Universitat Autònoma de Barcelona

Doctoral Thesis
Doctorat Farmacología
Departament de Farmacología, de Terapéutica i de Toxicologia

DISTRIBUTION OF 3,4-METHYLENEDIOXYMETHAMPHETAMINE (MDMA) IN NON CONVENTIONAL MATRICES AND ITS APPLICATIONS IN CLINICAL TOXICOLOGY

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Doctoral thesis submitted by Simona Pichini as a partial fulfillment of the requirements for the degree of Doctor by the Universitat Autònoma de Barcelona. The studies included in this thesis have been realized under the direction of Dr. Rafael de la Torre Fornell and Dr. Magí Farré Albaladejo at the Pharmacology Unit of the Institut Municipal d'Investigació Mèdica (IMIM), Barcelona, Spain; and at the Drug Research and Control Department of the Istituto Superiore di Sanità, Roma, Italy. Doctorate Program of the Universitat Autònoma de Barcelona.

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Yo soy yo y aquéllos a quienes amo

Jorge Bucay

To my friends, treasure of my life

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ABBREVIATIONS

AUC, area under the concentration curve

C_{max}, maximum plasma concentration

CNS, central nervous system

DA, dopamine

DEA, Drug Enforcement Administration

EIA, enzyme immuno assay,

FDA, Food and Drug Administration

GC, gas chromatography

GC-MS, gas chromatography coupled to mass spectrometry

HHA, 3,4-dihydroxyamphetamine

HHMA, 3,4-dihydroxymethamphetamine

HMA, 4-hydroxy-3-metoxyamphetamine

HMMA, 4-hydroxy-3-metoxymetamphetamine

5-HT, 5 hydroxytryptamine, serotonin

ISS, Istituto Superiore di Sanità

IMIM, Institut Municipal d'Investigació Mèdica

IS, internal standard

LOD, limit of detection

LOQ, limit of quantification

MAO, monoaminooxidase

MBDB, *N*-methyl-1-(1,3-benzodioxole-5-il)-2-butanamine

MBTFA, *N*-methyl-bis-(trifluoroacetamide)

MDEA, 3,4-methylendioxyetilamphetamine, MDE, Eve

MDA, 3,4-methylendioxyamphetamine, love drug

MDA- D_5, deuterated 3,4-methylendioxyamphetamine

MDMA, 3,4-methylendioxymetamphetamine, ecstasy

MDMA-D_5, deuterated 3,4-methylendioxymetamphetamine

MDPA, 3,4-methylendioxypropylamphetamine

MS, mass spectrometer

SD, standard deviation

SIM, Selected Ion Monitoring

SPE, solid phase extraction,

$t_{1/2}$, elimination half-life

t_{max}, time to reach maximum concentrations

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Justification

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Justification

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is a synthetic amphetamine derivative with potent effects on several neurotransmission systems (i.e. dopaminergic, noradrenergic) but more specifically on the serotonergic one. The drug, synthesized in 1912 but never marketed, became popular in the 1970s and 1980s and due to the induction of feelings of euphoria, friendliness, closeness to others, and empathy after its use was classified in a new drug class known as "entactogens". MDMA has become increasingly popular in Europe, North America and Australia over the past 15 years. It is primarily consumed by young people in large dance and music environments ("raves") and sometimes in small social settings. Undesirable acute effects associated with the recreational use of MDMA include loss of appetite, jaw clenching, trismus, bruxism, headache, nausea, sweating, muscle aches, fatigue, and insomnia. MDMA can also cause acute toxic reactions with tachycardia, hypertension, arrhythmia, panic attack, and psychosis up to severe intoxication and death. Acute medical complications include malignant hyperthermia, seizures, cerebral haemorrhage, hepatitis, rhabdomyolisis, disseminated intravascular coagulation, and acute renal failure. Animal studies in rats and primates have shown that MDMA acts as serotoninergic neurotoxin. Upon these observations, it has been hypothesised that MDMA might produce long-term reductions in serotoninergic activity and degeneration of serotoninergic neurons in humans. Some studies associate chronic heavy use of ecstasy with persistent psychological deficits and cognitive impairment. Differently from classical amphetamines that once ingested seem to restore fatigue-induced impairment and decrease the reaction time, MDMA, especially at high doses (> 75 mg), contributes to a decline in psychomotor performance. Indeed, MDMA has been implicated in fatal traffic accidents probably due to impairment in driving related tasks and potentiation of risky driving.

MDMA analysis in biological fluids has been confined up to date to clinical pharmacology studies and to forensic science cases. New developments in clinical toxicology including roadside on-site testing analysis of potentially intoxicated drivers and drug testing at the workplace require new analytical approaches and the use of alternative biological matrices for the detection of MDMA consumption. Objectives of these new clinical toxicology applications include the possibility of establishing whether individuals have consumed the drug, when and how much and/or if they have been acting under the effect of the drug.

Although blood and urine sampling have been consistently used for these purposes, drawbacks associated with these bodily fluids (invasive procurement, privacy issues, availability of techniques to tamper positive results) have questioned their utility in those situations, in which other biological fluids could provide the required information.

Saliva, sweat and hair are alternative biologic matrices, which have been extensively and successfully used to assess recent and past and/or acute and chronic exposure to drugs of abuse.

In fact, saliva is the only fluid that has been used as an alternative to blood in several pharmacokinetic and pharmacotoxicologic studies and there is evidence that when a given drug is detected in salivary specimens, there is a high likelihood for a subject being under the pharmacologic effects of the drug. This last statement can be true also in case of sweat. Indeed sweat is another alternative matrix for drugs of abuse testing, that, depending on the approach of sample collection (cotton wipe *vs.* sweat patch), may be oriented to detect recent drug use or monitoring drug consumption for time-windows wider than those provided by urine. On the other hand, hair analysis can provide information of past use of a drug up to year(s) even on monthly basis.

Although a number of scientific articles came out in recent years, which showed the detection of MDMA and its principal metabolites in saliva, sweat and hair of drug consumers, the results obtained were referred as a simple evidence of active exposure to the drug at present or in the past. In fact, due to the lack of clinical studies correlating analytical findings to drug effects, no definitive answer could be given regarding the association of the analytical data to the moment of drug consumption, to the consumed dose and to the eventual acting under the influence of the drug itself.

Furthermore, the new mandatory guidelines of the U.S. Substance abuse and Mental Health Services Administration (SAHMSA) on the use of saliva, sweat and hair for drug testing at the workplace, the need of several European Countries (U.K., Belgium, Germany, Spain, Italy) of validated methodologies for non invasive specimens collection for both drug testing inside and outside medical settings advocated for systematic studies on the distribution profile of illicit drugs in the above mentioned biological matrices after controlled (or monitored) administration. These kinds of studies are needed to solve the problem of analytical results interpretation and to provide generalized evaluation criteria that could be applied not only to single cases, but also to larger segments of the population (e.g. workers, drivers). This issue is of relevance for the possible legal implications of positive findings in these settings.

Within this context, the Department of Pharmacology of the Institut Municipal d'Investigació Mèdica (Barcelona, Spain) in an international cooperation with the Department of Drug Research and Control from the Istituto Superiore di Sanitá (Rome, Italy) set up a series of studies during the last four years regarding the usefulness of saliva and sweat testing for the detection of MDMA after a single controlled drug administration and the suitability of hair testing to monitor past chronic exposure to MDMA in a follow-up study of consumers.

On one hand, this thesis illustrates the results obtained in the clinical trials, which aimed to correlate MDMA kinetics in saliva and sweat after a single drug administration to subjective effects discussing the meaning of the qualitative and quantitative analytical data in the light of possible influence of the drug on individual behaviour.

On the other, the thesis illustrates the results of segmental hair analysis of MDMA in a cohort of consumers during a follow-up study, which aimed to look for an objective biomarker of the history of drug consumption and its eventual relationship with evolution of cognitive performance and psychopathology prevalence.

Analytical methods for confirmatory purposes, such as specific devices for sample collection and on site qualitative testing have been set up and validated to identify and quantify the parent drug and eventual metabolites present in the mentioned matrices.

Eventual association of drug concentration in saliva, sweat and hair with psychomotor performance, subjective effects and self-reported consumption is also discussed together with potential applications of data obtained in clinical toxicology.

1. Introduction

1. Introduction

1.1 Drug distribution in humans: nonconventional fluids and matrices

To produce its characteristic effects, a drug must be present in appropriate concentrations at the site(s) of action. Although a function of the quantity of drug administered, the drug concentrations attained also depend upon the ADME cycle of the drug itself: the extent of drug absorption, distribution in different matrices and fluids, biotransformation and excretion (Figure 1) (Rowland, 1989).

Up to the Eighties, the presence and the disposition of a drug inside the human body, and eventual association with clinical/subjective effects had been attained by plasma and urine testing, since it was not always possible or desirable (because difficult and/or invasive) to sample other biological matrices and fluids. Nonetheless, in the last two decades measurement of drug concentration in fluids and matrices other than blood and urine (the so called "nonconventional fluids and matrices") gained increasing importance (Pichini, 1996). On one hand, improved technology (non invasive sample collection, dedicated devices for sample collection, different possibilities of extraction procedures and new analytical methods) has made possible the measurement of minute quantities of substances extracted from complex biological matrices. On the other hand, it appeared that the determination of drug and metabolite concentrations in nonconventional human body materials may be useful for two principal applications: firstly the possibility of determining pharmacokinetic parameters at the target organ and target concentration intervention; secondly and most importantly the application of the information obtained by drug testing in non conventional fluids and matrices in clinical and forensic toxicology.

