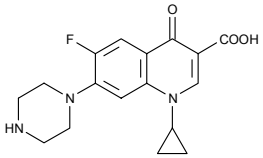
2. Enrofloxacin (ENR)



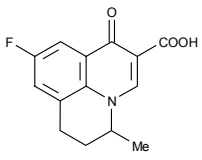
3. Danofloxacin (DAN)



4. Marbofloxacin (MAR)



5. Ciprofloxacin (CPR)



6. Flumequine (FLU)

Fig. 1. Structures of the seven quinolones studied.

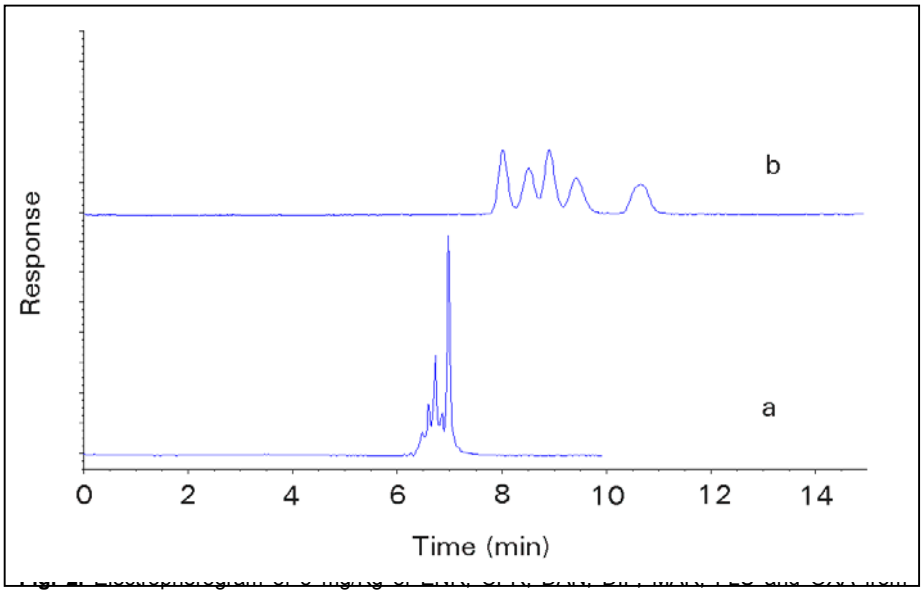
For these reasons we chose a mixture of MeOH/ACN as a medium. Ammonium acetate is the most common electrolyte and this is the one we chose.

As we saw earlier, Barbosa et al. [17,18,27] described how these compounds behave in organic solvent/water systems, but there are no studies of how quinolones behave in a completely nonaqueous medium.

We began our study by using 20 mM ammonium acetate as BGE, dissolving this and standard solutions in MeOH/ACN (50:50,v/v). First, we tested several pH* between 5 and 11. We adjusted acidic medium using 0.1 M acetic acid (HAc) and basic medium using 0.1 M NaOH. At pH* above 9, peaks were well-defined but not completely separated. At pH* between 5.5 and 9, the compounds were not separated and the peaks were not well-defined. At pH* below 5.5, there were five peaks but these were broad.

Fig. 2 compares the electropherograms of 5 mg/kg of these Qs from standard solutions analyzed at pH* 10 and pH* 5.4. In both cases, there were five peaks, but at the basic pH*, the peaks were more efficiency. We therefore chose pH* 10 to study how other parameters affected the separation.

We then studied whether we could change the proportions of MeOH and ACN in the nonaqueous system to improve the separation. We tested three mixtures MeOH/ACN, 50:50, 75:25 and 25:75 (v/v), and when we increased the MeOH content of the medium, there were broad peaks and the analysis time increased a little. On the other hand, when we increased the percentage of ACN in the medium, the analysis time dropped slightly and the peaks became more efficiency. However, increasing the percentage of ACN from 50 % to 75 % reduced the resolution. The best separation therefore was obtained with the initially mixture consisting of MeOH/ACN (50:50, v/v).



standard solution. The pH* was adjusted to 10 (a) and 5.4 (b). Background electrolyte: 20 mM ammonium acetate dissolved in MeOH/ACN (50:50,v/v). Injection: 50 mbar for 3 seconds. Capillary temperature: 25ºC. Separation voltage: 30 kV. The detector was set at 290 nm.

Other modifications

To improve separation, some papers recommend adding modifiers like sodium acetate (NaAc) and acetic acid to BGE to adjust the acid-base properties of the electrophoresis medium [22,25]. We tested what happens when several concentrations of sodium acetate (2.5, 5 and 20 mM) are added, and the best results were obtained when 5 mM of NaAc was added, although the improvement in separation was very small. As before, there were only five peaks, and these were not completely separated.

The reversal of EOF direction

As these compounds were not completely separated, we decided to change the operating conditions. Under normal operating conditions, the migratory direction of anions is opposite to the direction of the EOF. We added the polycation hexadimethrine bromide (HDB) to the nonaqueous electrophoresis medium to generate a suitable and stable system with reversed EOF. Then, when the polarity voltage is negative, both the migration direction of the anions and the EOF are towards the detector, which makes the separations rapid and highly efficient [16,22]. To reverse EOF, it is enough to add low concentrations of HDB (0.001 – 0.05 %). We therefore added HDB 0.002 % (w/v) and adjusted pH* to 10 using NaOH 0.1 N. At these conditions, although we observed only three peaks, these were efficient. As we have already stated, adding acetic acid to the BGE can improve the separation. We studied the addition of several percentages between 1 and 5 % (v/v) acetic acid, and when 1 % acetic acid was added to BGE (pH* 6.4), the separation of analytes improved slightly and when 4 % acetic acid was added practically all compounds except FLU and OXA were separated. This was because at these conditions the pH* value was 5.4, and all compounds are expected to be charged positively except FLU and OXA, which are non-ionic since they contain only one ionizable group while the others contain two ionizable groups. Therefore, they cannot be separated at these conditions. The elution order is FLU/OXA, DIF, ENR, DAN, MAR and CPR. To

separate the charged compounds with a good resolution and efficiency we increased the concentration of HDB to 0.004 % and maintained the concentration of acetic acid at 4 %, and Fig. 3 shows and electropherogram obtained in the optimum conditions in this study. If the method has to be applied to determine compound 6 in presence of compound 7 it will be necessary to use a mass spectrometer as detection system.

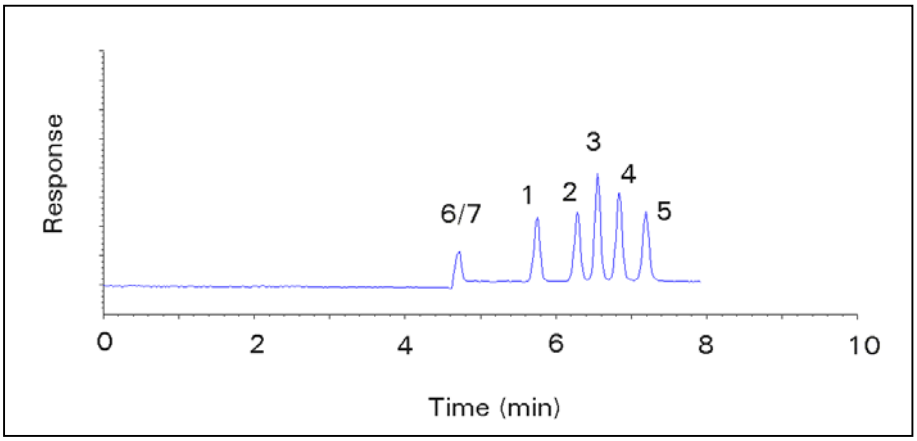


Fig. 3. Electropherogram of 5 mg/Kg standard solution. BGE, 20 mM ammonium acetate, 0.004 % HDB, 4 % acetic acid, pH* 5.4, in MeOH/CAN 50:50 v/v; separation voltage, -30 kV. For peak assignments see Fig. 1. For other conditions see Fig. 2.

Calibration parameters

After optimizing the separation we determined the calibration parameters. Linearity was between 250 and 4000 μg/kg for DIF and ENR, while for DAN, MAR and CPR it was between 250 and 5000 μg/kg. We injected these solutions five times, starting with the least concentrated solution and ending with the most.

The area values obtained were successively analyzed using ULC (Univariate Linear Calibration) software [28] to evaluate the correlation coefficient (r^2), the relative standard deviation (R.S.D.) within solutions and the limit of detection (LOD). The limit of detection (LOD) was calculated according to the method of Widefordner and Long [29] using the ULC program with K equal to 3, and also tested experimentally. For all compounds the LOD were less than 120 μg/kg . The R.S.D. of the areas were between 4.0 % and 11.6 % and the R.S.D. of the migration time were between 3.8 % and 4.2 % for ten repeated injections of standard solutions of 1000 μg/kg.

A fresh buffer was used after each sequence of three injections. These results are shown in Table 1.

Table 1

Calibration data and precision studied with standard solutions

Compounds	Linearity (μg/kg)	Correlation coefficient (r^2)	LOD (μg/kg)	LOQ (μg/kg)	% R.S.D.[a] (area)	% R.S.D.[a] (time)
DIF	250-4000	0.9966	101	202	4.0	3.8
ENR	250-4000	0.9945	119	238	8.4	4.0
DAN	250-5000	0.9989	104	208	11.6	4.0
MAR	250-5000	0.9970	111	222	10.0	4.1
CPR	250-5000	0.9991	57	114	9.5	4.2

[a)] Calculated for ten consecutive runs at 1000 μg kg^{-1}

APPLICATION

In the clinical and biomedical fields, CE is considered an alternative to existing standard techniques such as HPLC, GC, etc. Generally, to analyze biological samples by these techniques, particulate matter that may block the capillary must be removed from the sample. Under certain conditions, it can be injected

directly onto the capillary with no pretreatment. However, very often one form of sample pretreatment is needed: without it, there will often be interference when complex samples are analyzed, and the sensitivity of the detector is often unsuitable without pretreatment [16,31]. An important practical consequence of using NACE separation medium is that the organic phases resulting from the extraction of the analytes from matrix sample or from eluents from solid-phase extraction can be injected directly into the capillary electrophoresis system [22].

The applicability of this method is illustrated by analyzing tissue samples. These types of samples are by far the most complex matrices for drug extraction. In a previous study [30] in our laboratory we developed an analytical procedure for determining these quinolones in pig kidney samples. This involved extracting Qs from the matrix, and using SPE as the clean-up step and HPLC for the separation. With hydrochloric acid they were extracted efficiently and the extract was then passed through Baker C_{18} cartridges to obtain the analytes in a suitable medium for them to be injected into the instrument. With this system, recoveries for all compounds were over 80 %.

Fig. 4a shows the electropherogram of a blank tissue sample and Fig. 4b shows the electropherogram of a spiked plasma sample (2000 μg/Kg of DIF, ENR, DAN, MAR and CPR) after the SPE procedure has been carried out. As we can see, there was no interference at the analysis time for the quinolones we studied. However, the migration times increases slightly when the method is applied in the analysis of kidney samples. It could be due to the complexity of the sample. Therefore, we can use this pretreatment to analyze these compounds in these kinds of samples by NACE.

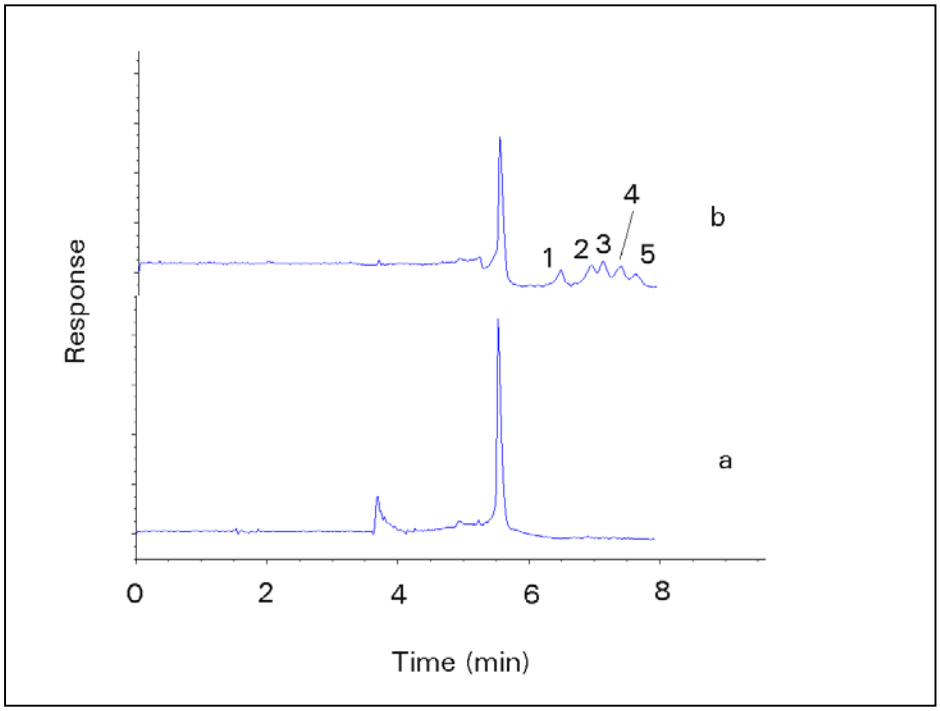


Fig. 4. Electropherogram of (a) a blank pig kidney sample and (b) a pig kidney sample spiked with 2000 μg/Kg of ENR, CPR, DAN, DIF and MAR. For other conditions see Figure 2.