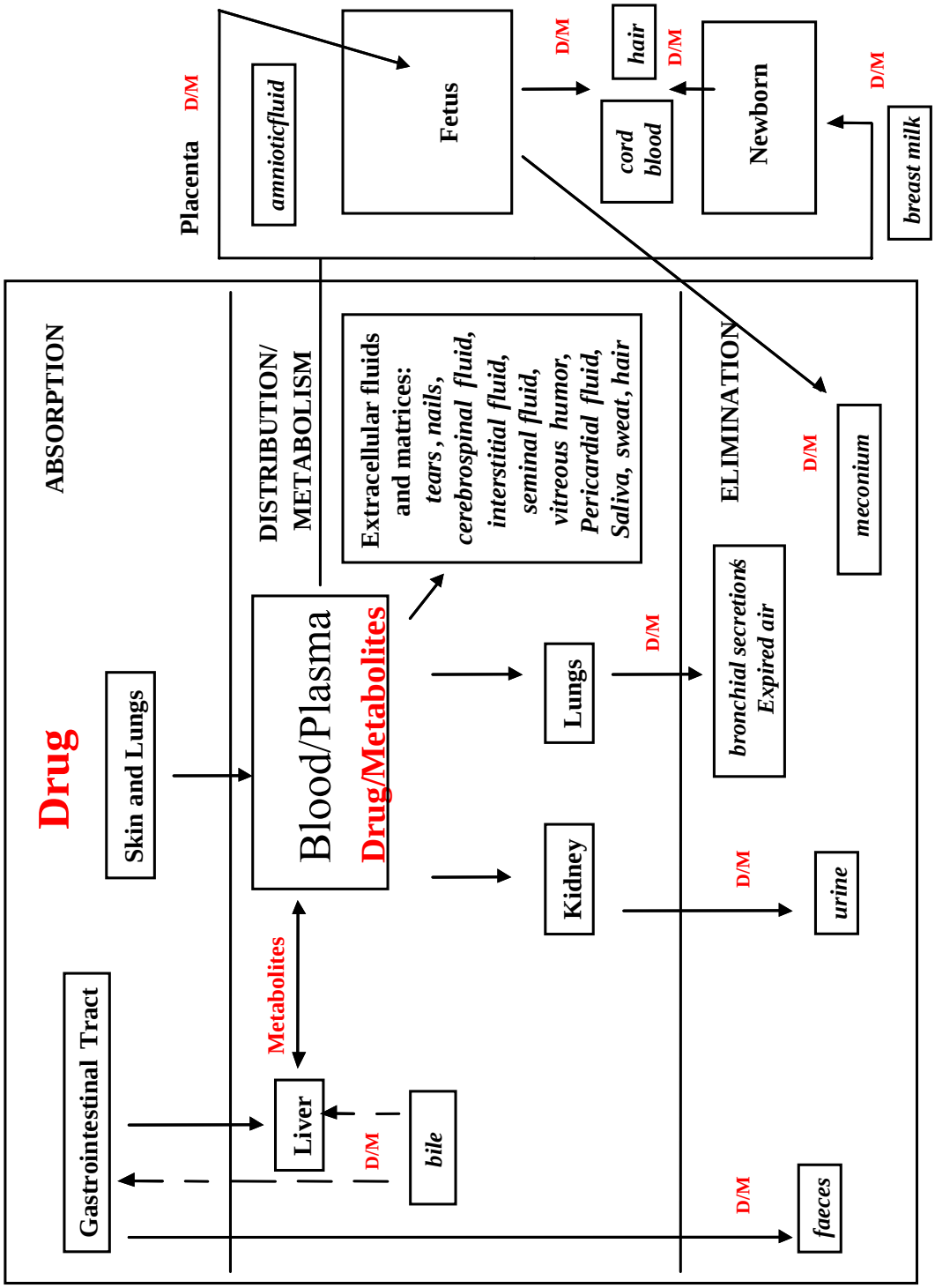


Figure 1. A scheme of drug disposition inside the human body (D/M=drug/metabolite(s))

For instance, concerning drug target intervention, the determination of drug (and metabolites) concentration in matrices such as tears, nails, cerebrospinal fluid, bronchial secretion, peritoneal fluid, seminal and interstitial fluid is important as these reveal the presence of a drug in the target organ or at the site of action with eventual related physiological and morphological changes; others such as bile and faeces provide information on excretion route (entero-hepatic circulation) of drugs and metabolites different from that measurable in urine. In the field of toxicology: vitreous humour, pericardial fluid together with cadaveric blood collected at different body sites are generally sampled for forensic purposes (Drummer, 2004). Conversely, matrices such as hair or nails can give information on past ingestion of drugs over a period of weeks up to years and can be used in forensic cases (looking for a cause or a secondary cause of death) and clinical cases (looking for a cause of a chronic clinical effect). Saliva and sweat can account for recent consumption and drug testing in these matrices can be associated with acute subjective effects. Some other matrices and fluids related to gestation/delivery period are also important for toxicological purposes: drug presence in foetal or cord blood and amniotic fluid or foetal hair and meconium can account for intrauterine acute and chronic exposure to xenobiotics.

Among the above-mentioned fluid and matrices, there are some, which show specific and restricted areas of interest, while others can give information useful for several applications in toxicology (Table 1). Furthermore it cannot be forgotten that other attractive aspects of alternative matrices are the need of a minimal technology for collection (and there is no need for a medical environment) and further processing and the possibility of performing on-site tests. As shown in Table 1, for their characteristics (possibility of different application in forensic and clinical toxicology, lack of invasiveness and difficulties at the moment of collection) saliva, sweat and hair have been the alternative biologic substrates

most commonly used for the analysis of drugs and particularly drugs of abuse in the field of forensic and clinical toxicology. For this reason, these three matrices will be described in details in the following sections, focusing on their composition, the physiology of their production, advantages, and limitations and application of provided information. Conversely, the other matrices and their fields of application will be briefly mentioned, together with the theory of mechanisms underlying the passage of a drug from blood to extravascular fluids and matrices.

Fluid/matrix	Clinical Pharmacology/ Pharmacokinetics/ TDM	Forensic Toxicology	Clinical Toxicology/ Acute toxicity/TDM	Correlation with plasma concentration	Correlation with pharmacological/ Toxicological effects	Invasiveness	Difficulty of procurement
Plasma	+++	+	+++		+++	++	+/-
Urine	+	+	+++	+/-	+++	+	-
Tears	+/-	-	-	-	-	+	-
Nails	+/-	++	-	-	-	-	+/-
Cerebrospinal fluid	++	-	-	+	++	+++	++
Bronchial secretions	+	-	+	-	++	-	-
Interstitial fluid	+	-	+	++	+	+++	++
Seminal Fluid	+	-	++	+/-	++	-	++
Bile	+/-	+	-	+/-			
Faeces	+/-	-	-	-	-	-	+
Meconium	-	+	+	-	++	-	++
Cord blood	-	-	++	++	++	+	+/-
Amniotic fluid	-	+	+	+/-	++	+++	+++
Breast milk	+	-	++	+	++	-	++
Vitreous humour	-	+++	-	+/-	+	+++	+++
Pericardial Fluid	-	+++	+	+/-	++	+++	+++
Saliva	+++	+	+++	++	+++	-	-
Sweat	++	+	++	+/-	+++	-	+/-
Hair	++	+++	+	+/-	++	-	-

Table 1: Suitability of nonconventional matrices in forensic and clinical toxicology

1.1.1 Mechanism of the passage of a drug to extravascular fluids and matrices

The passage of a drug from the blood or plasma to another fluid (or matrix) has been extensively studied for saliva, cerebrospinal fluid (CSF), bile, amniotic fluid, milk and semen, but remains uncertain or unknown for many other matrices (Rowland M, 1989; Goodman and Gilman, 1992; Clarke, 1986).

In general, drug passage into a fluid (or matrix) occurs by passive diffusion and appears to be regulated by the physicochemical characteristics of the drug (such as molecular weight, molecular volume, dissociation constants, lipid solubility and protein binding). For a lipid-soluble compound, the ratio of total drug concentration (ionised and unionised) in a fluid, which is separated from the plasma by a lipid barrier, may be expressed by a modified version of the Henderson-Hasselbach equation:

$C_F/C_P = 1+10(pH_F-pK_a)/1+10(pH_P-pK_a)$ (for a weak acid)

$C_F/C_P = 1+10(pK_a-pH_F)/1+10(pK_a-pH_P)$ (for a weak base)

Where C_F and C_P represent the total concentration of each drug in a fluid and plasma, respectively, pK_a is the negative logarithm of the acid dissociation constant for each drug, and pH_F and pH_P are the negative logarithms of the hydrogen ion concentration in fluid and in plasma, respectively.

According to these equations, unbound drugs that are weak bases will concentrate in fluids whose pH is lower that that of the plasma; these drugs will only achieve concentrations similar to those in plasma in fluids with a pH approaching that of plasma. The opposite situation occurs for drugs that are weak acids. The fluid/plasma concentration will be near

unity for drugs that are neither acidic nor basic. It has to be said that principal drugs of abuse, but particularly psychostimulants such as cocaine, amphetamines and "designer drugs" are weak bases with pKa higher than 8-9. For this reason, and due to the fact that several body fluids and matrices show a pH lower than plasma (e.g. saliva, sweat, cerebrospinal fluid, seminal fluid, etc.) the excretion of these substances in those fluids and matrices is at least theoretically relevant and potentially interesting for purposes of clinical and forensic toxicology.

1.1.2 Tears

The development of sensitive techniques for drug analysis have made it possible to measure minute amounts of drugs in small volumes of biological fluids as tears (Van Haeringen, 1985). Similarly to saliva, tear samples can be obtained without discomfort to patients or volunteers, and drug concentrations in tears represent the non-protein bound fraction of a drug. Furthermore tear fluid, especially after stimulation, can be more homogeneous and constant in its composition than saliva (Haeckel and Hanecke, 1993). Nonetheless, for many compounds (sulphonamides, salicylic acid, benzylpenicillin), the tear/plasma concentration ratio neither has a good correlation nor is near unity, and hence is useless for target concentration intervention or bioavailability studies (Van Haeringen, 1985). These discrepancies are mainly due to tear's pH, which is the chief variable determining the concentration of drugs in tears. In practice, theoretical calculations predict that unionised and acidic drugs with low pKa values distribute in tears in a predictable manner. Drug measurement in tears has been proposed for monitoring oculomucocutaneous syndrome caused by practolol (Van Haeringen, 1985).

1.1.3 Nails

Like head and body hair, nails are a biological matrix in which drugs tend to accumulate during long-term exposure. Drugs are incorporated in nails by a double mechanism: (i) deposition into the root of the growing nail via the blood flow in the nail matrix: and (ii) incorporation via the nail bed during growth from the lunula to the beginning of the free margin. Together, these mechanisms account for a wide retrospective window of drug detection (Palmeri et al., 2000). Although nails are subject to external contamination, they are an interesting substrate for the investigation of long-term drug abuse and forensic examination. Some authors have reported the accumulation of methamphetamine in the nails of habitual users and the presence of cocaine in the same quantity as that found in hair, and that of the cocaine metabolite benzoylecgonine in a larger amount than in hair (Suzuki and Inoue, 1989). Both stimulants were more concentrated in toenails than in fingernails (Suzuki, 1984). MDA and MDMA were also found in fingernails of users with concentrations slightly higher than in head hair (Cirimele et al., 1995).

1.1.4 Cerebrospinal fluid

Investigation into drug distribution in the cerebrospinal fluid (CSF) is of great importance, for two main reasons:

(1) drug and adverse effects on the CNS have unique clinical relevance because of the severity of the situations that the drugs are supposed to either cure or cause; and

(2) the blood-brain barrier, which drugs must cross to reach the specific sites of action or toxicity, is characterized by particular properties of permeability.

For this second reason, drug measurement in CSF is necessary to establish if the concentration required for the desired pharmacological effect in the CNS has been reached, or when physiological, pathological and anatomical modifications to the blood-brain barrier allow the penetration of molecules, which may severely affect the CNS. Although the CSF compartment cannot be sampled easily (collection is by invasive techniques, i.e. lumbar puncture), several drugs such as anticonvulsants, psychoactive drugs, antineoplastics and opioids have been monitored in this matrix (Bonati et al., 1982; Pichini et al., 1992; Maurer, 1998).

1.1.5 Bronchial secretions

Monitoring antibiotic concentrations in bronchial secretions may provide indirect evidence of parenchymal concentrations of drugs used in bronchial infections. Indeed, this monitoring can support local drug administration in spite of systemically one, since drugs administered systematically may not always reach those parts of the body where they are most needed (Pichini et al., 1996). Sample collection is very invasive and requires suitably trained staff to perform it. Patients usually have a tracheotomy or an endotracheal tube fitted, allowing access to the tracheobronchial tree; a suction catheter is the used to collect sputum samples. In particular, the measurement of antibiotic concentrations in bronchial secretions has proved to be useful in evaluating the penetration of the blood-bronchoalveolar barrier by ampicillin, cefalothin, gentamicin and beta lactams administered during episodes of pneumonia or bronchitis (Ehrlich et al., 2001).

1.1.6 Interstitial fluid

Drugs are delivered transdermally when administered: topically in skin diseases (psoriasis, dermatosis, etc.); in form of transdermal delivery systems to ensure constant release of low concentration of drugs for a whole day (e.g. nicotine, nitroglycerin and theophylline patches) and most importantly when they are used in neonatal therapy (Kalia et al., 1998; Barrett et al., 1994).

In this last case, by using transdermal drug delivery it is possible to circumvent some of the normal difficulties encountered with oral or intravenous drug administration in neonatal therapy. In order to verify drug penetration through neonatal skin and to assess the feasibility of such an administration route, the interstitial fluid of neonates has been collected by use of a transcutaneous collection systems and monitored for the drugs (e.g. theophylline or buprenorphine) transdermally administered for apnoea or chronic lung disease associated with prematurity (Murphy et al., 1990; Barret et al., 1994).

1.1.7 Seminal fluid

It is known that drugs can be transported to the seminal fluid and can directly affect sperm cells, resulting in functional, physiological and metabolic changes (Pichini et al., 1994). Furthermore, exposure of spermatozoa to xenobiotic agents can potentially have adverse effects on the male gametes, and may cause male-mediated teratogenic effects and adverse effects on the outcome of a pregnancy (Friedler, 1985). To better evaluate the influence of drugs on seminal fluid, the presence of several drugs and drugs of abuse in the male accessory gland and the extent to which a drug distributes from blood into the male genital tract have been investigated (Smith, 1981, Pacifici et al., 1993; Pichini et al., 1994; Pacifici et al., 1995; Cone et al., 1996) and eventually associated to changes in semen

functionality. Finally, as previously stated, the measurement of drug concentrations in semen is important not only to study the effects on reproductive physiology but also to evaluate the pharmacological effects and adverse drug reactions in women exposed to drugs excreted in semen, with possible consequences on pregnancy outcome (Friedler, 1985).

1.1.8 Bile

Biliary excretion is an important route of elimination of some drugs and metabolites in humans (Rollins and Klaasesen, 1979; Chandra and Brouwer 2004). The effect of this excretion on drug pharmacokinetics is unique, since drugs cleared by the biliary route may undergo enterohepatic circulation or elimination in the faeces. The lack of reliable information regarding the biliary excretion of drugs in humans is partly due to the relatively inaccessibility of the human biliary tract. Nonetheless, several drugs (such a as penicillins, cephalosporins, aminoglycosides and cardiac glycosides) have been determined in bile and the bile-to-plasma ratio calculated as an indication of the extent to which the drug is concentrated in bile (Karachalios and Charalabopoulos , 2002).

1.1.9 Faeces and meconium

The use of faeces as a biological matrix for drug concentration monitoring is usually limited to studies in new drug development and to when initial pharmacokinetic parameters need to be defined for a drug and any metabolites in human and animal models (Nave et al., 2004; Sandborn and Hanauer, 2003) In addition, quantification of drug and metabolites in faeces specimens can provide information regarding enterohepatic circulation and biliary excretion of a drug, difficult to achieve by bile analysis because of the inaccessibility of human biliary tract. A particular application of drug monitoring in faeces is determination of

xenobiotics in meconium. Meconium is the first faecal matter passed by a neonate. Its formation starts between the 12th and 16th week of gestation and usually accumulates in foetal bowel until birth and the neonate passes it one-five days after birth. For this reason, meconium analysis allows the detection of maternal drug use to approximately the last 20 weeks of gestation and consequently provides information of foetal chronic exposure to drugs, being more informative than urine for the detection of drug exposure in pregnancy (Pichini et al., 1996; Ostrea et al., 1988; Koren et al., 2002). In recent years, drug testing in meconium has been successfully applied to assess intrauterine exposure to drugs of to provide the basis for appropriate treatment and follow-up of newborns, which can present symptoms of drug withdrawal and impairment in physical and mental development (Pichini et al., 2003a; Pichini et al., 2003b; Pichini et al., 2004; Garcia-Algar et al., in press)

1.1.10 Cord blood and amniotic fluid

Drugs administered to pregnant women have the potential to cross the placenta and reach the foetus (Szeto, 1993; Pacifici and Nottoli, 1995). Measuring concentrations in foetal blood and/or amniotic fluid can show transplacental passage of drugs and metabolites during pregnancy; furthermore, comparison of the values found with drug concentrations in maternal blood allows calculation of the extent of such passage and a rough estimation of the quantity of foetal exposure to drugs. Since both amniotic fluid and foetal blood collection are invasive procedures that can be harmful to the foetus, the development of new techniques for the sampling of blood from the cord vein and artery has permitted sampling of cord blood from mid-gestation to term of gestation and preferably at delivery. The presence of a drug or metabolite in amniotic fluid and/or cord blood may also explain the toxicity of agents whose administration during pregnancy has been associated with foetal diseases, such as the case of

drugs of abuse. Indeed, many authors investigated these biological matrices for the presence of illicit drugs (Ripple et al., 1992; Dempsey et al., 1998; Jauniaux et al.,1999; Winecker et al., 1997; Pichini et al., 2000). Nonetheless, differently from information obtained by testing meconium or foetal hair, amniotic fluid but especially cord blood objectively assesses an acute exposure, which can account (or cannot) a chronic exposure.

1.1.11 Breast milk

The major reason for drug investigation in human milk is to calculate excretion of drug in this fluid and, consequently, the approximate dose ingested by breastfeeding infants (Atkinson et al., 1988). The dose received by a breastfeeding infant can be estimated by performing a standardised pharmacokinetic study designed for this purpose. This information is important not only to protect nursing infants from untoward effects of maternal medication, but also to allow effective pharmacologic treatment of breastfeeding mothers (American Academy of Paediatrics Committee on Drugs, 2001).

It should be noted that for many drugs the percentage of the maternal dose received via breast milk is below 1 % or between 1 and 10 %; there are, however, some for which the percentage exceeds 50% (e.g. phenobarbital, theophylline) (Atkinson et al., 1988; Pons et al., 1994). It must also be taken into account that pharmacokinetic parameters of children are continuously changing, particularly in premature neonates.

Some drugs of abuse (cocaine, morphine, phencyclidine, amphetamine tetrahydrocannabinol) have been determined in the breast milk of addicted mothers (Perez-Reyes and Wall, 1982; Steiner et al., 1984; Robieux et al., 1990; Pons et al., 1994; Winecker et al., 2001) and high concentration of illicit drugs was found in case of phencyclidine or amphetamine. These data and the lack of definitive information regarding heroin and

metabolites excretion in milk prompted the statement that mothers using those drugs should not breastfeed (Pons et al., 1994).