CONCLUSIONS

This paper reports a nonaqueous capillary electrophoresis system for assaying several quinolones in pig kidney samples. This system involves extracting them from the sample matrix in acidic medium and using solid-phase extraction with Baker C_{18} cartridges as the clean-up step. They were analyzed using the NACE system with reversal electroosmotic flow. This provided good selectivity and analysis time was short.

The LODs are similar to those of other methods of analyzing quinolones by aqueous capillary electrophoresis. This is an alternative to HPLC methods

because of its short analysis time. However, it is a little less sensitive than HPLC. We will continue working to increase the sensitivity of the CE method with other preconcentration techniques.

Also, the relatively low current generated in organic solvents, the volatility of the solvents and the use of ammonium acetate as electrolyte seem promising for CE – MS experiments and improve the potential of qualitative analysis in real samples.

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III.7. Determinació de ciprofloxacina, enrofloxacina i flumequina en plasma de porc mitjançant un sistema d'isotacoforesi capil·lar - electroforesi capil·lar

Un dels objectius de la tesi és l'estudi de sistemes que permetin determinar antibiòtics en mostres biològiques, tal com s'ha esmentat en els treballs anteriors. Un altre dels objectius és desenvolupar sistemes que permetin augmentar la sensibilitat en electroforesi capil·lar de manera que es puguin determinar antibiòtics a baixes concentracions.

En aquest treball s'ha desenvolupat un sistema que permet determinar tres quinolones, la flumequina, l'enrofloxacina i la ciprofloxacina, en plasma de porc per CE. La flumequina i enrofloxacina han estat àmpliament utilitzades en veterinària, i la ciprofloxacina és el principal producte de degradació de l'enrofloxacina, pel que resulta necessària la seva determinació en teixits animals. Els valors de MRL per aquests compostos en teixits de porc es mostren a la Taula 1.

Taula 1.

Valors de MRL de la enrofloxacina i flumequina en diferents teixits de porc.

Substància farmacològicament activa	Residu	MRL ($\mu g \cdot Kg^{-1}$)	Teixit diana
Enrofloxacina	Enrofloxacina + ciprofloxacina	100 100 200 300	Múscul Pell + greix Fetge Ronyó
Flumequina	Flumequina	200 300 500 1500	Múscul Pell + greix Fetge Ronyó

La presència d'aquests antibiòtics en plasma no està regulada.

Primer de tot es desenvolupa el sistema per dur a terme la separació d'aquests compostos per CZE. Com a tampó s'utilitza fosfat sòdic 10 mM i clorur sòdic 5 mM a pH 9.0. El clorur sòdic s'afegeix per tal de millorar l'eficàcia i la resolució

dels pics. En aquestes condicions els tres compostos estan completament separats [1].

Posteriorment s'estudia un sistema per tal d'augmentar la sensibilitat del mètode. El sistema escollit es coneix com a isotacoforesi capil·lar (ITP) i permet preconcentrar la mostra *on-column* és a dir, dins el mateix capil·lar [2,3]. Es tracta d'un sistema electrolític discontinu ja que s'utilitzen dos electròlits diferents, el *leading* (L) i el *terminating* (T). Els ions del *leading* tenen elevada mobilitat, i els del *terminating* tenen baixa mobilitat, de manera que els ions de la mostra tenen una mobilitat intermitja, és a dir entre els dos electròlits. En aquest sistema s'escull com a *leading* el tampó fosfat sòdic 10 mM, clorur sòdic 5 mM a pH 9.0 (tampó que també s'utilitzarà posteriorment per du a terme la separació electroforètica). Com a *terminating* s'utilitza β-alanina 10 mM a pH 9.0.

ITP és un sistema que permet la injecció de grans volums de mostra, la qual posteriorment és preconcentrada en l'interior del capil·lar en zones isotacoforètiqques, tal com s'ha descrit en el segon capítol, per tal d'augmentar la sensibilitat del mètode. En ITP primer té lloc la injecció de la mostra. Un cop la mostra és injectada es posa en l'extrem d'entrada del capil·lar un vial que contingui *terminating* i en el de sortida un vial que contingui *leading* i llavors s'aplica simultàniament pressió positiva i voltatge negatiu. La pressió positiva s'utilitza per impedir la pèrdua dels analits per l'extrem d'entrada del capil·lar, mentre que el voltatge negatiu s'utilitza per preconcentrar la mostra dins el capil·lar. Un cop la mostra s'ha preconcentrat es canvia el vial d'entrada per un vial que contingui també *leading* i s'aplica únicament voltatge negatiu per tal de desplaçar la mostra a l'inici del capil·lar, i eliminar així tant la matriu de la mostra com *terminating* que pugui quedar present en l'interior del capil·lar.
Ha estat necessari optimitzar varis paràmetres en el procés de ITP, com són el volum injectat, el temps de durada de la preconcentració, i el temps que s'aplica el voltatge negatiu, així com la longitud i el diàmetre intern del capil·lar. Les

condicions òptimes han estat la injecció de mostra d'un 30 % (del total del capil·lar), la durada de la preconcentració ha estat de 1 minut aplicant una pressió de 50 mbar i 10 kV de voltatge negatiu, la matriu de la mostra s'ha eliminat aplicant un voltatge negatiu de 10 kV durant 1 minut, i les dimensions del capil·lar han estat 90 cm de longitud i 100 μm de diàmetre intern.

Amb aquest sistema, ITP-CZE, els límits de detecció han estat de 50 $\mu g \cdot l^{-1}$ per la ciprofloxacina i flumequina i de 10 $\mu g \cdot l^{-1}$ per l'enrofloxacina, i s'ha aconseguit augmentar la sensibilitat del mètode unes 40 vegades en relació a la injecció hidrodinàmica convencional.

Posteriorment aquest sistema s'ha aplicat a la determinació d'aquests compostos en plasma. Com en els estudis anteriors, s'utilitza la SPE com a sistema de pretractament. En aquest cas, s'estudia la possibilitat d'utilitzar els sorbents Oasis HLB. Aquest sorbent ja l'hem utilitzat tant en el segon com en el cinquè treball per a la determinació de tetraciclines en diferents matrius biològiques on s'aconsegueixen majors recuperacions que utilitzant sorbents C_{18}. Això és degut principalment a que aquests sorbents tenen una major capacitat de càrrega (és a dir, major volum de ruptura) que els sorbents C_{18}, a més de no haver-hi la possibilitat de que el sorbent s'assequi i que els analits no puguin quedar retinguts en el sorbent, o de que hi hagin problemes de repetitivitat del procediment.
En aquest treball s'utilitza un patró intern per a tenir una acurada quantificació. El compost que es tria com a patró intern és una altra quinolona, la lomefloxacina, per tant és d'esperar que tingui un comportament similar a les quinolones objecte d'estudi. En aquestes condicions quan es du a terme l'extracció en fase sòlida les recuperacions són pròximes al 100 %. Per tant és un sistema adequat per du a terme el pretractament de la mostra.

En aquest sistema s'han aconseguit LOD de 70, 85 i 50 $\mu g \cdot l^{-1}$ per la ciprofloxacina, enrofloxacina i flumequina, respectivament. Per tant aquest

sistema es caracteritza tant per la seva simplicitat com sensibilitat. És una bona alternativa als mètodes de cromatografia de líquids en l'anàlisi de quinolones en mostres biològiques, i determinació de residus en teixits animals a nivell de MRL.

Els resultats obtinguts en aquest estudi poden trobar-se a continuació, ja que s'adjunta una còpia del treball publicat a la revista Journal of chromatography B, 772 (2002) 163-172.

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DETERMINATION OF CIPROFLOXACIN, ENROFLOXACIN AND FLUMEQUINE IN PIG PLASMA SAMPLES BY CAPILLARY ISOTACHOPHORESIS – ZONE ELECTROPHORESIS

ABSTRACT

Quinolones are a group of synthetic antibiotics that are widely used in veterinary medicine. Their residues may remain in tissues, milk, etc. intended for human consumption. The European Union fixes the maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin. Analytical methods are therefore needed to determine them in biological samples. In this study, we describe capillary isotachophoresis – zone electrophoresis (ITP – CZE) to analyze three quinolones, enrofloxacin (ENR), ciprofloxacin (CPR) and flumequine (FLU), in pig plasma samples. We used solid-phase extraction with Oasis HLB cartridges as a sample pretreatment clean-up step. Capillary zone electrophoresis (CZE) requires low amounts of sample and is not as sensitive as one would wish. ITP – CZE is an easy way to increase the sample loadibility and sensitivity. With this system sensitivity increases 40-fold. The detection limits for CPR, ENR and FLU were 70, 85 and 50 $\mu g\ l^{-1}$, respectively, which were lower than their MRLs in different kinds of samples. This method is simple and sensitive, and is therefore an alternative tool to the existing HPLC methods for analyzing the residuals of these quinolones in biological samples.

keywords: Capillary isotachophoresis – Capillary zone electrophoresis; Quinolones; Plasma Samples; Solid-phase Extraction

INTRODUCTION

Quinolones (Qs) are an important group of synthetic antibiotics with bactericidal action. They are derived from nalidixic acid, a naphthyridine derivative introduced for clinical applications in the livestock and farming industries usually to treat urinary, pulmonary and digestive infections [1]. Their bactericidal activity involves the selective inhibition of DNA gyrase [2].

Quinolones, however, are not limited to clinical applications. They are also widely used to treat and prevent veterinary diseases in animals intended for human consumption and commercially farmed fish such as salmon and catfish. Several agents have been specifically developed for veterinary medicine. For example, danofloxacin, enrofloxacin and sarafloxacin are used to treat respiratory and enteric bacterial infections in cattle, swine, chicken and turkey, and diseases in aqua-cultured fish [2,3]. The widespread administration of these drugs in veterinary medicine represents a potential risk because there may be residues of the drugs in edible tissues. Antibiotic residues in food are a cause for concern because resistant human pathogens are emerging and because there may be allergic hypersensitivity reactions in humans [4]. Restrictive measures and regulatory levels for quinolones are not widely established in veterinary infection despite calls for them by regulatory agencies. European Union, on the recommendation of its Committee for Veterinary Medicinal Products, included a number of quinolones (enrofloxacin, ciprofloxacin, sarafloxacin, difloxacin, marbofloxacin, danofloxacin, oxolinic acid and flumequine) in Council Regulation (EC) No. 2377/90 [5], which establishes the maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin [3,6]. These limits demand analytical methods that can determine the presence of quinolone residues in biological samples and commercial products. The MRLs values are in the range 100-300 □g/kg for the sum of enrofloxacin and ciprofloxacin and in the range 200-1500 □g/kg for flumequine in pig tissue samples. For plasma samples MRLs are not fixed.

Current methods of analyzing Qs in biological samples are based on liquid chromatography (LC) [7,8]; few studies have determined quinolones by capillary electrophoresis (CE). CE is a good alternative to LC in drug analysis [9-15]. It combines high resolution and easy automation with modest sample requirements and low solvent consumption [16,17]. CE has a very good sensitivity based on mass detection. This is important when the size of the sample is very limited, as when analyzing a single cell. Thus a minute amount of sample is sufficient. However, CE is not sufficiently sensitive when based on concentration especially compared to high-performance liquid chromatography (HPLC). In most practical applications the latter feature is far more important, especially when routinely analyzing compounds present at low concentrations. To take full advantage of the separation power of CE for trace analysis of biological samples, we considered several methods of improving determination limits. One of these is to increase detection sensitivity. Several highly sensitive detection systems, such as a laser-induced fluorescence detection and electrochemical detection, have been reported [18,19].

Another method is to increase the sample loadability of the system. Electrophoretic analyte–focusing techniques are an elegant way of increasing loadability in CZE. These techniques, such as the coupling of isotachophoresis (ITP) with CZE and field – amplified injection procedures [20,21], are based on applying local differences in electrical field strength during the injection or focusing step to enable the analyte ions to stack [21-25]. ITP uses a discontinuous electrolyte system made up of two different electrolytes: the leading electrolyte and the terminating electrolyte. The leading electrolyte contains ions with high mobility whereas the terminating electrolyte contains ions with low mobility. The sample ions therefore have an intermediate mobility, so in the capillary there are stacked isotachophoretic zones with sharp boundaries. During separation, the concentration of analytes in the sample changes until the sample ions get at equilibrium with respect to the concentration of the leading ions. When equilibrium is reached the leading and sample ions are moved at the

same velocity. Moreover, in this zone, ion mobility is inversely proportional to the electric field [22,38]. This technique can be used with many compounds, ranging from small charged analytes to proteins. The main interest of ITP – CZE system lies in analyzing samples that are not only dissolved in water but that have some conducting ions, as in biological samples [28]. The concentrating and separating power of ITP means that detection limits (LOD) could be lowered by at least two orders of magnitude [28].