1.1.12 Vitreous humour

The vitreous humour is a clear gel that occupies the posterior compartment of the eye, located between the crystalline lens and the retina and occupying about 80% of the volume of the eyeball. Light initially entering the eye through the cornea, pupil, and lens, is transmitted through the vitreous to the retina. Vitreous humour has the following composition: 1. water (99%), 2. a network of collagen fibrils, 3. large molecules of hyaluronic acid, 4. peripheral cells (hyalocytes) 5. inorganic salts, 6. sugar, 7. ascorbic acid (Rodieck, 1988). Vitreous humour can be collected easily by direct aspiration during autopsy, is generally less subject to post-mortem changes because of its compartmentalization, and may be present in decedents when other biological fluids are not available (e.g. decomposed bodies). Drugs and drug metabolites passively diffuse into the vitreous humour and analyte concentrations are often similar to the drug concentrations in the circulating blood (Hepler and Isenschmid, 1998). Several drugs of abuse, therapeutic drugs, poisons and/or their metabolites have been determined in humour vitreous of decedents to assess exposure to the above-mentioned substances and to look for possible causes/concomitant causes of death (Ferrara et al., 1995; Maurer, 1998; Chronister et al., 2001). Indeed, the use of vitreous humour as an alternative sample to blood in post-mortem samples was investigated for the detection of heroin abuse by quantifying levels of morphine and 6-monoacetylmorphine (6-MAM) (Scott and Oliver 1999; Wyman and, Bultman, 2004) and for the identification and quantification of cocaine and metabolites (Mackey-Bojack et al., 2000, Chronister et al., 2001). Finally, MDMA and MDA levels were determined in vitreous humour in post mortem cases to investigate the

redistribution of the parent drug and its metabolite in fatal overdoses (Clauwaert et al., 2000; de Letter et al., 2002; de Letter et al., 2004).

1.1.13 Pericardial fluid

Pericardial fluid, also defined intrapericardial fluid, is an amount of 5-10 ml fluid, which bathes the coronary vessels and myocardium. Although classically defined as an ultrafiltrate of plasma, pericardial fluid actually varies significantly from plasma with respect to its composition and concentration of a range of factors. This is so because it is locally conditioned by by vascular tissues as well as myocardiocytes and mesothelium (Dickson et al., 1999). Sodium and chloride distributions were found to be not markedly different from the ratio predicted for a passive distribution; calcium and magnesium were distributed in a manner expected from a passive ultrafiltrate of plasma and the potassium concentration was higher than that of plasma. It was suggested that the elevated potassium concentration of pericardial fluid might reflect the lability of the cardiac intracellular potassium during cardiac contraction. Pericardial fluid was found to contain between one quarter and one third of the protein of plasma with a far higher proportion of albumin to other proteins with thee osmolality slightly lower than that of plasma, and a similar pH (Gibson and Segal, 1978). Pericardial fluid has been traditionally used as a as an alternative specimen to blood for post-mortem toxicological analyses and resulted as a good sample for quantitative confirmation of analyses performed on blood samples or a quantitative alternative to blood in exsanguinated victims (Moriya and Hashimoto, 1999). Fairly good correlations were observed between blood and pericardial fluid for several illicit drug drugs: both neutral and basic drugs and acidic drugs with fluid to plasma ratio always approaching the unit (Moriya and Hashimoto, 1999). Opiates, barbiturates, benzodiazepines, amphetamines and “designer drugs” including

MDMA have been detected in pericardial fluid from fatalities (Moriya and Hashimoto, 2000; Dams et al., 2003)

1.1.14 Saliva

Saliva is a complex fluid produced by a number of specialized glands which discharge into the oral cavity of the glands of mammalian vertebrates. Most of the saliva is produced by the major salivary glands (parotid, submandibular, and sublingual), but a small contribution is made by the numerous small labial, buccal, and palatal glands that line the mouth (Van Dam and Van Loenen, 1978; Vining and McGinley, 1986) (Figure 2).

Parotid glands are located in the palate and secrete a type of serous saliva directly coming from plasma, which shows a high concentration of electrolytes; sublingual glands, which constitute the 70% of salivary glands are located at the side of buccal cavity and their serous and mucus saliva contain amylase, mucoproteins and mucopolisaccarides. Finally, submandibular gland, located behind the tongue produce a serous and mucus fluid (Huestis and Cone, 1998; Kidwell et al., 1998). Indeed, the parotid glands are "serous" glands, for their acinar cells contain only serous-secreting cells, whose secretions are devoid of mucin compared to that of the submandibular and sublingual glands, which contain both serous- and mucin-secreting cells (Davenport, 1977). The viscosity of the submandibular saliva usually decreases with increasing flow rate since the serous cells have a greater response to stimulation than do the mucin-secreting cells. The sublingual gland contains predominantly mucin-secreting cells and thus their secretion has a thick, viscous nature (Vining and McGinley, 1986).

Salivary glands have a high blood flow (Haeckel, 1990). The external carotid arteries enter the submandibular and sublingual glands along with the main ducts and nerves, thereby

creating a hilum, although this hilum is not as clearly defined as in larger organs such as the kidney. The direction of the blood flow is countercurrent to the direction of the salivary flow (Davenport, 1977).

Clearly, before any drug circulating in plasma can be discharged into the salivary duct it must pass through the capillary wall, the basement membrane and the membrane of the glandular epithelial cells. The rate-determining step for this transportation is the passage of the drug through the lipophilic layer of the epithelial membrane. Physicochemical principles dictate that for such a passage to occur, drug must show a degree of lipophilicity.

However, saliva is not a simple ultrafiltrate of plasma, as has sometimes been suggested, but rather a complex fluid formed by different mechanisms: by a passive diffusion process, by an active process against a concentration gradient, by ultrafiltration through pores in the membrane, or by pinocytosis (Caddy, 1984). An active transport mechanism clearly operates for many electrolytes and for some proteins such as IgA. An active transport mechanism has also been suggested for some drugs. However, most mechanisms of active transport are not well understood.

Salivary secretion is a reflex response controlled by both parasympathetic and sympathetic secretomotor nerves. Stimulation of sympathetic fibres to all glands causes vasoconstriction; in man, stimulation of the sympathetic trunk in the neck or injection of epinephrine causes secretion by the submaxillary but not by the parotid glands.

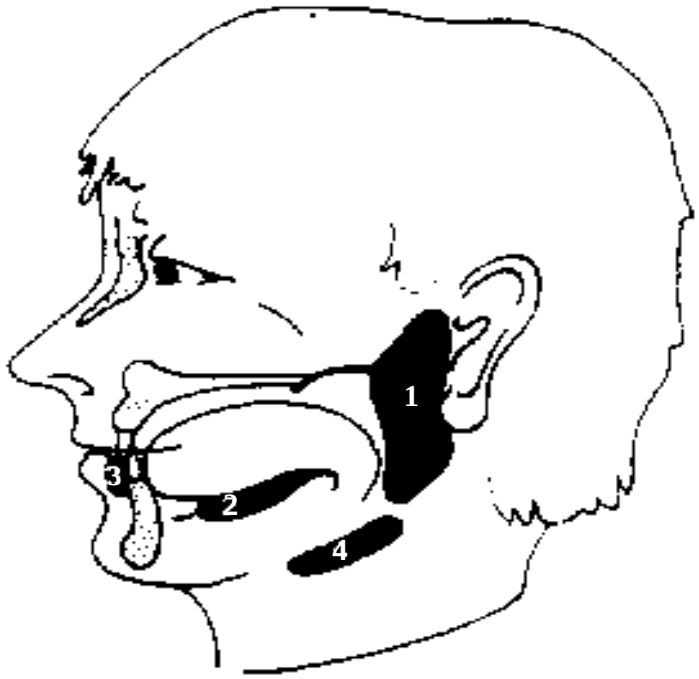


Figure 2. Topography of the salivary glands: 1. glandula parotis; 2. glandula sublingualis; 3. glandula labialis; 4. glandula submandibularis. (From: Van Dam and Van Loenen, 1978).

During embryologic life, the parotid gland, the first of the three major glands to appear, is seen by the 6th week. It derives from the ectoderm as a primitive oral epithelium. In the submaxillary gland, the primordia appear at the end of the 6th week and, unlike the parotid, are probably of endodermal origin. The sublingual glands are the last of the three major salivary glands to appear. They are, like the submandibular gland, probably endodermal in origin. Cell differentiation starts in the secretory ducts with the progressive transformation of ciliated cells into columnar, squamous, and goblet cells. Intralobular ducts and acinar differentiation, including myoepithelial cell formation, begins about the 8th month. Saliva

production starts at this time as a mucinous liquid; however, several studies in rodents suggest that full maturation is completed only after birth (Martinez-Madrigal and Micheau, 1989).

Saliva, like other body fluids, is a dilute aqueous fluid containing both electrolytes and protein with an osmolality less than or equal to that of plasma. Also present in saliva is a certain amount of cell debris arising from the epithelial cells of the mouth together with food residues (Caddy, 1984). The osmolality is principally determined by the type of gland and by secretory activity, whose degree is affected by many factors including sex, age, nutritional or emotional state, season of the year (Mandel, 1974), darkness (Shannon and Suddick, 1973), and a variety of diseases and many pharmacological agents. Circadian variations have been shown in unstimulated and stimulated saliva for flow-rate, pH and some salivary constituents (Ferguson and Fort, 1974).

The total volume of saliva produced each day in adults is 500 to 1500 ml with a flow arte between 0.1-10 ml/min (Lentner, 1981). Mixed saliva, which is the most accessible and most frequently used for drug analysis, consists mainly of the secretions of submandibular (65%), parotid (23%), and sublingual (4%) glands, the remaining 8% being provided by the minor numerous glands (Caddy, 1984). These proportions are a function of the type, intensity and duration of stimulation. The important stimulus for secretion is the presentation and ingestion of food; the quantity and quality of the secretion vary with the nature of nutrition. A comparison of the compositions of saliva and plasma is given in Table 2 (From: Ritschel and Thompson, 1983). Water is the major saliva constituent (90-99%). Other components are: proteins (and above all enzymes) amylase, glucose, urea, lipids and hormones (Kidwell et al, 1998; Huestis and Cone, 1998).

The bicarbonate concentration of saliva is highly dependent upon the gland type, nature of the stimulation, and flow rate; it may be larger than or less than the plasma

concentration. As a result of a concomitant increase in bicarbonate concentration, the salivary pH rises with increasing rates of secretion. Saliva pH can range from 6.2 to 7.4, with the higher pH exhibited upon increased secretion (Drobitch and Svensson, 1992).

Table 2: Parametric correlation of saliva to plasma

Parameter	**Mixed Saliva**	**Plasma**
Volume	500-1500 ml/day	4.3% of BW*
Rate of flow	0.6(0.1-1.8) ml/min	
pH	6.7(5.6-7.9)	7.4
Water [%]	98(97-99.5)	91.5(90-93)
Total protein [g/100 ml]	0.3(0.15-0.64)	7.3 (6-8)
Albumin [g/100 ml]		4.5(4-5)
Mucin [g/100 ml]	0.27(0.08-0.6)	
Amino acids [mg/100 ml)	0.1-40	0.98
Electrolytes [mMol/l]		
Potassium	8-40	3.5-5.5
Sodium	5-100	135-155
Calcium	1.5-2	4.5-5.2
Phosphate	5.5-14	1.2-2.2
Chloride	5-70	100-106
Cholesterol [mg/100 ml]	7.5(3-15)	150-300
Dry Substance [g/l]	6(3.8)	80
*BW = Body Weight		

The large variations in some constituents of saliva are the result of different collection techniques, devices and flow rates. Drugs are secreted in saliva by passive diffusion, produced by gradient concentration and drugs that are liposoluble and with a low molecular weight are the main candidates for this type of secretion. Minor mechanisms of secretion are: active transport, controlled by CNS and ultrafiltration for molecules with higher molecular weight (> 300 Da).

Saliva is undoubtedly the nonconventional matrix most commonly used in place of blood for the concentration monitoring of many drugs, and for pharmacokinetic and pharmacotoxicological studies and there is evidence that when a given drug is detected in salivary specimens, there is a high likelihood for a subject being under the pharmacologic effects of the drug (Pichini et al., 1996). This is because of some distinct advantages:

(1) saliva is easily collected without the need for specials skills; hence, drug monitoring using saliva may be more cost effective that that using blood;

(2) drug concentration monitoring in saliva is especially important for studies in children, elderly patients and pregnant women, and in all situations where samples need to be obtained without exposing a patient to discomfort, skin irritation and risk of infection;

(3) a non-invasive fluid collection is preferred when multiple serial samples are required; and, in the case of outpatients, samples can be collected at home;

Furthermore, the rationale for measuring the concentration of a drug in saliva is that the pharmacological action depends on the unbound fraction of the drug in the plasma; this fraction is usually excreted by the salivary gland into the saliva.

Some disadvantages, however, have to be mentioned:

(1) collected volume is generally small, unless salivation is not stimulated;

(2) oral contamination from certain routes of administration (smoking, snorting, oral ingestion) can influence drug concentration;

(3) the method of sample collection may influence salivary drug concentrations as a result of changes in pH and flow rate (Kidwell et al., 1998).

The primary determinants that appear to govern the potential utility of drug measurements for target concentration intervention are the following:

(1) the drug and any metabolites must be excreted in the saliva and remain stable in this fluid, with minimal effects of saliva pH or of salivary flow stimulation;

(2) saliva flow can be stimulated to ensure adequate sample volume;

(3) saliva offers less possibilities of adulteration as compared with urine specimens ;

(4) a correlation between drug concentration in plasma and that in saliva over a wide concentration range is needed;

(5) the saliva/plasma drug concentration ratio must remain invariate with regard to single-dose, interdose interval and steady-sate estimates;

(6) the pharmacokinetic parameters in saliva must be similar to those in plasma.

Several methods have been described for the collection of mixed saliva. In many studies claiming to have utilized unstimulated whole saliva, the subjects have usually been asked to spit directly into a collection tube. This spitting itself is usually a sufficient stimulus to elicit a flow of about 0.5 ml/min. Although most patients prefer donating saliva rather than blood, a substantial social barrier exists to "spitting". For this and other reasons, subjects often experience decreased salivary secretion (dry mouth) if asked to provide a sample. Many researchers have found it advantageous to further stimulate salivation and a number of stimuli have been used. Chewing paraffin wax, Parafilm®, rubber bands, pieces of Teflon or chewing gum (Dawes and Macpherson, 1992) will usually elicit a flow of 1 to 3 ml/min. The use of

acid lemon drops or a few drops of 0.5 mol/l citric acid are among the most potent of taste stimuli and will generally induce a maximal secretion of 5 to 10 ml/min (Vining and McGinley, 1986). For studies requiring high saliva flow rates for extended periods of time, secretion-stimulating drugs, such as the parasympathomimetic drug pilocarpine, have sometimes been used orally, subcutaneously, or intravenously. However, in doses sufficient to produce very high flow rates, parasympathomimetic drugs have undesirable side effects such as flushing, palpitations, colicky abdominal pains, and an urgent desire to micturate (Höld et al., 1995a).

There are several advantages of stimulating salivary flow:

(1) Large volumes of saliva can be obtained within a short time;

(2) The pH of stimulated saliva mostly lies within a narrow range around the value of 7.4, whereas the pH of unstimulated saliva shows a larger variability, that may be of importance for the salivary secretion of weak acidic and basic compounds (Ritschel and Thompson, 1983);

(3) The intersubject variability in the S/P ratio may be diminished when stimulated saliva is used, as it has been reported for digoxin. On the other hand, it is possible that the concentration of drugs in saliva is affected by stimulating salivary flow. For instance, any physical or chemical stimulus used during the collection of saliva must not absorb or modify the compounds to be measured, nor must it introduce interfering factors into the assay procedure (Danhof and Breimer, 1978).

Cooper et al. (1981) were the first to use a dental cotton roll to collect saliva in order to monitor desipramine. Over the years their method has undergone some improvements, and the dental cotton roll is nowadays available as the Salivette® (Sarstedt, Germany). The procedure for collection is as follows: the Salivette® is used to absorb the saliva into a dental cotton roll

after chewing for 30-45 seconds with or without stimulation. After being soaked with saliva, the dental roll is placed in a container that is closed with a plastic stopper. The container fits into a polystyrene tube that is then centrifuged for 3 minutes at about 1000 g. During centrifugation the saliva passes from the cotton roll into the lower part of the tube. The container is then taken out of the tube and the clear saliva is poured out of the tube. Cellular particles are retained at the bottom of the tube in a small sink compartment. A disadvantage of the Salivette® is that the dental cotton roll interferes with several hormone and drug assays, such as that for testosterone. The advantage of the Salivette® over many other sampling devices is that it reliably absorbs a relatively large volume of saliva (1.5 ml) in a short time (Höld et al., 1995b). The OraSure® (OraSure Technologies, Inc., Bethlehem, PA, USA) and Oralscreen® (Avitar Inc., Canton, MA, USA) are two collection devices (made by a suckling pad of absorbent material) that absorb only 1.0 ml sample and, moreover, collects a mixture of gingival crevicular fluid and saliva rather than saliva alone, since the pad is placed between cheek and gums. The term "oral sample" is used rather than saliva when these devices are used (Niedbala et al., 2001a). These devices, recently developed for saliva testing of drugs of abuse (including marijuana, cocaine, opiates, amphetamines, methamphetamine, PCP and alcohol) have been especially designed to be coupled with microplate enzyme immunoassays for on site detection of illicit drugs (www.orasure.com, www.rapiddetect.com) (Niedbala et al., 2001a; Niedbala et al., 2001b; Barret et al., 2001; Kidwell et al., 2003), but collected saliva by can also be used for confirmation analysis by chromatography/mass spectrometry.

Previous studies on drugs in saliva (Cone, 1993) have shown that weak bases, such as cocaine, opiates, benzodiazepines, or nicotine tended to concentrate in saliva because its pH is slightly acidic as compared with that of plasma (Kidwell et al., 1998; Moolchan et al., 2000). Although some metabolites have been detected, the parent drug is usually the main analyte

found in saliva (Wolff et al., 1999). Recently, saliva testing of drugs of abuse has been extensively used as an alternative matrix to blood and urine in clinical and forensic toxicology of drug of abuse (Kintz and Samyn, 2002). Indeed, all the principal drugs of abuse and among them amphetamines have been determined in the saliva of known users (Cone, 2001; Hoffman, 2001), saliva have been proposed as a matrix for workplace drug testing (Caplan and Goldberger, 2001) and finally on-site saliva testing has been recently applied for "driving under the influence of drug"(DUID) controls at the roadside, with subsequent confirmation by gas chromatographic-mass spectrometric analysis (Samyn and Van Haeren 2000; Samyn et al., 2002). These assays on saliva proved to be sufficiently sensitive for the detection of recent abuse in drug users. However, in case of amphetamine and related substances, few reports have presented systematic studies on the excretion profile of these compounds in saliva after controlled administration (Cone 1993; Wan et al., 1978; Kintz and Samyn, 1999; Kintz 1997).

1.1.15 Sweat

Sweat secretion is an important mechanism for maintaining a constant core body temperature (Huestis and Cone, 1998). Following sympathetic nerve stimulation, sweat is excreted onto the surface of the skin and evaporated to release body heat. Sweat is secreted from two types of sweat glands, eccrine and apocrine glands (Figures 3 and 4). These glands originate deep within the skin dermis and terminate in excretory ducts emptying onto the skin or developing hair follicles. Eccrine glands are located on most skin surfaces, while apocrine glands are restricted to skin of the armpit, genitalia and anus. Water is the primary constituent of sweat, approximately 99 %, and sodium chloride is the most concentrate solute (Robinson and Robinson, 1954).