Two techniques have been described for coupling ITP to CZE. The first one uses on-column transient ITP in a single capillary, and the second one couples a second CZE column to the ITP column [21,23-29]. The loadability in the second one is lower than in the first one but the instrumentation is much simpler using a single capillary. Several types of automated ITP – CZE have been developed for anionic and cationic separations in a single capillary using backpressure programming [28,29]. A hydrodynamic pressure was used to remove the terminating electrolyte before the CZE started. The ITP conditions prevented excessive zone broadening.

Because the complexity of biological samples they cannot generally be injected directly to the analytical instrument. A sample pretreatment must be performed to obtain them without interferences and in a suitable medium. The bibliography contains several pretreatment methods to analyze quinolones in biological samples; for example, sample deproteinization [11,12,30], liquid-liquid extraction (LLE) [7,10,22,38] and solid-phase extraction (SPE) [9,20,28,34-37]. Deproteinization, with a mixture of phosphoric acid and phosphate-buffered saline, achieved high recoveries (around 80% in tissue [30] and over 90% in plasma and urine [11,12]), but at these conditions the sample matrix is not suitable for ITP – CZE. LLE, however, obtained low recoveries for ciprofloxacin (CPR), mainly because such multiple extraction steps cause loss for molecules with low recoveries. Moreover, when the extraction step was performed using organic solvents like ethyl acetate or methylenechloride, the recoveries were

very low for Qs with piperazinyl moiety (<40%), like CPR and enrofloxacin (ENR) [37]. On the other hand, SPE was more selective and simpler to operate than LLE when pretreating complex matrices [9,20,28,34-37]. In a previous study we determined several quinolones in pig plasma samples using SPE with C18 cartridges as sample pretreatment and we have obtained high recoveries (90 – 100%) [9]. To our knowledge, apart from our previous study, there have been no other studies of SPE as a pretreatment for determining Qs in biological samples by CZE.

The main purpose of this paper was to find conditions for isotachophoretic preconcentration in a single capillary using a hydrodynamic backpressure programming to enhance the loadibility of the sample and thus achieve lower detection limits for determining three quinolones, ciprofloxacin (CPR), enrofloxacin (ENR) and flumequine (FLU) in pig plasma samples. As far as we know, this system has not yet been used to analyze Qs. Before carrying out the analysis, we had to clean-up the sample by solid-phase extraction with Waters Oasis HLB cartridges.

EXPERIMENTAL

Instrumentation

Electrophoretic experiments were performed using a Hewlett Packard 3D CE (Waldbronn, Germany), with a DAD detector (Diode Array Detection). Data were collected with the software provided with the HP 3D Chemstation (Hewlett-Packard), which was operated under Windows NT (Microsoft). The capillary was fused-silica (64.5 cm $\times$ 75 μm i.d.) supplied by Supelco (Bellefonte, PA, USA). A detection window was prepared by burning off the polyimide coating 56 cm from the capillary inlet.

Reagents and standards

Ciprofloxacin (CPR) and enrofloxacin (ENR) were donated by Cenavisa (Reus, Spain), and lomefloxacin and flumequine were purchased from Sigma (Sant Louis, USA). Standard stock solutions of 1000 mg l^{-1} were prepared in NaOH 0.1 N and stored under refrigeration. Standard working solutions were prepared weekly or daily, depending on their concentration, by diluting the standard stock solution with water which had been purified by a Milli-Q system (Millipore, Bedford, MA, USA).

Disodium hydrogen phosphate (Probus, Barcelona, Spain), sodium chloride (Sigma Chemical Co. Inc., MO, USA) and β-Alanine (Sigma Chemical Co. Inc., MO, USA) were used to prepare the electrophoretic solutions. Sodium hydroxide (Prolabo, Bois, France) and phosphoric acid (Probus, Barcelona, Spain) were used to adjust the pH of the electrolytes. Methanol (MeOH) (SDS, Peypin, France) was used during solid-phase extraction.

Capillary Isotachophoresis – Zone Electrophoresis Conditions

Before use, the capillary was rinsed with 1 M NaOH (Probus SA, Barcelona) (1000 mbar pressurized flow) for 15 min, and then with H_2O Milli-Q for 15 min. Finally it was flushed with running buffer for 10 min. Before each analysis, the capillary was rinsed with the running buffer for 3 min.

During Isotachophoresis, we used two different electrolytes: the leading one was 10 mM sodium monohydrogen phosphate and 5 mM NaCl (pH 9.0, adjusted with diluted phosphoric acid) and the terminating one was 10 mM β-Alanine (pH 9.0, adjusted with 0.1 N NaOH). Sample injection was performed hydrodynamically at a pressure of 50 mbar for 110 s. In the focusing step 10 kV were applied as negative voltage in conjunction with 50 mbar of positive pressure for 1 min. In the fourth step of the isotachophoretic procedure, we applied a negative voltage of 10 kV for 1 min in the analysis of standard solutions and we applied a negative voltage of 10 kV for 3 min in the analysis of pig plasma samples. Electrophoretic separation was developed using the leading electrolyte as the background electrolyte (BGE). The detector was set at 260 nm. The capillary temperature was 25 ºC and the separation voltage was 25 kV.

Electrophoretic conditions

As before, 10 mM sodium monohydrogen phosphate and 5 mM NaCl (pH 9.0, adjusted with diluted phosphoric acid) were used as BGE. Sample injection was performed hydrodynamically at a pressure of 50 mbar for 6 s. Running buffer was then introduced to the capillary at 50 mbar for 6 s to diminish the dispersion of the sample during the analysis. The detector was set at 260 nm. The capillary temperature was 25 ºC and the separation voltage was 25 kV.

Sample pretreatment

Solid-phase Extraction (SPE) was used for pretreatment with Waters Oasis HLB cartridges (60 mg). The cartridge was activated with 5 mL of MeOH followed by 5 mL H_2O Milli-Q at a flow rate of 1 - 2 mL/min using a water aspirator as a vacuum source connected to the cartridge pack. 1 mL of sample was passed through the cartridge. The cartridge was washed with 1 mL H_2O Milli-Q and the compounds were eluted from the cartridge with 1 mL aliquot of MeOH.

Pig plasma samples was used in all experiments.

RESULTS AND DISCUSSION

ITP-CZE system

Capillary electrophoresis (CE) is characterized by the low amount of sample that needs to be injected (between 10 and 100 nl). Higher injections lengthen the injection volume and consequently broaden the band. Therefore, the injection length of the sample can be reduced by stacking procedures, which narrow the sample zone before separation. This makes the CE method more sensitive. In this paper we have used an on-column stacking system prior to capillary electrophoresis separation. The stacking system we used was ITP, which is based on a discontinuous electrolyte system made up of two different electrolytes: the leading electrolyte and the terminating electrolyte. The difference between the electrolytes are their mobilities.

The ITP system involves the following steps:
1) The inlet and outlet vials contain leading electrolyte (L). The capillary is filled with L.
2) The inlet vial is replaced with the sample vial. The sample is injected hydrodynamically.

3) The sample vial is replaced with the terminating electrolyte vial (T) . The focusing step is then started: negative voltage and positive hydrodynamic backpressure are applied. The term “positive backpressure” means that the backpressure induces a flow in the direction of the capillary outlet. “Negative backpressure”, on the other hand, induces a flow in the opposite direction. The negative voltage resulted in an electroosmotic flow in the direction of the capillary inlet, while the positive backpressure opposed this movement to produce the stack of sample analytes. Moreover, because of the characteristics of the ITP zones, the peak did not broaden.
4) The T vial is replaced with the L vial, and then a negative voltage is applied. As a result, the concentrated analyte ions approach the capillary inlet and T is removed before the CZE separation.
5) CZE separation is begun.
A scheme of this system is shown in Fig. 1.

During the ITP step the ions with more mobility than the leading ions and those with less mobility than the terminating ions are removed from the capillary. This considerably cleans the sample [28]. It is therefore important to choose a suitable electrolyte system in which stable isotachophoretic zones are formed and the analytes are completely separated. Several papers have used phosphate electrolyte as background electrolyte (BGE) for analyzing quinolones and obtained a good separation [13-15].

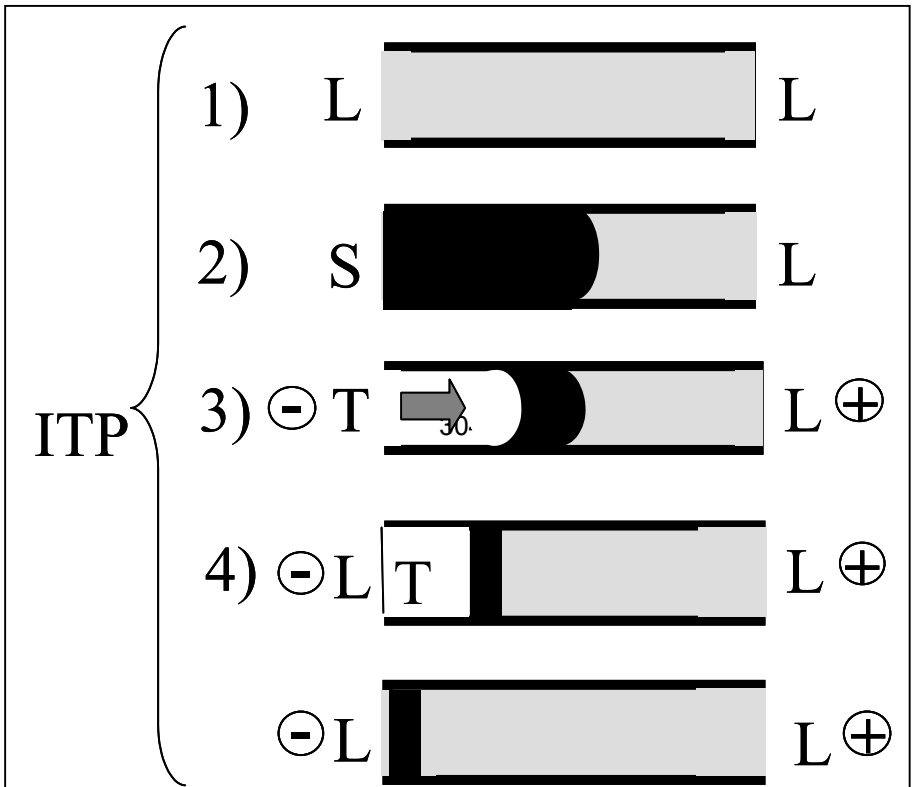


Fig. 1. Scheme of ITP – CZE system. The voltage is indicated by the negative and the positive symbols, which mark the cathode and the anode, respectively. The arrow marks the sense of the pressure applied. Nomenclature: S: Sample, L: Leading electrolyte vial and T: Terminating electrolyte.

Phosphate is a fast ion, faster than quinolone compounds, and is therefore considered a suitable leading ion. We then had to choose a terminating electrolyte (T). β-alanine is often used and is the recommended as terminating electrolyte in anionic ITP [23,28]. It has also less mobility than quinolone compounds. For this reason we chose it as terminating electrolyte.

For capillary electrophoretic separation we can use a terminating electrolyte (T), a leading electrolyte (L) or an electrolyte that is different from T and L as background electrolyte. For several reasons, we chose the L as the background electrolyte in CZE. Firstly, it is better for the sample clean-up because the sample ions with less mobility than the terminating ions – including neutral species and counter ions – are removed in the inlet vial during the focusing step. Secondly, as the conductivity of L is always higher than that of analyte ions, band broadening will be less when L is used as the BGE in the CZE step than when T is used.

Thirdly, with L it is easy to monitor the current for precisely timing when to switch from ITP to CZE because, when it is switched from ITP to CZE, the current will be high. If we had used T as BGE, the current would have been considerably lower. Also, it would have fluctuated due to ripples in the power supply and made it difficult to time the moment precisely [29].

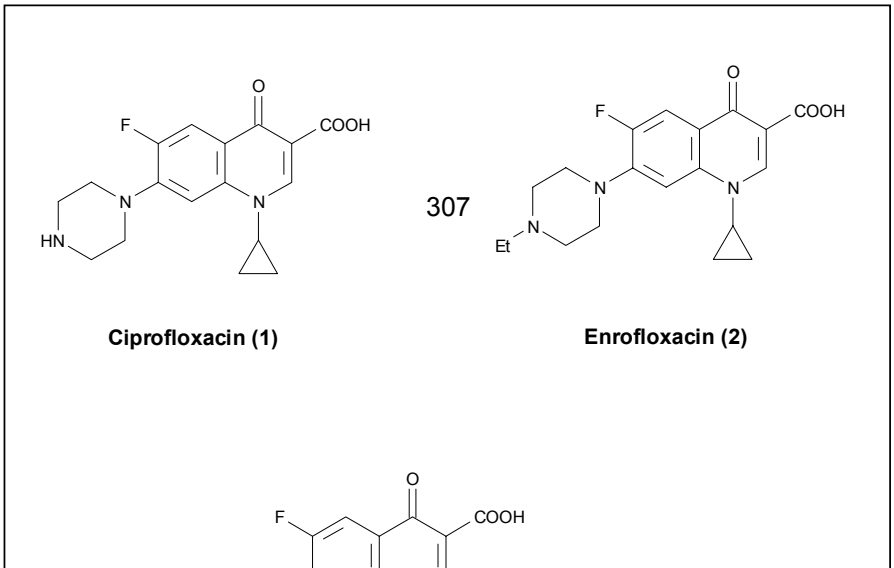
ITP is a stacking system that is used before CZE analysis to improve the sensitivity of capillary electrophoresis. In the bibliography [21,23-25,28,29], there are descriptions of improvements in determination limits as a result of using ITP as the stacking system. However, the loadability is limited by the capillary volume. To establish the optimum conditions we must study the following parameters: injection volume, voltage and pressure applied during the third step (focusing step) and the duration of this step.