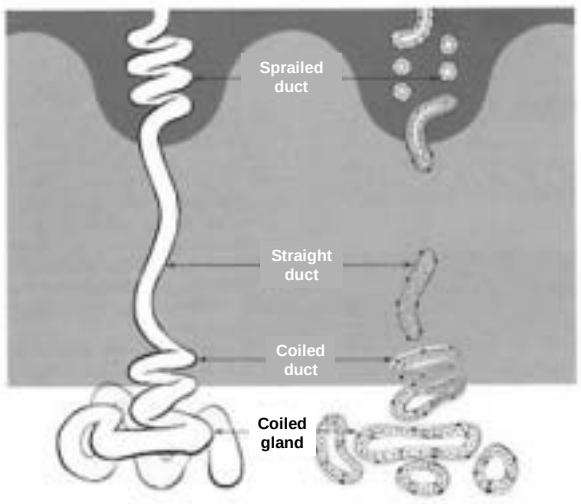


Figure 3. The eccrine gland: eccrine units consist of three portions: (1) the acrosyringium or intraepidermal spiralled duct; (2) the coiled and straight intradermal duct; and (3) the secretory coiled gland.

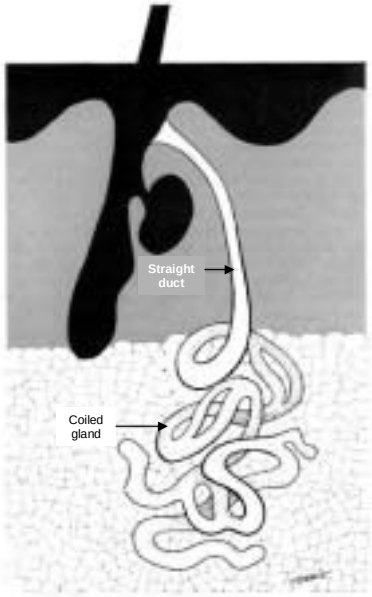


Figure 4. The apocrine unit: apocrine units consist of a secretory-coiled gland and a straight duct that traverses the dermis and empties into the isthmus (uppermost portion) of a hair follicle.

Sweat also contains albumin, gamma globulins, waste products, trace elements, drugs, and many other substances found in blood. The rate of sweating is highly dependent upon environmental temperatures. Above 31 ºC, humans begin to sweat and may excrete as much as 3L/h over short periods of time (Randall, 1953). The average pH of sweat of resting individuals was reported to be 5.82. Following exercise, the pH was found to increase with increasing flow rate and was reported to be between 6.1 and 6.7 (Doran et al., 1993). Approximately, 50 % of sweat is generated by the trunk of the body, 25 % from the legs, and the remaining 25 % from the head and upper extremities (Randall, 1953).

Multiple mechanisms have been suggested for the incorporation of drugs into sweat including passive diffusion and transdermal migration (Nielsen and Rasmussen, 1975; Pichini et al., 1996). Passive diffusion of drugs from blood to sweat is favoured for lipid-soluble substances. Non-ionized basic drugs diffuse into sweat and become ionized as a result of the lower pH of this biological fluid. Basic substances may accumulate in sweat as compared to blood due to the pH differential between the two matrices. Some drugs may migrate across the dermal and epidermal layers into the stratum corneum.

Sweat testing is relatively non-invasive and identification of drug in sweat may serve as a means of monitoring recent drug use with a window of detection which can be somewhat wider than that provided by urine testing (Kidwell et al., 1998) Although the use of sweat for drug testing has been hampered by difficulties in sample recovery and sensitivity of analytical methods (Haeckel and Hanecke, 1993), there are some distinct advantages in using this matrix:

(1) matrix collection is non invasive and this fact can allow the application of rapid qualitative on site tests (Samyn and Van Haeren, 2000),

(2) sweat testing can provide a means to obtain both information on recent drug use and a cumulative estimate of drug exposure over a period of several weeks, depending on the device applied for collection,

(3) the process of sample adulteration is quite difficult.

Disadvantages, however, have to be mentioned:

(1) collected volume is generally small (from few µL in normal conditions to 1-5 mL when sweating is induced by physical exercise),

(2) the production of a quantitative result in drug testing is difficult, due to the fact that the volume of sweat collected is not measurable when traditional collections systems (cotton wipes, sweat patches) are used.

Two methodological approaches are currently applied for drug testing in sweat (de la Torre and Pichini, 2004).

(1) The first one is directed to demonstrate a recent use of drugs (less than 24 hours) and consists of a punctual sweat collection coupled to an immunochromatographic test which provide a qualitative result (Kintz et al., 1998a; Samyn and Van Haeren, 2000) or by a cotton wipe, subsequently extracted and subjected to confirmatory analysis (Samyn et al., 2002). This approach is mainly oriented to detected subjects that act under the influence of drugs.

(2) The second one is based on patch technology and allows monitoring of illicit drug use for time-windows wider than those provided by urine testing. This is because the patches can be worn for up to one week, drugs tend to accumulate in the collection device and no drug degradation seems to occur during this time-interval (Caplan and Goldberger, 2001). Patch technology is mainly used for the follow-up of drug-addicts

under treatment to verify abstinence. Both approaches benefit from a low invasiveness and pose less ethical problems for sample collection when compared to blood or urine.

Sweat testing may serve as a useful tool in surveillance of individuals in treatment and probation programs due to the fact that monitoring illicit drugs in sweat on a weekly basis may provide sufficient detection sensitivity (Kintz, 1996; Kintz et al., 1996a; Kintz et al., 1996b). Another important application of sweat testing, which has found its major diffusion in the last years, has been the roadside on-site testing of potentially intoxicated drivers (Samyn and Van Haeren, 2000; Samyn et al., 2002).

One of the major difficulties of working with sweat has been its collection and the measurement of the volume excreted in a defined time period. The amount of sweat excreted is highly variable both between individuals and within a single person, and is dependent upon their daily activities, emotional state and environment. Systematic collection of specimens is difficult because of the unequal distribution of sweat glands. Sweat can be collected non-invasively with gauze or filter paper, or with specialized collection devices. In 1980, Phillips reported for the first time the development of an adhesive patch for the long-term collection of sweat (10 days). This patch was occlusive in design, trapping both the solute and water components permitting the determination of analyte concentration in sweat. A disadvantage of the occlusive patch design was the limited time the patch could be worn. A linear uptake rate of 18 to 47 mg/day of sweat was observed. It was suggested that it might be possible to monitor drug-taking behaviour through use of the patch. This sweat patch was later utilized in the validation of self-reports of alcohol use (Phillips, 1984).

In the last decade, a new non-occlusive sweat collection device, the PharmChem® Sweat Patch from PharmChem Laboratories (Menlo Park, CA, USA) has been developed and

successfully applied to sweat testing for drugs of abuse (Kintz et al., 1996b; Fay et al., 1996; Fogerson et al., 1997; Huestis et al., 2000)

This patch can be worn for an extended time period and concentrates solutes on a collection pad while allowing water to evaporate from the patch. The device consists of an adhesive layer on a thin transparent film of surgical dressing to which a rectangular absorbent pad is attached. Sweat concentrates on the absorbent pad while oxygen, carbon dioxide, and water vapour escape through the transparent film. Larger molecules are excluded by the molecular pore structure of the plastic membrane. The skin must be thoroughly cleaned with isopropyl alcohol prior to affixing the patch to prevent contamination of the patch or interference with the deposition or detection of analytes on the patch. Attempts to remove the patch prematurely or tamper with the device are readily visible to personnel trained to remove the sweat patch. Care must be taken not to contaminate the absorbent pad when removing and storing the patch prior to analysis (Huestis and Cone, 1998).

Although a variety of drugs of abuse, and among them amphetamines and MDMA, have been detected in sweat of consumers (Vree et al., 1972; Burn and Baselt, 1995; Kintz et al., 1996a, Fay et al., 1996; Fogerson et al., 1997; Kintz, 1997; Kintz and Samyn, 1999; Huestis et al., 2000), little information is available on the excretion profile of drugs of abuse and particularly amphetamines and related compounds in sweat after controlled administration (Vree et al., 1972; Kintz et al., 1997; Pacifici et al., 2001).

1.1.16 Hair

Hair consists of five morphological compounds: cuticle, cortex, medulla, melanin granules and cell membrane complex (Figures 5a and 5b). Each is distinct in morphology and chemical composition. The number of hair follicles ranges from 80,000 to 100,000 follicles on the human head, but these decrease with age. Hair follicles are embedded in the dermis of skin and are highly vascularized to nourish the growing hair root or bulb.