During the optimization of the ITP, it is important to take into account when to stop the negative voltage applied in the fourth step and start CZE. If we start

CZE too early, some terminating ions remain inside the capillary, which makes the electric field inhomogeneous and affects the efficiency and migration time during CZE. Therefore, if CZE starts so late, all or part of the sample ions are removed from the capillary. We could control this step by monitoring the electric current [29]. The voltage is stopped when the electric current generated is 90% of the electric current generated when the capillary is full of L. At this moment, we consider the sample matrix is almost completely out of the column.

These parameters were optimized with standard solutions of CPR (1), ENR (2) and FLU (3) dissolved in MilliQ water. Fig. 2 shows their structures. For developing their analysis it was used phosphate electrolyte at pH 9 as BGE. At this pH there were obtained a completely separation of several quinolones [13]. We therefore chose sodium monohydrogen phosphate as the leading electrolyte.

The first parameter to optimize was the injection volume. We fixed the maximum plug of sample that could be injected and then determined the best conditions of focusing. The volume injection was increased from 10 to 30%, while the parameters of the third step were fixed at 10 kV (negative voltage) and 50 mbar (positive backpressure) for 1 min. The sample amount that can be focused isotachophorelly depends on the compounds and the sample matrix. In the best conditions, the maximum sample volume can be injected in ITP – CZE systems [23,28,29] is 55% of the total capillary volume. However, in this study injections above 30% obtained broadening peaks and in this case, the optimum injection volume was 30%.



Ciprofloxacin (1)

Enrofloxacin (2)

Fig. 2. Structures of the three quinolones.

After fixing the injection volume at 30%, and fixing the third step at 10 kV (negative voltage) and 50 mbar (positive backpressure), we studied the duration of the third step. The time necessary to accomplish the preconcentration was evaluated by increasing the focusing time until the peaks ceased to get higher in the CZE step. We studied several focusing times: 1, 5, 10 and 20 min. Lower focusing times (1 and 5 min) obtained well-defined peaks. For the highest focusing times (10 and 20 min) the sample is not more stacked, moreover both resolution and efficiency became worse. Fig. 3 shows the resolutions at the different focusing times. As the resolutions and the responses were similar for 1 and 5 min, and the stacking was not improved by increasing the focusing time from 1 min to 5 min, we chose 1 min as the optimum. However, at these conditions quinolones were not completely separated.

As we have already explained, during the fourth step the analyte ions are stacked approach the capillary inlet and the terminating ions are removed from

the capillary. The inlet vial and the outlet vial contain leading electrolyte. This step takes 1 min at the optimum experimental conditions.

After optimizing the parameters that affect the stacking step, we had to finish optimizing the electrophoretic separation. It was important remember that all modifiers we added to the BGE could affect the stacking step.

To improve electrophoretic separation we studied several parameters: adding an organic solvent, adding a salt (NaCl) to the BGE, and changing the analysis voltage.

When we add an organic solvent to the separation buffer several of its characteristics are modified, i.e. selectivity, viscosity and zeta-potential. It is difficult to predict how adding organic solvents affects electrophoretic mobility, but in general, adding 2-propanol (IPA) or methanol (MeOH) seems to raise electrophoretic mobility and accentuate differences in mobility [16,38]. So we added up to 10% (v/v) of MeOH and IPA; not only was separation not improved but there were broadening peaks.

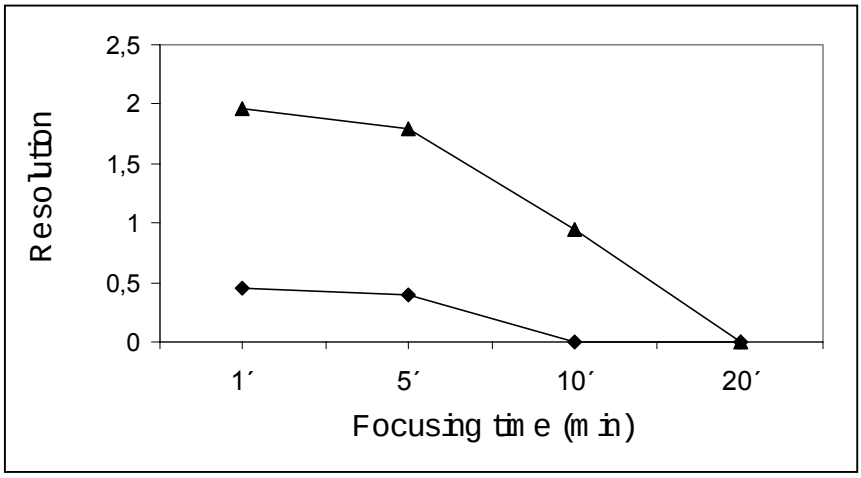


Fig. 3. Representative focusing time versus resolution from standard solution with 1 mg l^{-1} of CPR, ENR and FLU. The buffer system was: 10 mM sodium monohydrogen phosphate (pH 9.0, adjusted with diluted phosphoric acid) as l
eading electrolyte and 10 mM β-Alanine (pH 9.0, adjusted with NaOH 0.1 N) as terminating electrolyte. Background electrolyte (BGE) was the same as the leading one. Injection: 50 mbar for 6 s. Capillary temperature: 25ºC. Separation voltage: 25 kV. Symbols: ◆: Ciprofloxacin/Enrofloxacin, ▲: Enrofloxacin/Flumequine.

Adding a salt increased the efficiency of the peaks. We added several concentrations of NaCl to the BGE: 2.5, 5 and 10 mM. Results were best when 5 mM NaCl was added. The addition of a salt could affect the stacking step. This is because the salt contains chloride which is a fast ion. However, its addition not make worse the separation and adding 5 mM NaCl to the leading electrolyte (BGE) and applying 15 kV during the CZE step all peaks were efficiency and had high resolution, as we can see in Fig. 4.

After optimizing the separation we determined the calibration parameters. Linearity was between 100 and 1500 μg l^{-1} for ciprofloxacin and enrofloxacin, while for flumequine it was between 100 and 1000 μg l^{-1}.

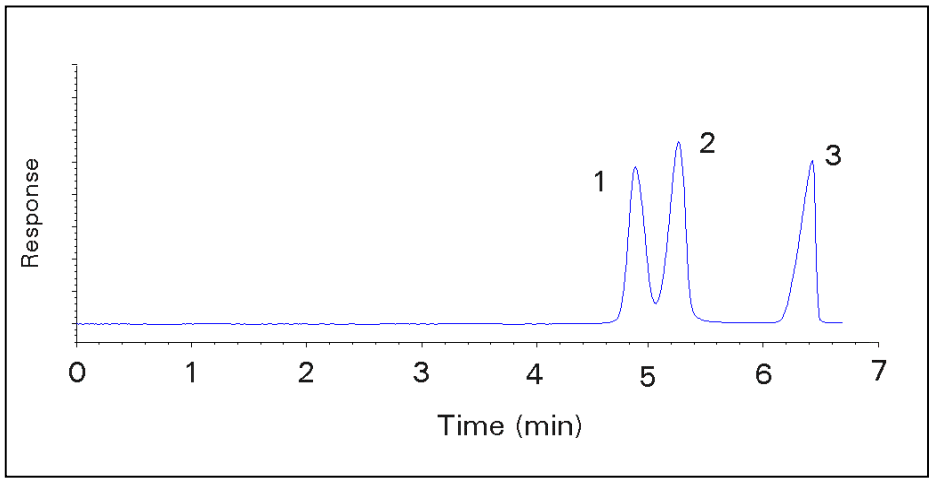


Fig. 4. Electropherogram of 1 mg l^{-1} of CPR, ENR and FLU from standard solution. The separation voltage during CZE analysis was 15 kV. The buffer system was: 10 mM sodium monohydrogen phosphate (pH 9.0, adjusted with diluted phosphoric acid), 5 mM NaCl. For other conditions see Fig. 3.

We injected these solutions five times, starting with the least concentrated one and ending with the most. The area values obtained were successively analyzed using ULC (Univariate Linear Calibration) software [39] to evaluate the correlation coefficient (r), the relative standard deviation (R.S.D.) within solutions and the limit of detection (LOD). Linearity was good, and the correlation coefficient was over 0.999. The detection limit (LOD) was calculated according to the method of Widefordner and Long [40] using the ULC program with K equal to 3. LOD was 50 μg l^{-1} for ciprofloxacin and flumequine, and 10 μg l^{-1} for enrofloxacin. The R.S.D. of the areas were between 0.6% and 2.9% and the R.S.D. of the migration time were between 1.1% and 4.5% for ten repeated injections of standard solutions of 1000 μg l^{-1}. A fresh buffer was used after each sequence of three injections. These results are shown in Table 1.

Table 1

Calibration data and precision studied with standard solutions.

Compounds	Linearity ($\mu g\ l^{-1}$)	Correlation coefficient (r^2)	LOD ($\mu g\ l^{-1}$)	LOQ ($\mu g\ l^{-1}$)	%R.S.D.[a] (area)	%R.S.D.[a] (time)
CPR	100-1500	0.9996	50	100	0.6	0.2
ENR	100-1500	1.0000	10	20	2.8	0.3
FLU	100-1000	0.9973	50	100	2.9	0.5

[a)] Calculated for ten consecutive runs at 1000 $\mu g\ l^{-1}$

This study shows that CZE can be used to trace quinolone antibiotics. As far as we know, this is the first time that ITP – CZE has been used to decrease the limits of detection of quinolones by CE. Fig. 5 compares the electropherogram from analyzing 8 mg l^{-1} of standard solution by conventional CZE (Fig. 5a) with the electropherogram from analyzing 0.2 mg l^{-1} of standard solution by ITP – CZE system (Fig. 5b). The signals for each compound are similar. However, in the ITP – CZE system the analysis time increased for all compounds.

With this system the sensitivity of the CE method increased 40-fold. Coupling ITP with CZE in a single capillary is therefore suitable for analyzing enrofloxacin, ciprofloxacin and flumequine at low levels.

We have therefore improved detection limits so that they are similar to those of liquid chromatography methods with UV detection [34,37,41].

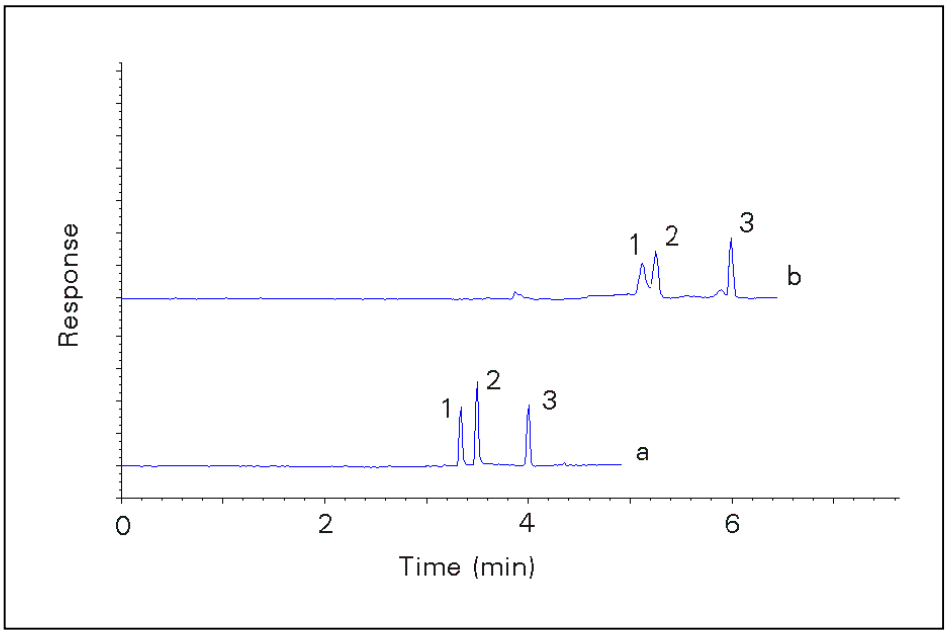


Fig. 5. Electropherogram of 8 mg l^{-1} of CPR, ENR and FLU from standard solution analyzed by CZE system (a) and a standard solution of 0.2 mg l^{-1} analyzed by ITP – CZE system (b). For other conditions see Fig. 4.

Analysis of plasma samples

CE has a number of advantages over liquid chromatography and other techniques. For example, less sample preparation is needed, analysis is less expensive and there is less interference in the assay. CE methods sometimes analyze drugs or antibiotics in serum and plasma samples by directly injecting them [42]. Analysis time and the cost of consumables are therefore smaller. However, as plasma or serum samples contain large amounts of proteins, this could generate interfering peaks. They also contain many constituents at higher concentrations, so during ITP long zones are formed and, when CZE separation begins the analytes start from very different positions [42]. Consequently, the sample must be pretreated.

As a sample pretreatment we studied solid-phase extraction to remove the sample matrix. This type of pretreatment prepares samples for a wide range of analytes in complex matrices because it is more selective and simpler to operate, and because it uses less than other types of pretreatments like liquid-liquid extraction or deproteinization [16,17,38].

In a previous study [9] we determined several quinolones in pig plasma samples using SPE as the pretreatment and CE to carry out analysis. We used C_{18} cartridges for the SPE, and obtained high recoveries. As far as we know, no other study of quinolones has used studies SPE as the pretreatment and CE as the analysis. In this paper, we used Waters Oasis HLB cartridges to carry out the SPE pretreatment. This is because they contain a macroporous copolymer with lipophilic retention characteristics that provide the reversed-phase properties that are needed to retain the analyte. They also have hydrophilic retention characteristics that avoid the wettability problems of the C_{18} packings.

As we know, this kind of cartridge has not been used to determine quinolones, but recoveries are high when they are used to determine antibiotics in pig plasma samples [41,44].

The parameters optimized for SPE pretreatment were the clean-up and elution steps. One mL of plasma sample was passed through the cartridge, and several volumes of water were tested (between 0.5 and 2 mL) to obtain a clean sample with good recoveries for all quinolones. One mL of Milli-Q water was enough for the clean-up step. To optimize the elution step we used MeOH. We also studied several volumes of MeOH for the elution of Qs from the Waters Oasis HLB cartridge. One mL of eluent was enough to carry out this step and recoveries were high (around 100%) for all compounds.

For accurate quantification we used an internal standard. This makes accurate injections unnecessary because a reference standard is included in each sample analysed. We choose an internal standard with a migration time that enables quinolones to be eluted in a suitable time and does not allow compounds to interfere in the electropherogram. We therefore analyzed a test sample containing known amounts of each component plus a predetermined amount of the internal standard (I.S.). The precision of the analysis depends on accurately measuring the peak areas. The internal standard should have similar physicochemical properties to the analyte. For this reason we investigated three quinolones: marbofloxacin, danofloxacin and lomefloxacin. The best results were for lomefloxacin, which was efficiently extracted from plasma samples, and had a migration time that correctly separated the three compounds in this study.

During the analysis of pig plasma samples by ITP – CZE system described, the time of duration of the fourth step increased. This is because the complexity of the sample makes more difficult the approach of analyte ions to the capillary inlet. This step endures 3 min.

Fig. 6a shows the electropherogram of a blank plasma sample and Fig. 6b shows the electropherogram of spiked plasma sample (1500 μg l^{-1} of CPR, ENR and FLU) after the SPE procedure has been carried out. In Fig. 6b we can see that the analysis time increased slightly. This was because the complexity of the sample matrix increased in the fourth step, when the sample was concentrated near the capillary inlet. As we can see, there was no interferences at the analysis time of quinolones which are studied. We can therefore conclude that the pretreatment studied can be used to analize these compounds in these kinds of samples.

Next we determined the calibration parameters in pig plasma samples, spiking blank pig plasma with different standard concentrations. Linearity was between 100 and 1500 μg l^{-1} for ciprofloxacin and enrofloxacin, and for flumequine it was between 100 and 1000 μg l^{-1}. The LOD was 70 μg l^{-1} for enrofloxacin, 85 μg l^{-1} for ciprofloxacin and 85 μg l^{-1} for flumequine (see Table 2).

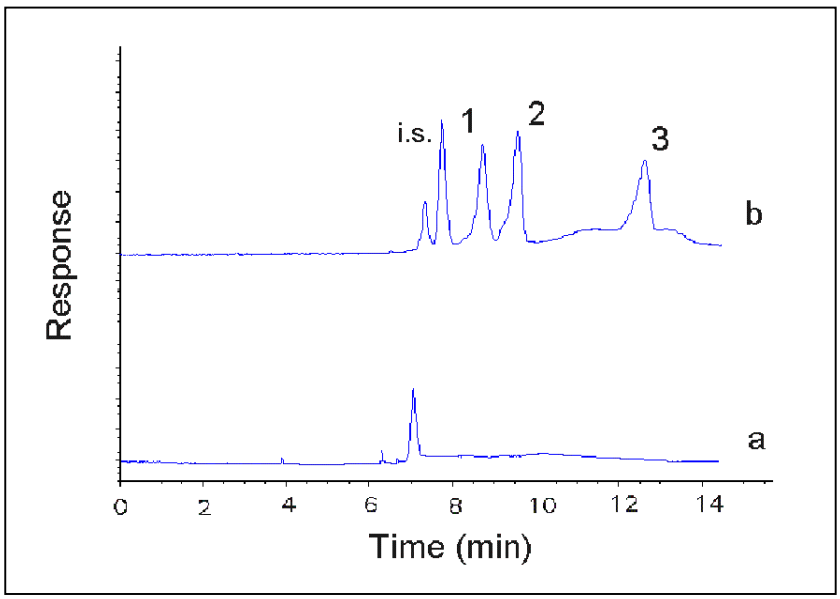


Fig. 6. Electropherogram of a blank plasma sample (a) and a plasma sample spiked with 1.5 mg l^{-1} of CPR, ENR and FLU (b). For other conditions see Fig. 4.

Table 2

Calibration data and precision studied in several spiked plasma samples.

Compounds	Linearity ($\mu g\ l^{-1}$)	Correlation coefficient (r^2)	LOD ($\mu g\ l^{-1}$)	LOQ ($\mu g\ l^{-1}$)	%R.S.D.[a] (area)	%R.S.D.[a] (time)
CPR	100-1500	0.9988	70	140	4.0	1.2
ENR	100-1500	0.9968	85	170	4.0	1.3
FLU	100-1000	0.9958	50	100	5.0	1.8

[a)] Calculated for five consecutive runs at 1000 $\mu g\ l^{-1}$

For pig plasma samples spiked with different concentrations of each quinolone, the recoveries were above 90% for all compounds. The R.S.D. values were between 5% and 7% for pig plasma samples spiked with 200 $\mu g\ l^{-1}$, and between 4 and 5% for pig plasma samples spiked with 1000 $\mu g\ l^{-1}$ (see Table 3).

Table 3

Recoveries (%R) and repeatibility (%RSD) for CPR, ENR and FLU from pig plasma samples at several spiked level preconcentrations.

	Spiked level preconcentration			
	200 $\mu g\ l^{-1}$		1000 $\mu g\ l^{-1}$	
Compounds	**%R**	**%RSD[a]**	**%R**	**%RSD[a]**
CPR	128	6	90	4
ENR	126	5	101	4
FLU	98	7	92	5

[a)] Calculated for five consecutive runs at each concentration spiked with CPR, ENR and FLU standards, respectively.

Based on the good results of LODs obtained for plasma samples, the developed method could be applied for tissue samples after a sample pretreatment, which is now under study in our laboratory.

CONCLUSIONS

This study shows that an ITP – CZE system in a single capillary can be used to determine enrofloxacin, ciprofloxacin and flumequine in pig plasma samples. With this system, sensitivity is 40 times higher than with CZE. We used Oasis HLB cartridges to carry out SPE as a clean-up step. Recoveries were around 100%. As far as we know, this kind of cartridge has never been used to determine quinolones.

This system is simple and sensitive. It is also characterized by its repeatability as the low relative standard deviations (RSD) obtained throughout the procedure demonstrate.

We therefore conclude that this is a good alternative method to HPLC for analyzing quinolones in biological samples at low levels.

ACKNOWLEDGEMENTS

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III.8. Estudi de diversos sistemes d'*stacking on-line* per l'anàlisi de marbofloxacina per electroforesi capil·lar

L'objectiu d'aquest treball és desenvolupar un sistema que permeti determinar una quinolona, la marbofloxacina, en plasma de porc per CE. Un cop el sistema establert s'estudien diferents sistemes de preconcentració *on-column*, per tal d'augmentar la sensibilitat d'aquest mètode per poder determinar la marbofloxacina a baixos nivells de concentració.

Els sistemes de preconcentració estudiats són ITP i FASI [1,2]. Ambdós sistemes els hem estudiat en treballs anteriors. El sistema de FASI l'hem aplicat en el primer treball per a l'anàlisi d'amoxicil·lina en plasma de porc, en el qual s'aconsegueix augmentar la sensibilitat unes 35 vegades, mentre que en el treball anterior s'ha estudiat el sistema de ITP per l'anàlisi de tres quinolones, enrofloxacina, ciprofloxacina i flumequina en plasma de porc, aconseguint augmentar la sensibilitat unes 40 vegades.

En el present treball es pretén comparar el potencial de la ITP amb el de FASI, per veure en quin dels dos casos s'aconsegueix augmentar més la sensibilitat del mètode quan el que s'analitza és marbofloxacina en plasma de porc.

La marbofloxacina és una quinolona àmpliament utilitzada en veterinària que també està regulada per la EU. Com a tampó per dur a terme la separació s'utilitza fosfat sòdic 10 mM a pH 9.0, com en l'estudi anterior.

En el sistema isotacoforètic s'utilitza com a *leading* el mateix que el tampó. Pel que fa al *terminating* se n'estudien tres de diferents: la β-alanina, tris(hydroxymethyl)aminomethane (TRIS) i trietanolamina (TEtOHA). Tots aquests electròlits es caracteritzen perque tenen una baixa mobilitat [2,3].

S'optimitzen els diferents paràmetres del procés d'ITP pels diferents *terminating*, com són el volum de mostra injectada, pressió i voltatge a aplicar, el temps de duració de la preconcentració, així com la longitud i el diàmetre intern del capil·lar.

Les millors condicions obtingudes han estat quan s'ha utilitzat β-alanina com a *terminating*, injectant un 50 % (del volum total del capil·lar), l'aplicació de 50 mbar i –10 kV durant 5 minuts en el procés de preconcentració i, –10 kV durant 3,5 minuts per desplaçar la mostra preconcentrada al principi del capil·lar. El capil·lar és de 90 cm de longitud i 100 μm de diàmetre intern. Amb aquestes condicions l'augment del senyal és d'unes 75 vegades, respecte la injecció hidrodinàmica convencional.

També s'ha estudiat el sistema de FASI com a sistema de preconcentració. A diferència de la ITP només s'utilitza un electròlit, el mateix que s'utilitza en el procés de separació per CE. Després d'optimitzar els diferents passos d'aquest sistema, com són el volum d'injecció (85 %), el temps d'stacking (quasi 9 min) i les dimensions del capil·lar (90 cm de longitud i 100 μm de diàmetre intern), s'observa que l'augment del senyal és d'unes 65 vegades.

En els dos casos hi ha un augment important del senyal, malgrat això es tria com a millor sistema de preconcentració la ITP degut a que l'augment de la sensibilitat és superior.

Posteriorment el sistema ha estat aplicat a l'anàlisi de marbofloxacina en mostres de plasma. Com en el treball anterior s'utilitzen sorbents Oasis HLB per extreure la marbofloxacina, aconseguint recuperacions pròximes al 100 %, a més de l'eliminació d'interferències presents en la matriu.

Mitjançant aquest sistema el LOD és de 20 $\mu g \cdot l^{-1}$. Per tant cal destacar la gran sensibilitat d'aquest mètode i la possibilitat del seu ús en l'estudi de residus ja que els valors de MRL per aquest compost en diferents teixits animals és igual o superior a 50 $\mu g \cdot Kg^{-1}$.

Els diferents resultats d'aquest treball han estat publicats a la revista Chromatographia 55, (2002) 585-590 i se n'adjunta la corresponent còpia a continuació.

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STUDY OF SEVERAL ON-LINE STACKING PROCEDURES FOR ANALYZING MARBOFLOXACIN BY CAPILLARY ZONE ELECTROPHORESIS

ABSTRACT

This paper describes the analysis of marbofloxacin (MAR) in pig plasma samples at low levels by capillary electrophoresis (CE). We studied two on-column stacking systems to increase the sensitivity of CE: field-amplified sample injection (FASI) and isotachophoresis (ITP), and optimized several parameters to obtain the best sensitivity with each system. Conditions were best with an ITP-CZE system, with 10 mM sodium monohydrogen phosphate (pH 9.0) as the leading and background electrolyte (BGE) and 10 mM β-alanine (pH 9.0) as the terminating electrolyte. This system was 75 times more sensitive than conventional CE and the enhanced detection limit (LOD) was 20 $\mu g \cdot Kg^{-1}$. A sample of pig plasma was analyzed with a clean-up step that involved solid-phase extraction onto Oasis HLB cartridges followed by elution with methanol. Recoveries with this system were around 100 %.

keywords: Capillary zone electrophoresis; On-line Stacking procedure; Marbofloxacin

INTRODUCTION

Marbofloxacin (MAR) (Figure 1) is a new fluoroquinolone antibiotic widely used to treat and prevent veterinary diseases. It acts by inhibiting bacterial DNA-gyrase and is used to treat bovine respiratory diseases (oral or parenteral administration to cattle including lactating dairy cattle), respiratory diseases in pigs, and in sows for parenteral treatment of Mastitis Metritis Agalactiae syndrome. Marbofloxacin is a compound that is excreted principally in the urine. In all species, unmetabolized marbofloxacin is the main component of the residues in tissues and excreta [1].