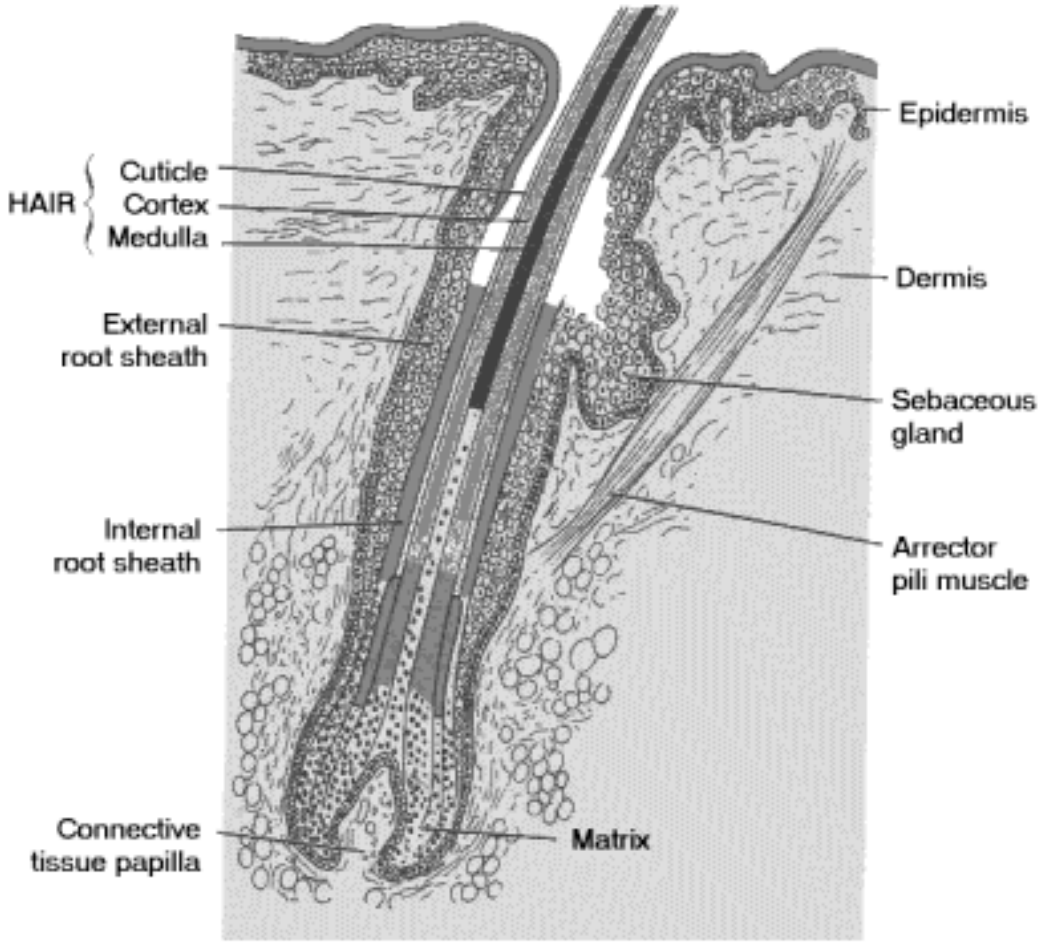


Figure 5a: Morphology of hair

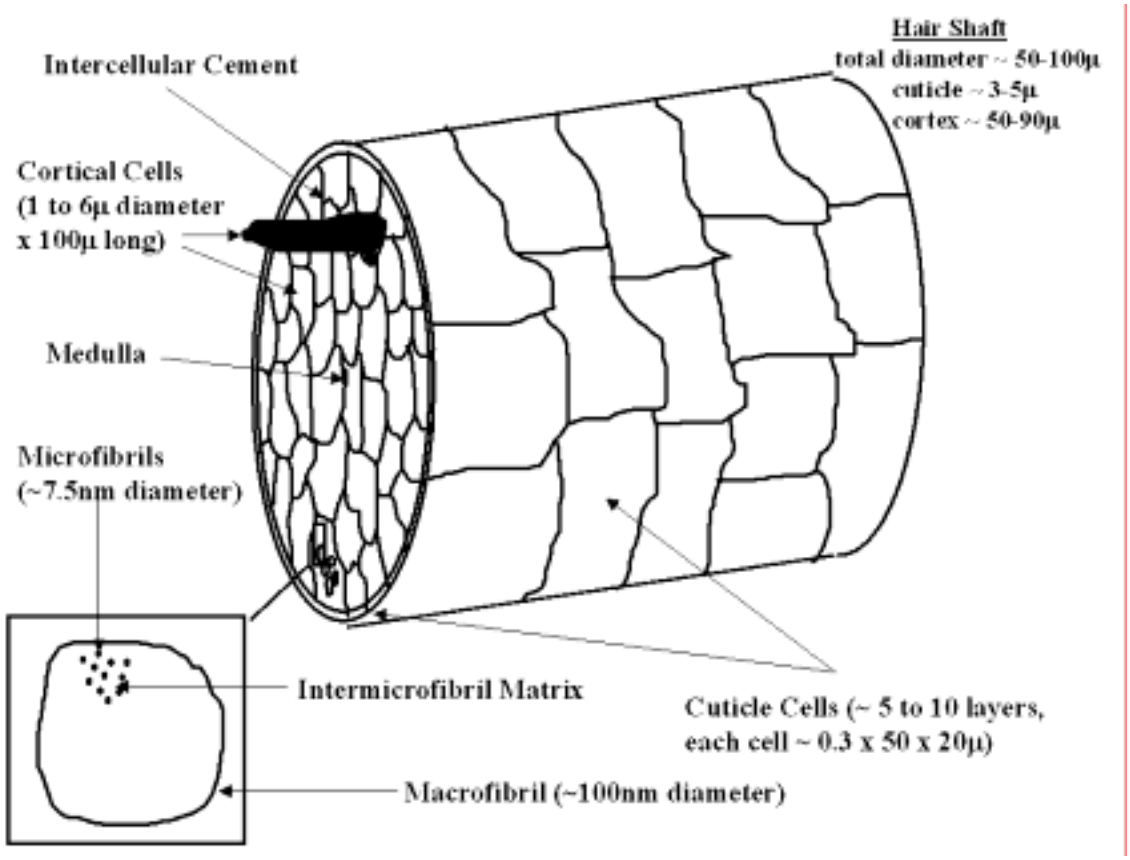


Figure 5b: Anatomy of a hair shaft

The bulb at the base of the follicle contains matrix cells that give raise to the layers of the hair shaft including the cuticle, cortex, and medulla. Matrix cells undergo morphological and structural changes as they move upward during growth to form different layers of the hair shaft. These layers can often be distinguished by qualitative and quantitative differences in their proteins and pigments (Harkey, 1993).

Hair is composed of approximately 65 to 95 % protein, 1 to 9 % lipid, and small quantities of trace elements, polysaccharides, and water (Harkey, 1993). The proteins synthesized within the matrix cells determine the durability and strength of the hair shaft. Matrix cells also may acquire pigment or melanin during differentiation into individual layers of hair. The pigment present in hair cells determines the colour of the hair shaft. The primary

structure of hair consists of two or three α-keratin chains wound into strands called microfibrils. Microfibrils are organized into larger bundles of macrofibrils that comprise the bulk of the cortex. Hair strands are stabilized and shaped by disulfide and hydrogen bonds giving the microfibrils a semi-crystalline structure. Cytochrome P-450 and other enzymes have been identified in the hair follicle providing evidence that drug metabolism may occur within this structure; however, as hair becomes fully keratinized in three to five days, this capability is presumed to be greatly reduced. (Harkey, 1993).

A protective layer of epithelial cells called the cuticle surrounds the cortex. The cuticle is the outermost layer of hair, the innermost region is the medulla, and the hair cortex lies between these components. The overlapping cuticle cells protect the cortex from the environment. As hair ages, there is a gradual degeneration of the cuticle along the shaft due to exposure to ultraviolet radiation, chemicals and mechanical stresses. The cuticle may be partially or totally missing in cases of damaged hair (Okamoto et al., 2003) Hair damaged by cosmetic treatments and/or ultraviolet radiation may influence the deposition and stability of drug in the hair (Jurado et al., 1997; Skopp et al., 1997).

Hair follicles continue to grow for a number of years and undergo different phases during a normal growth cycle (Sachs, 1995). Approximately 85 to 90 % of the hair is in the anagen or growth phase at any single time. A small portion of mature hair lasts for a period of 2 to 3 weeks and is immediately followed by the telogen phase, the resting phase of hair. During this phase, no growth occurs. Approximately 10 to 15 % of head hair is usually in the telogen phase at all times. The hair strand may not be shed for several months prior to replacement by a new strand. Hair growth rates vary according to body location, sex and age.

Head hair grows at an average rate of 1.3 cm/month although there is some variation according to sex, age and ethnicity (Wennig, 2000). Conversely, axillary and pubic hairs show a slower growth rate (Ebling, 1987).

It is well known that drugs, including drugs of abuse, can be incorporated in hair, where they remain indefinitely stable (Kintz, 1996b)

Although many authors suggested that drugs may enter hair from multiple sites, via multiple mechanisms, and at various times during the hair growth (Kidwell and Blank, 1995), possible pathways for drug incorporation into hair including cycle can be summarized as follows:

(1) passive diffusion from blood into the hair follicle;

(2) excretion onto the surface of hair from sweat and sebum;

(3) from external contamination.

Drugs and their metabolites are distributed throughout the body primarily by passive diffusion from blood. High lipid solubility, low protein binding, and physicochemical factors that favour the unionized form of the drug in blood generally facilitate distribution across membranes. Diffusion of drug from arterial blood capillaries to matrix cells in the base of the follicle is considered a primary means for drug deposition in hair. Presumably, drug binds to components in the matrix and to pigments. As the cells elongate and age, they gradually die and coalesce forming the non-living hair fibre. Drug that may be present is embedded in the hair matrix (Huestis and Cone, 1998).

The predominant analytes generally found in hair and sweat are the parent compounds, rather than their more polar metabolites, which usually predominate in urine (Kintz, 1996). Sweat and sebum have been implicated in the deposition if drugs in hair and may account for the lack of dose-concentration relationships reported in some studies and for

the presence of drug in distal segments of hair that do not correlate with the time of drug exposure (Cone, 1996).

The spectre of racial bias has been raised in regard to hair testing due to apparent variation in binding affinity for drugs between different ethnic hair types (Joseph et al., 1996). Evidence at present suggests that protein and melanin are the principle components involved in binding. There are different types of melanin granules in hair including: eumelanins found in dark coloured hair; pheomelanins in light colour hair; and erythromelanin in red hair. Distinct chemical and physical properties have been described for the different types of melanin, e.g., pheomelanins have higher sulphur content, and for eumelanins of different hair colours; melanin granules in black hair are at least twice the size of those in brown hair. The differential binding of drugs to hair of different colour and ethnic origin has been the subject of several in vivo investigations, suggesting that excretion of drug was closely linked with the presence of melanin (Gygi et al., 1996).

In the last two decades, the analysis of hair for drugs and particularly drugs of abuse has received enormous attention, largely because of several advantages over drug testing methodologies employing body fluids such as urine or serum (Kintz, 1996; Pichini et al., 1996). In fact,

(1) drugs and metabolites remain sequestered in the hair shaft with no observed time degradation, providing a window of detection which is much wider (of the order of weeks to several months) than that of serum or urine, in which drug levels decrease rapidly over a relatively short period of time (hours to days),

(2) hair collection is simple, non-invasive and replicable for eventual confirmation of the original results,

(3) there is low potential for evasion or manipulation of results from hair testing,

(4) there is a low risk of disease transmission in the handling of samples,

(5) hair samples are stable indefinitely and very difficult to manipulate to alter their drug content.