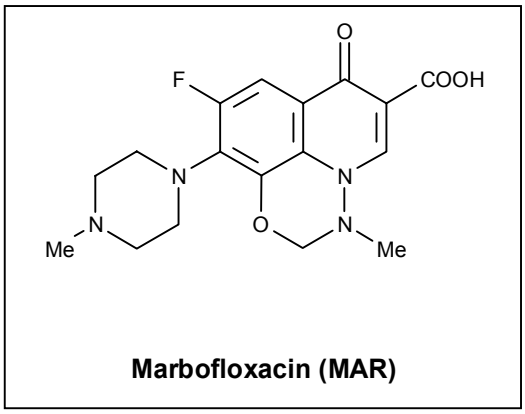


Fig. 1. Structure of marbofloxacin.

The residues of marbofloxacin may remain in tissues and milk etc. intended for human consumption. To prevent health problems with consumers, therefore, and on the recommendation of its Committee for Veterinary medicinal products, the European Community has included marbofloxacin and other antibiotics in the Council Regulation (EC) No. 2377/90, which establishes the maximum residue limits (MRLs) of these antibiotics in livestock products [2]. It is important therefore to determine it at low levels in biological samples. In this study we have determined MAR in pig plasma samples at low levels by CZE.

Some authors have published analytical methods for determining MAR in biological samples based on HPLC [1,3-6]. These methods can determine several quinolones in animal samples and are characterized by their sensitivity, which is essential in these studies because the MRLs are fixed at $\mu g \cdot Kg^{-1}$. CE is therefore a good alternative to HPLC methods because it combines high resolution and easy automation with modest sample requirements and low solvent consumption. CE has been used to determine quinolones in pharmaceutical formulations and in standard solutions [7-12] using different electrolytes, but only a few have been applied to biological samples [13,14]. The bibliography contains a few studies on the use of capillary electrophoresis to determine marbofloxacin. These were developed by Barrón et al. [15,16] who determined the pKa values and mobilities of several quinolones, including MAR, in an aqueous medium or in mixtures of organic solvents and water. As far as we know, all these studies were developed in standard solutions and CE has not been used to determine marbofloxacin in biological samples.

One of the main drawbacks of CE is its low sensitivity, which is due to the small separation column, and only a small amount of the sample can be loaded and the concentration sensitivity of the UV detector is low. There are several ways to increase sensitivity. One is to use highly sensitive detection systems such as a laser-induced fluorescence detection and electrochemical detection. Some systems can inject high amounts of sample and are efficient for on-column sample concentration, such as field-amplified sample injection (FASI) and isotachophoresis (ITP). With the FASI system, sample stacking occurred when large volumes of sample were introduced into the capillary and the sample matrix was removed by pumping it out of the column using the electroosmotic flow. Therefore, in the bibliography [17,18] systems in which an ITP preconcentration is combined with capillary zone electrophoresis (CZE) are described. These systems can be developed in a single capillary. In this case, the whole capillary is filled with sample and the zone is isotachophoretically

preconcentrated because the movement of the analytes is balanced by a hydrodynamic backpressure to prevent the sample from leaving the capillary.

Unlike FASI, ITP is a discontinuous electrolyte system made up of two different electrolytes: the leading electrolyte (L) and the terminating electrolyte (T). The leading electrolyte must have a leading ion, the mobility of which is higher than that of any analyte. The terminating ion of the terminating electrolyte must be the slowest one to be able to close the train of analyte zones. The sample ions therefore have an intermediate mobility, so in the capillary there are stacked isotachophoretic zones with sharp boundaries. During separation, the concentration of analytes in the sample changes until the sample ions reach equilibrium with respect to the concentration of the leading ions. When this equilibrium is reached, the leading ions and sample ions are moved at the same velocity. Moreover, in this zone, ion mobility is inversely proportional to the electric field [19,20].

ITP is particularly useful when samples are not simply dissolved in water but have some conducting ions, such as biological samples [21].

Stacking systems are therefore used to inject the largest possible sample into the narrowest band and so increase sensitivity and produce the highest resolution. Generally, large-volume stacking using hydrodynamic injection has the best reproducibility. However, stacking may be limited because of peaks broadening when large amount of sample was injected. On-column ITP-CZE can achieve the highest stacking capability, but it is limited because it is complex. FASI is easy to implement and automate but the sample matrix can cause bias in the injected amount. So, depending on the application, suitable injection and stacking methods should be chosen. For this reason, the development of a study to determine which type of stacking system is better for determining marbofloxacin in plasma samples is interesting [19,20,22,23].

This kind of sample is characterized by its complexity, so it must be treated before the CE analysis by deproteinization [2], liquid-liquid extraction (LLE) [1,5], solid-phase extraction (SPE) [4,6], etc.

The aim of this paper is to determine marbofloxacin in pig plasma samples by capillary electrophoresis (CE) at low levels. We have made a comparative study of two types of on-column stacking systems (FASI and ITP), and assessed how they can be used with samples of pig plasma. We have tested several parameters in the two systems, such as capillary dimensions, injection volume and focusing time. Also, in the ITP – CZE system we have studied several electrolyte systems in order to obtain good sensitivity.

Finally, using Oasis HLB cartridges. because of the large variations in the composition of the sample matrix, a pretreatment step is likely to improve both the reproducibility and the efficiency in the CZE analysis [24]. So, before applying ITP or FASI the sample must be pretreated, and in this work this pretreatment was made using Oasis HLB cartridges.

EXPERIMENTAL

Instrumentation

Electrophoretic experiments were performed using a Agilent 3D CE (Waldbronn, Germany) with a DAD detector (Diode Array Detection). Data were collected with the software provided with the HP 3D Chemstation (Agilent), which was operated under Windows NT (Microsoft). The capillary was fused silica (64.5 cm x 75 μm i.d. and 90 cm × 100 μm i.d.) supplied by Beckman Coulter, Inc.

(Fullerton, USA). A detection window was prepared by burning off the polyimide coating 81.5 cm from the capillary inlet.

Reagents and standards

Marbofloxacin (MAR) was provided free by Vétoquinol (Lure, France). Standard stock solutions of 1000 $\mu g \cdot Kg^{-1}$ were prepared in NaOH 0.1 N and stored under refrigeration at 4 ºC. Standard working solutions were prepared weekly or daily, depending on their concentration, by diluting the standard stock solution with water that had been purified by a Milli-Q system (Millipore, Bedford, USA).

Disodium hydrogen phosphate (Probus, Barcelona, Spain), β-Alanine (Sigma Chemical Co. Inc., USA), tris(hydroxymethyl)aminomethane (TRIS) (Sigma Chemical Co. Inc., USA) and triethanolamine (TEtOHA) (Sigma Chemical Co. Inc., USA) were used to prepare the electrophoretic solutions. Sodium hydroxide (Prolabo, Bois, France) and phosphoric acid (Probus, Barcelona, Spain) were used to adjust the pH of the electrolytes. Methanol (MeOH) (SDS, Peypin, France) was used during solid-phase extraction.

Capillary Zone Electrophoresis Conditions

Before use, the capillary was rinsed with 1 M NaOH (Probus SA, Barcelona) for 15 min, with Milli-Q water for 15 min, and finally was flushed with running buffer for 10 min. Before each analysis, the capillary was rinsed with the running buffer for 3 min. In all cases, the capillary was rinsed by 1000 mbar pressurized flow.

10 mM sodium monohydrogen phosphate (adjusted to pH 9) was used as BGE. Sample injection was performed hydrodynamically at a pressure of 50 mbar for 6 seconds. Running buffer was then introduced to the capillary at 50 mbar for 3 seconds to diminish the dispersion of the sample during the analysis. The

detector was set at 290 nm. The capillary temperature was 25 ºC and the separation voltage was 25 kV.

Field-Amplified Sample Injection Conditions

10 mM sodium monohydrogen phosphate (adjusted to pH 9) was used as BGE. Sample injection was performed hydrodynamically at a pressure of 50 mbar for 300 seconds. During sample stacking a voltage with a reverse polarity (-10 kV outlet positive) was applied for 570 seconds. See above for the other conditions.

Capillary Isotachophoresis Conditions

During the isotachophoresis step, we used a system with two different electrolytes: the leading electrolyte (10 mM sodium monohydrogen phosphate, adjusted to pH 9) and the terminating electrolyte (10 mM β-alanine, adjusted to pH 9). Under these conditions, the sample was injected hydrodynamically at a pressure of 50 mbar for 330 seconds. In the focusing step 10 kV was applied as negative voltage in conjunction with 50 mbar of positive pressure for 5 min. In the fourth step of the isotachophoretic procedure a negative voltage of 10 kV was applied for 210 seconds.

Sample Pretreatment

Solid-phase Extraction (SPE) was used for pretreatment with Waters Oasis HLB cartridges (60 mg). The cartridge was placed on vacuum manifold and activated with 5 mL of MeOH followed by 5 mL Milli-Q water. 1 mL of sample was passed through the cartridge. The cartridge was washed with 1 mL Milli-Q water, the compounds were eluted from the cartridge with 1 mL of MeOH, and an aliquot

was analyzed by capillary electrophoresis. Pig plasma samples were used in all experiments.

RESULTS AND DISCUSSION

Field-Amplified Sample Injection – Capillary Zone Electrophoresis

The first on-column stacking system we studied was field-amplified sample injection (FASI), which comprised the following two steps. The sample was first injected hydrodynamically. The injection volume was the amount of sample that filled the capillary as far as the detection window. This was the maximum amount of sample that could be injected. A negative voltage was then applied across the capillary. The electrical field was therefore stronger in the sampling zone than in the separation zone because the concentration of ions was lower. We controlled this step by monitoring the electric current [24]. The voltage current was stopped when the electric current generated was 95 % of that generated when the capillary was full of BGE. At that moment, we considered the sample matrix to be almost completely outside the column. Finally, the polarity was reversed and the separation was performed.

In FASI, therefore, several parameters must be taken into account (the injection volume and the time to stop the voltage current). These parameters are not independent, so when one of them changes, the others also change. So, after testing all these parameters, using 10 mM sodium monohydrogen phosphate (adjusted to pH 9) as BGE, the best conditions were when the sample was introduced hydrodynamically (50 mbar for 280 seconds, i.e. 85 % of the total capillary volume), the sample vial was changed to a BGE vial and a voltage with a reverse polarity (-10 kV outlet positive) was applied. After 530 seconds, the voltage was stopped, the polarity switched back to the normal position for CZE (15 kV outlet negative) and CZE separation is begun. In this way, sensitivity was

20 times greater than with conventional CZE (in which the sample is introduced hydrodynamically and the sample volume injected is around 1%).

To make the method more sensitive, therefore, we tested two parameters: the length (64.5 and 90 cm) and inner diameter (75 and 100 μm) of the capillary. When both parameters increase, a large amount of sample is injected and the signal of marbofloxacin increases. The results so far explained were obtained with a capillary 64.5 cm in total length, 56 cm effective length (corresponding to the length up to the detection window) and 75 μm inner diameter. Optimization was developed using 10 mM sodium monohydrogen phosphate (adjusted to pH 9) [10] as BGE and standard solutions of 1000 $\mu g \cdot Kg^{-1}$. The optimization is performed as before but with a capillary of different dimensions.
The best conditions were therefore obtained with a capillary with a total length of 90 cm and an inner diameter of 100 μm, into which the sample was introduced hydrodynamically (50 mbar for 300 seconds, i.e. 80 % of the total capillary volume). During the second step, 10 kV of negative voltage was applied for 570 seconds. Under these conditions the sensitivity was 65 times higher than with conventional CZE.

We can therefore conclude that changing the capillary dimensions considerably increased sensitivity and that the efficiency of the peak was high.

Figure 2 shows that the sensitivity when 1000 $\mu g \cdot Kg^{-1}$ of MAR from standard solution is analyzed with FASI under its optimum conditions (Figure 2b) is greater than when it is analyzed with conventional CZE (hydrodynamic injection, 50 mbar for 6 seconds) (Figure 2a).

Capillary Isotachophoresis – Capillary Zone Electrophoresis

The other type of on-column stacking we studied was Isotachophoresis (ITP), which we applied in a single capillary.

First, the capillary was filled with the leading electrolyte (L) and then the sample was injected hydrodynamically.

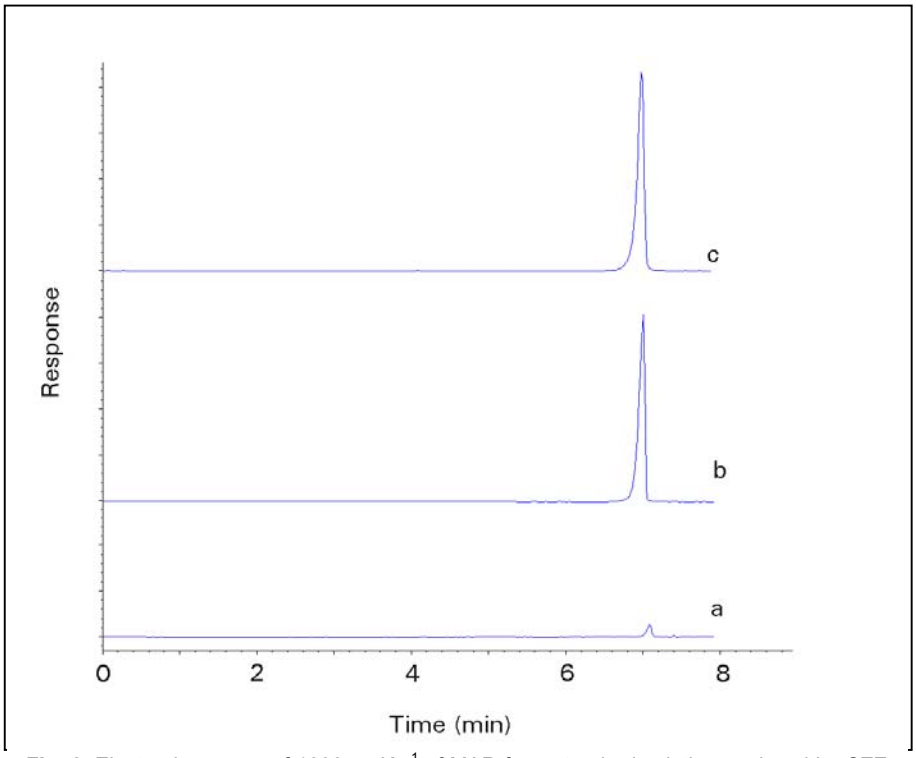


Fig. 2. Electropherogram of 1000 μg·Kg^{-1} of MAR from standard solution analyzed by CZE with injection of 50 mbar for 6 seconds and using fused silica capillary (a), analyzed by FASI – CZE system with injection of 50 mbar for 300 seconds (b) and analyzed by ITP – CZE system with 10 mM β-alanine (adjusted to pH 9) as terminating electrolyte and with injection of 50 mbar for 330 seconds and focusing time of 5 min (c). 10 mM sodium monohydrogen phosphate (adjusted to pH 9) was used as leading electrolyte and background electrolyte. Capillary temperature: 25 ºC. Separation voltage: 25 kV. The detector was set at 290 nm. Capillary dimensions: 90 cm × 100 μm.

As explained in the bibliography [10,24,25], the maximum amount of sample that can be injected into ITP systems is 55 %, since any injections that are higher than this broaden the band. While the inlet vial contained T and the outlet vial contained L, the analytes were focused on-column by applying a negative voltage in conjunction with a positive hydrodynamic backpressure. The term "positive backpressure" is used to explain that the backpressure induces a flow in the direction of the capillary outlet, whereas a negative backpressure induces a flow in the opposite direction. The negative voltage induced an electroosmotic

flow in the direction of the capillary inlet, while the positive backpressure opposed this movement and produced the stack of sample analytes. Moreover, the characteristics of the ITP zones meant that the peaks did not broaden. Next, both the inlet vial and the outlet vial contained L. A negative voltage was applied to stack analyte ions near to the capillary inlet and at the same time remove the matrix sample and T. As in the FASI system, this step was controlled by monitoring the electric current.

Finally, we began CZE separation by applying a positive voltage. As we have already stated, ITP is a discontinuous system made up of two electrolytes: the leading electrolite (L) and the terminating electrolyte (T). The L has a leading ion whose mobility is higher than that of any analyte. The terminating ion of the T has to be the slowest one in order that the train of analyte zones can be closed. The sample ions therefore have an intermediate mobility. Therefore, the ions with more mobility than the leading ions and those with less mobility than the terminating ions are removed from the capillary. This system cleans the sample considerably [24]. It is therefore important to choose a suitable electrolyte system in which stable isotachophoretic zones are formed and the analytes are completely separated. Theoretically, anions must always form the last zone in the system. In this work we used 10 mM sodium monohydrogen phosphate (adjusted to pH 9) as the leading electrolyte (L) and BGE because phosphate is a fast ion, faster than quinolone compounds, and is therefore considered a suitable leading ion. We tested three terminating electrolytes (T): β-alanine, tris(hydroxymethyl)aminomethane (TRIS) and triethanolamine (TEtOHA), because these are recommended in the bibliography as suitable terminating electrolytes [10,19,26]. All these buffers generate a low current, which favors rapid migration of the analyte ions, and are stacked between the two electrolytes. The ionic mobility depends on both the ionic strength and the temperature of the solution. Table 1 shows the ionic mobilities of these electrolytes [27,28]. Optimization was carried out with a standard solution of 1000 $\mu g \cdot Kg^{-1}$ of MAR.

Table 1

Ionic mobilities of the selected substances

Substance	u_{tab} (10^{-9} m^2 V^{-1} s^{-1})
β-Alanine	36.7
TRIS	29.5
TEtOHA	30.1

With ITP – CZE, there are several parameters to optimize. These parameters are not independent, so we must remember that if one parameter is changed, all the others are affected. First we studied the maximum plug of sample that could be injected. After testing several conditions, the parameters of the focusing step were fixed at 10 kV (negative voltage) and 50 mbar (positive backpressure) for 1 min. The duration of the fourth step therefore depended on the current generated and was controlled by monitoring the current. We tested several injection volumes (30, 40, 50 and 55 % of the total capillary volume) at the same conditions. The optimum injection volume in each case was 50 % (50 mbar for 160 seconds) for β-alanine, 30 % (50 mbar for 100 seconds) for TRIS and 55 % of the total capillary volume (50 mbar for 180 seconds) for TEtOHA, respectively. Signal therefore increased 16-fold, 15-fold and 20-fold, respectively, with respect to conventional CZE. With TRIS, broad peaks were obtained for injections above 30 %. Stacking was therefore highest when we used TEtOHA as the terminating electrolyte.

By fixing the volume injection in the best conditions for each terminating electrolyte, we optimized the focusing time in the third step. As before we fixed 10 kV (negative voltage) and 50 mbar (positive backpressure) in the third step. We tested several focusing times: 1 min, 5 min and 10 min. As before, we controlled the fourth step by monitoring the current generated. For TEtOHA and TRIS, focusing times of over 5 min produced broad peaks. For this reason we did not analyze focusing times of over 5 min. When we applied a focusing time

of 5 min for β-alanine, the increase in signal was 25-fold and higher focusing times not increase the stacking. These results are shown in Figure 3.

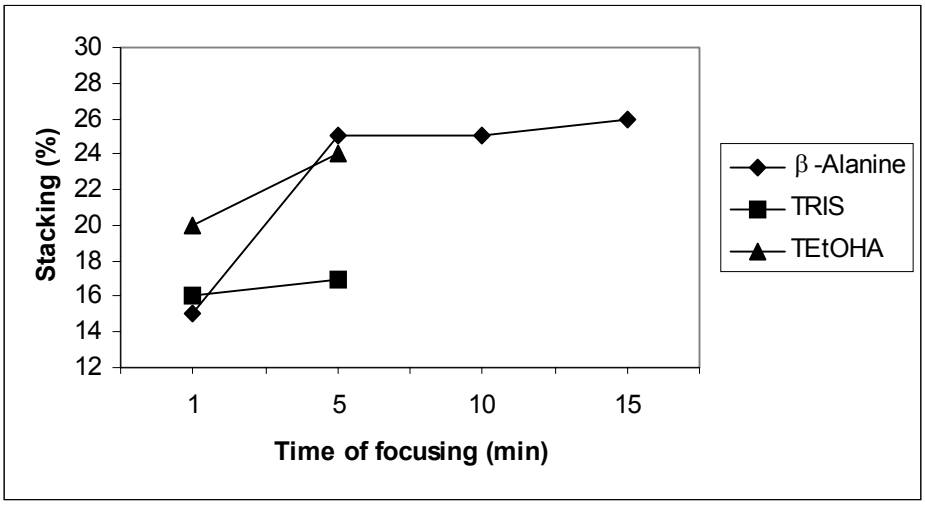


Fig. 3. Stacking (%) versus time of focusing (min) from standard solution with 1000 μg·Kg^{-1} of MAR. It was analyzed by ITP – CZE system. Three terminating electrolytes were tested: β-Alanine; TRIS and TEtOHA. The capillary dimensions were 64.5 cm × 75 μm. Injection volume: 50 % (50 mbar for 160 seconds) for β-Alanine, 30 % (50 mbar for 100 seconds) for TRIS and 55 % (50 mbar for 180 seconds) for TEtOHA. Focusing step: -10 kV and 50 mbar for 5 min. For other conditions see Figure 2.

As before, to increase the sensitivity of the method we tested two parameters: the length (64.5 and 90 cm) and inner diameter (75 and 100 μm) of the capillary. As with stacking using the FASI system, the highest increase in signal was with a capillary of 90 cm in total length and 100 μm in inner diameter (75-fold for β-alanine, 45-fold for TRIS and 55-fold for TEtOHA). Figure 2c shows the electropherogram from the analysis of 1000 μg·Kg^{-1} of MAR from standard solution by the optimum conditions obtained using ITP.

Figure 4 shows the increase in signal obtained with several capillary dimensions in the FASI and ITP – CZE systems, respectively. In all cases, β-alanine produced the highest increase. In all systems, the highest increase was obtained

with a capillary 90 cm in total length and 100 μm in inner diameter, because a large amount of sample was injected.

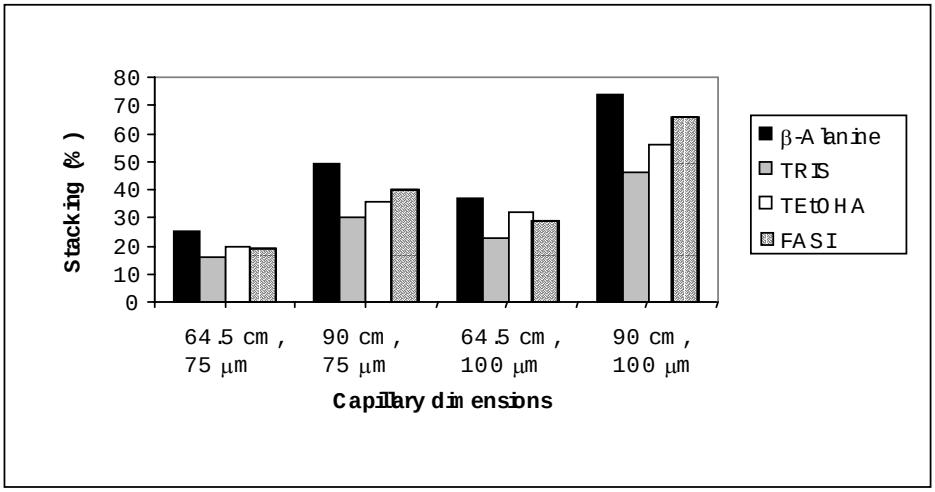


Fig. 4. Dependence of stacking (%) on capillary dimensions for a 1000 μg·Kg^{-1} standard solution of marbofloxacin. Three terminating electrolytes in ITP were tested: β-Alanine, TRIS and TEtOHA. The injection volume was 50 % (50 mbar for 160 seconds) for β-Alanine, 30 % (50 mbar for 100 seconds) for TRIS and 55 % (50 mbar for 180 seconds) for TEtOHA. The focusing step was -10 kV and 50 mbar for 5 min. Other conditions were as for Figure 2.

From these results we can conclude that the simplest and most sensitive way of determining marbofloxacin by CE is to use ITP – CZE in a capillary that is 90 cm in total length and 100 μm in inner diameter; disodium monohydrogen phosphate as the leading and BGE electrolyte, and β-alanine as the terminating electrolyte; 50 % of the injection volume of the total capillary length (i.e. 50 mbar for 330 seconds). During the third step 5 min is established as the focusing time. During the fourth step, the negative voltage applied is 10 kV for 210 seconds. Sensitivity with this method is 75 times higher than with conventional CE.

Next we established that by this method the linearity of marbofloxacin in standard solutions was between 25 and 1000 μg·Kg^{-1}. These solutions were

injected five times, starting with the least concentrated solution and ending with the most concentrated solution. The area values were successively analyzed using ULC (univariate linear calibration) software [29] to evaluate the correlation coefficient (r), the relative standard deviation (R.S.D.) within solutions and the limit of detection (LOD). Linearity was good; the correlation coefficient was above 0.999. We calculated the detection limit (LOD) and the quantification limit (LOQ) by the method of Widefordner and Long [30] using the ULC program with K equal to 3 and K equal to 6, respectively. The LOD was 12 $\mu g \cdot Kg^{-1}$ and the LOQ was 24 $\mu g \cdot Kg^{-1}$. The R.S.D. of the areas were between 2.3 % and 5.3 % and the R.S.D. of the migration time were between 1 % and 1.4 % for ten repeated injections of standard solutions of 1000 $\mu g \cdot Kg^{-1}$. A fresh buffer was used after every three injections.

Analysis of Plasma Samples

In this paper we also studied whether this method could be used to determine marbofloxacin in pig plasma samples. Biological samples are complex so, as they cannot be injected directly into the analytical instrument, a pretreatment step is needed before analysis. We chose solid-phase extraction (SPE) as the clean-up step because it is more selective and easier to use than other pretreatments, and recoveries are higher.

C_{18} cartridges are the most common sorbents. They are surface-bonded porous silica particles which are characterized by their hydrophobic alkyl groups. They are not hydrophilic, or water-wettable, so it is very important that the sorbent is wet before the aqueous sample is loaded as this is the main cause of low recoveries and poor repeatability and reproducibility.

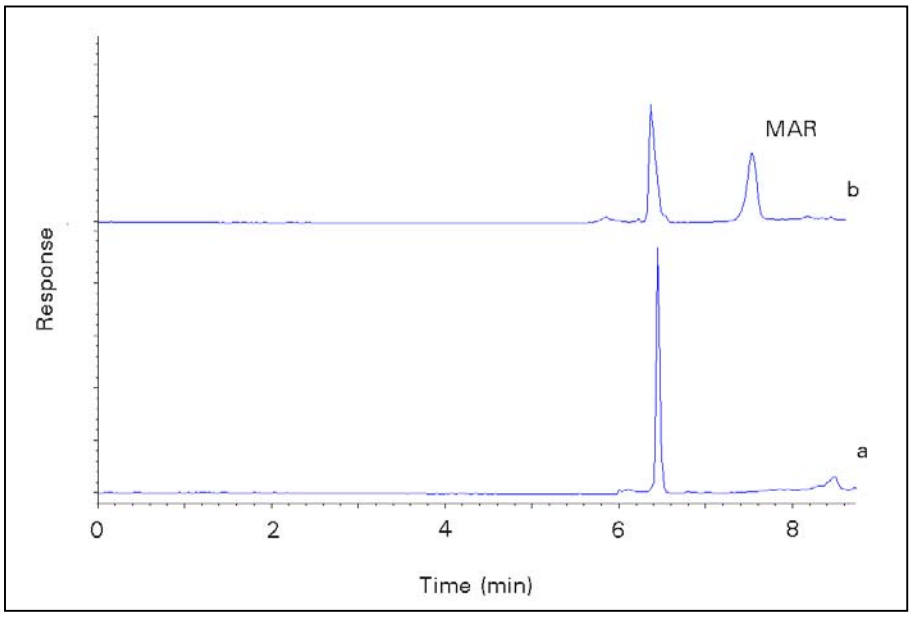
Oasis HLB cartridges, on the other hand, contain a polymeric reversed-phase sorbent. This is a macroporous copolymer which has both hydrophilic and lipophilic retention characteristics. They therefore have two important abilities; they remain wet with water and retain a wide spectrum of both polar and nonpolar compounds. In the bibliography, this type of cartridge has been used to extract antibiotics from pig plasma samples and has obtained high recoveries [31,32].

One mL of plasma sample was passed through the cartridge and one mL of Milli-Q water was enough for the clean-up step to obtain good recoveries for marbofloxacin. This compound was eluted from the cartridge using one mL of MeOH. In this way the recovery was around 100 %. The samples were analyzed by the previously developed ITP-CZE system.

Figure 5a shows the electropherogram of a blank plasma sample and Figure 5b shows the electropherogram of spiked plasma sample with 500 $\mu g \cdot Kg^{-1}$ of MAR.

There was no interference at the analysis time of MAR. This is therefore a good way to pretreat plasma samples that contained MAR before the analysis began.

We then determined the calibration parameters. Linearity was between 50 and 1000 $\mu g \cdot Kg^{-1}$. The LOD was 20 $\mu g \cdot Kg^{-1}$ and the LOQ was 40 $\mu g \cdot Kg^{-1}$. The R.S.D. of the areas was between 4 % and 5 % for pig plasma samples spiked with 100 $\mu g \cdot Kg^{-1}$, and between 7 and 9 % for pig plasma samples spiked with 1000 $\mu g \cdot Kg^{-1}$.



500 $\mu g \cdot Kg^{-1}$ of MAR (b). Analyzed by ITP – CZE system, using 10 mM β-alanine (pH 9, adjusted with 0.1 N NaOH) as terminating electrolyte. Injection: 50 mbar for 330 seconds. Focusing step: -10 kV and 50 mbar for 5 min. For other conditions see Figure 2.

The sensitivity obtained with this CE method is similar to that obtained by liquid chromatographic methods [1,3,5,6].

The European Community establishes MRLs of several antibiotics in livestock products to prevent health problems with consumers. For plasma samples MRLs are not fixed, but in most of pig tissue samples MRL are fixed at 150 μg/Kg for marbofloxacin. Therefore, the developed method for pig plasma samples could be applied in tissue samples after a sample pretreatment, which is now under study in our laboratory.

CONCLUSIONS

This study reports a simple and sensitive method for analyzing MAR in pig plasma samples by ITP – CZE in a single capillary. With this system, sensitivity was 75 times higher than with conventional CE.

We used Oasis HLB cartridges to perform SPE as a clean-up step. Recoveries were around 100 %. The method was also characterized by its repeatability, as is demonstrated by the low relative standard deviations (RSD) obtained throughout.

This method is simple and sensitive and is therefore an alternative to existing HPLC methods of analyzing residual marbofloxacin in plasma samples.

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CAPÍTOL IV. CONCLUSIONS

Les conclusions derivades del treball realitzat en la present Tesi Doctoral són les següents:

1. L'electroforesi capil·lar és una tècnica útil i adequada per determinar antibiòtics degut a que permet separar diferents compostos de manera simple i ràpida.

2. Tant l'electroforesi capil·lar per zones com la cromatografia capil·lar micel·lar electrocinètica són dues modalitats d'electroforesi capil·lar que permeten duu a terme la separació de diferents grups d'antibiòtics i la seva anàlisi en mostres biològiques.

3. L'electroforesi capil·lar permet realitzar el control d'un sol compost, com per exemple la determinació d'amoxicil·lina, oxitetraciclina i kanamicina i també de diferents famílies d'antibiòtics (tetraciclines, quinolones, etc...) en diferents tipus de mostres biològiques de manera simple i ràpida.

4. Mitjançant l'electroforesi capil·lar es poden determinar diferents antibiòtics en mostres de teixit animal a nivells de concentració inferiors als fixats per la normativa europea.

5. Quan la separació dels analits no és completa aquesta es pot millorar mitjançant l'addició d'un cert percentatge de solvent orgànic en el tampó de separació o bé dissolent el tampó de separació en únicament solvents orgànics. L'elevada selectivitat d'aquests sistemes, anomenats electroforesi capil·lar en medi no aquós (NACE), és deguda a l'àmplia varietat de solvents orgànics i les seves diferents propietats físiques i químiques (com la constant dielèctrica, polaritat, densitat, viscositat, propietats àcid-base, etc...).
6. L'ús de l'extracció en fase sòlida és cada dia més habitual per realitzar el tractament de les mostres biològiques en l'anàlisi d'antibiòtics,

concretament en l'etapa de *clean-up* de les mostres. Els sorbents més utilitzats per l'extracció són els C_{18}. L'electroforesi capil·lar per zones permet determinar l'oxitetraciclina en diferents teixits de porc (fetge i ronyó) als nivells de concentració establerts per la EU, si prèviament la mostra és homogeneïtzada i pretractada mitjançant extracció en fase sòlida utilitzant sorbents C_{18}.

7. Malgrat això, s'ha demostrat que en el cas de les matrius biològiques més complexes (com és el cas d'alguns teixits) s'obtenen millors resultats amb un altre tipus de sorbents, com per exemple sorbents polimèrics (LiChrolut EN, Oasis HLB, etc...). Un exemple seria en la determinació d'oxitetraciclina en mostres de múscul de porc per electroforesi capil·lar. En aquest cas quan s'utilitza un sorbent C_{18} per dur a terme el procés d'extracció en fase sòlida s'obtenen recuperacions d'un 20 %, mentre que quan s'utilitza sorbents Oasis HLB, les recuperacions passen a ser majors del 70 %.

8. També els sorbents d'intercanvi iònic poden ser utilitzats en el *clean-up* de les mostres. Un exemple és l'ús de l'intercanviador catiònic CBA que s'ha utilitzat en l'extracció de kanamicina de mostres de sèrum humà i la seva posterior anàlisi per electroforesi capil·lar per zones.

9. Una manera ràpida i simple d'augmentar la sensibilitat en electroforesi capil·lar és mitjançant l'ús de sistemes de preconcentració *on-column*, com són la injecció de grans volums de mostra (FASI) i la isotacoforesi capil·lar (ITP-CZE). Mitjançant aquests s'incrementa la sensibilitat en diversos ordres de magnitud.

10. L'electroforesi capil·lar per zones utilitzant la injecció de grans volums de mostra com a sistema de preconcentració *on-column*, permet analitzar l'amoxicil·lina en mostres de plasma de porc prèviament pretractades

mitjançant extracció en fase sòlida. L'aplicació permet rebaixar el límit de detecció de 7.5 $mg \cdot l^{-1}$ (corresponents a la injecció hidrodinàmica convencional) a 0.3 $mg \cdot l^{-1}$.

11. També és possible l'aplicació de l'electroforesi capil·lar a l'anàlisi d'antibiòtics en mostres d'origen humà. S'ha determinat kanamicina en mostres de sèrum humà mitjançant l'ús de l'extracció en fase sòlida com a procés de *clean-up* i posteriorment s'ha utilitzat un sistema d'injecció de grans volums de mostra per tal de disminuir els límits de detecció del sistema electroforètic. D'aquesta manera s'ha aconseguit augmentar la sensibilitat del sistema unes 20 vegades podent detectar concentracions de l'ordre de 0.1 $mg \cdot l^{-1}$.

12. En la determinació de diferents quinolones, ciprofloxacina, enrofloxacina i flumequina, en plasma de porc s'ha utilitzat un sistema d'isotacoforesi capil·lar – electroforesi capil·lar en un mateix capil·lar per tal de disminuir els límits de detecció del sistema. D'aquesta manera s'aconsegueix augmentar la sensibilitat 40 vegades, aconseguint uns límits de detecció de l'ordre de 0.1 $mg \cdot l^{-1}$.

13. És important escollir adequadament el sistema electrolític que s'utilitzarà per dur a terme un procés d'isotacoforesi capil·lar – electroforesi capil·lar. D'aquesta manera en l'anàlisi de marbofloxacina en plasma de porc mitjançant aquest sistema, s'observa que quan s'utilitza TRIS com a *terminating* l'augment del senyal és de 45 vegades, mentre que si el que s'utilitza és β-alanina l'augment del senyal passa a ser de 70 vegades.
14. Mitjançant l'electroforesi capil·lar per zones s'ha pogut determinar l'oxitetraciclina en diferents teixits de porc que han estat prèviament pretractats mitjançant extracció en fase sòlida. Mitjançant aquest sistema els límits de detecció són de 160, 120 i 85 μg d'oxitetraciclina per Kg de ronyó, fetge i múscul, respectivament. Per tant aquest sistema permet

determinar aquest compost a nivells de concentració més baixos als límits de màxim residu que estableix la normativa europea, els quals són de 600, 300 i 100 μg d'oxitetraciclina per Kg de ronyó, fetge i múscul, respectivament.

15. La separació i determinació de diferents quinolones en plasma de porc ha estat possible mitjançant l'ús de l'electroforesi capil·lar per zones pretractant prèviament les mostres mitjançant extracció en fase sòlida. Per tal d'augmentar la selectivitat del sistema s'ha addicionat metanol al tampó de separació aconseguint la completa separació de tots els compostos

16. Mentre la separació de diferents tetraciclines no ha estat possible mitjançant electroforesi capil·lar per zones en medi aquós, quan es treballa en medi no aquós s'aconsegueix la completa separació de tots els compostos. Aquest sistema s'ha aplicat a la separació i determinació d'oxitetraciclina, tetraciclina, 4-epioxitetraciclina i 4-epitetraciclina en plasma de porc pretractant prèviament les mostres mitjançant extracció en fase sòlida utilitzant sorbents Oasis HLB.

17. Una altra aplicació de l'electroforesi capil·lar en medi no aquós ha estat la determinació de diverses quinolones en ronyó de porc. En aquest cas ha estat necessari l'addició d'un modificador del flux electroosmòtic, el bromur d'hexadimetrina, i la inversió del voltatge de separació per aconseguir la completa separació de tots els compostos. Mitjançant aquest sistema s'obtenen límits de detecció de 120 $\mu g \cdot Kg^{-1}$ per a tots els compostos, valors inferiors als fixats per la normativa europea.

18. L'electroforesi capil·lar amb el tractament previ de la mostra mitjançant extracció en fase sòlida, i l'ús en alguns casos de sistemes de preconcentració *on-column*, es presenta com una alternativa per determinar antibiòtics en mostres biològiques. Si bé els resultats són

comparables als que s'obtenen per cromatografia de líquids, l'electroforesi capil·lar es caracteritza per la seva senzillesa i pels curts temps d'anàlisi. A més l'electroforesi capil·lar es caracteritza, perquè els sistemes de preconcentració *on-column* s'apliquen de manera ràpida i simple.

ANNEX

Els treballs sorgits de la present Tesi Doctoral. Inclosos en els capítols II i III, s´han publicat o estan pendents de publicació en les següents revistes:

- M. Hernández, F. Borrull, M. Calull, Trends Anal. Chem., (2002) (acceptat), (*apartat II.5*).
- M. Hernández, F. Borrull, M. Calull, J. Chromatogr. B, 731 (1999) 309-315, (*apartat III.1*).
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