(6) finally, result from a hair analysis can allow a distinction between low, moderate and heavy consumers of a specified substance.

Furthermore, as mentioned above, since hair grows at an average rate of 1.3 cm/month, it is theoretically possible to extrapolate a record of eventual drug usage by segmental hair analysis (Cirimele et al., 2002; Pichini et al., 2003). If this can be true in case of head hair, it is not in case of axillary and pubic hair. Axillary and pubic hairs have been used as well as head hair in forensic-toxicological investigations (Frison et al., 2003). Nonetheless, significant slower growth of axillary and pubic hair, usually shorter than head hair, and possible drug contribution from sweat, sebum and urine (this last in case of pubic hair) do not allow any relation between substance amount found in these two types of hair and time of consumption (Mangin and Kintz, 1993; Offidani et al., 1994) As a consequence of the above reported statements, some authors demonstrated that certain drugs of abuse become more concentrated in axillary and pubic hair than in head hair (Mangin and Kintz, 1993; Offidani et al., 1994;)

In any case hair testing has some drawbacks:

(1) environmental drug contamination, which can provide false positive results in non consumers (Kidwell and Blank, 1995);

(2) drugs migration along the hair shaft, which can provide a false positive result during drug cessation programs (Kidwell and Blank, 1995),

(3) lack of reference material to assist analytical laboratories in testing their performance (Pichini et al., 1996),

(4) several physiologic and non-physiologic biases (sweat and sebum participation in drug incorporation, racial effects, melanin binding, cosmetic treatments including hair care, permanents, dyes etc.) which affect quantitative result and eventual relationship between drug dose and hair concentration.

Since the major application of drug testing in hair has traditionally been past and/or chronic consumption of drugs of abuse, the crucial problem of differentiating passive exposure from consumption, especially in case of smoked drugs always coupled the history of hair testing for illicit drugs (Koren et al., 1992; Wang and Cone, 1995; Kidwell and Blank, 1995). However, at present the problem of external drug contamination appears to have been successfully overcome (Schaffer et al., 2002; Cairn et al., 2004a; Cairns et al., 2004b).

Another controversial issue in hair analysis is the interpretation of dose and time relationships. Whereas some authors presented data indicating a linear relationship between drug dose and amount of parent drug and/or metabolites found in hair (Mitzuno et al., 1993; Welp et al., 2003), some others showed the lack of dose-concentration association, advocating intersubject variability of drug incorporation in hair, drug moving along the hair shaft with time and finally incorporation of drug into hair by multiple mechanisms, as assessed above (Kintz et al., 1998. Wennig, 2000). Nonetheless, it seems always apparent that hair analysis can distinguish categorized patterns of drug consumption (Pepin and Gaillard, 1997).

Since Valente et al. (1981) reported the first evidence of illicit drugs in human hair of abusers by radioimmunoassay, hair testing for illicit drugs found a huge number of application in clinic and forensic toxicology ranging from alive and dead humans to foetuses and mummies (Bailey et al., 1997; Balabanova et al., 1992; Kintz, 1996; Segura et al., 1998;

Caplan and Goldberger, 2001; Cone, 2001; Koren et al., 2002; Kintz and Samyn, 2002; Drummer 2004). Traditional fields of application of hair testing have been:

(1) evidence (or non evidence) of chronic active and passive exposure to drugs in different court proceedings (criminal activity under the influence of drugs, suicides, homicides, overdose cases, child custody) (Huestis and Cone 1998),

(2) epidemiological studies of prevalence rates of drug use in selected populations (normal individuals, consumers, children, etc.) (Huestis and Cone 1998),

(3) workplace drug testing (Caplan and Goldberger, 2001),

(4) assessment of compliance in therapeutic drug monitoring (Nakahara, 1999),

(5) survey of applicants for driving license with an history of drug abuse (Montagna et al., 2000),

(6) exposure to drugs in intrauterine life (Koren et al., 2002),

As detailed in the previous lines and bibliography of this chapter, a wide variety of drugs of abuse and pharmaceuticals have been determined in consumer's hair and among them classical amphetamines and methylenedioxyderivatives (Nakahara, 1995; Nakahara et al., 1997; Kikura et al., 1997; Kintz, 1997; Kintz and Samyn 1999; Cairns et al., 2004a; Drummer 2004). This kind of illicit drugs has been successfully detected in hair samples even after a single dose (Nakahara et al., 1993; Nakahara and Hanajiri 2000) due to the basic and lipophilic nature of the compounds (Baselt and Cravey, 1995). Furthermore, an investigation on the possibility to use hair analysis of amphetamine derivatives to confirm self reported use of those drugs was firstly reported by Cooper et al. (2000) showing a certain concordance between reported data and substance levels detected in hair.

1.2 3,4-METHYLENEDIOXYMETHAMPHETAMINE , MDMA

1.2.1 Epidemiology of consumption

3,4-methylenedioxymethamphetamine, MDMA, or 'ecstasy' is a 'psychedelic amphetamine' that has gained popularity over the past 20 years because of its ability to produce strong feelings of comfort, empathy, and connection to others. (Wright et Pearl, 1995) This psychomotor stimulant (described as ‘entactogens’) has behavioural effects similar to those elicited by amphetamines and hallucinogens. (Nichols, 1986; Peroutka et al., 1988)

It most frequently comes in tablet form, although it is occasionally sold in capsules or as powder. It is most frequently used orally and rarely snorted or intravenously.

MDMA was first synthesized and patented by Merck pharmaceuticals in 1912 and patented in 1914, but it wasn't until the mid 1970s that articles related to its psychoactivity began to spread. In the late '70s and early '80s MDMA was used as a psychotherapeutic tool and also started to become available on the street. Its growing popularity led to it being made illegal in the United States in 1985 and its popularity has continued to increase since then (Gilman et al., 1990).

MDMA use is highest among individuals between the ages of 16 and 25, with the drug most strongly associated with dance and “rave” sub-cultures. However, ecstasy use is not limited to one age group or sub-culture. In the United States, prevalence of ecstasy use in the year 2002 was estimated to be 3.1% for eighth graders, and 8.2% for 12 graders in 2000, and estimated to be 5.5% for college students, and 3.6% for young adults (ages 19-28) in 1999, being methamphetamine the psychostimulant most frequently used throughout the country. On the other hand, Europe remains an important area for the production and use of

amphetamines and ecstasy but not methamphetamine (EMCDDA annual report 2003). After cannabis, the most commonly used drug in EU countries is usually either ecstasy or amphetamine, with rates of lifetime experience among the adult population generally ranging between 0.5 % and 5 %, being Portugal the country with the lowest consumption (0.5%) and Ireland the one with the highest (4.9%). Spain and UK set respectively at 3.9 and 4.6%. Although ecstasy use continues to be highly prevalent among Europe's urban youth – and studies show very high use in some groups, such as partygoers – a marked increase is not seen generally in the wider population. Deaths where ecstasy is mentioned, though widely highlighted in the media, remain relatively rare, although these are increasing. The drug is mentioned in less than 2% of all reports of total drug-related deaths (there were 8.756 drug-related deaths in the EU in 2000).

Amphetamines account for around one third of people treated for drug problems in Finland and Sweden and 9% in Germany – but elsewhere in the EU they account for typically less than 1%.

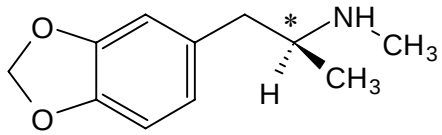
Both amphetamine and ecstasy seizures raised substantially in the EU in the last decade, although now seem more stable. Almost 99% of tablets analysed in the last reporting year in Germany, Spain, Portugal, the UK, and Norway contain MDMA or its close analogues MDEA and MDA, although a range of other substances was occasionally present. The average price of ecstasy tablets decreased in most countries in 2001.

1.2.2 Chemistry

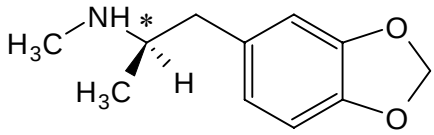
As shown in Figure 6, the basic structure of MDMA is a β-phenylisopropylamine group, with a methylenedioxy group forming a 5-membered ring including C-3 and C-4 of the benzene ring. The methyl group on α–carbon (R2) confers resistance to oxidative deamination of this compound and, therefore, an increased metabolic half- life (Cho and Segal, 1994).

MDMA has a chiral centre at the α- carbon, with a pair of enantiomers (Pizarro et al., 2002) (the racemic mixture is usually consumed) that show different pharmacological activity and body disposition, being the S-isomer of MDMA responsible for psychostimulant and entactogenic activities as compared to hallucinogenic-type properties of R-isomer. (Spitzer et al., 2001) Stereoselective metabolism for MDMA has been also reported (Pizarro et al., 2003).

Figure 6. Enantiomers of MDMA



(S)-(+)-MDMA



(R)-(-)-MDMA

MDMA is a basic drug with a pKa around 9.9 (data derived from methamphetamine, Baselt and Cravey, 1995), a molecular weight of 193, and from what is known for amphetamine and methamphetamine a protein binding around 20% and a moderately high volume of distribution. These properties confer an easy diffusion across cells membranes and

lipidic layers, and tissues or biological matrices with a more acidic pH than blood (Pichini et al., 1996).

1.2.3 Mechanism of action

MDMA is an indirect monoamine agonist producing release from presynaptic terminals of mainly serotonin (5-hydroxytriptamine, 5-HT) but also norepinephrine (NE) and dopamine (DA) in the CNS and at the peripheral level. (Parrott, 2001). It interacts with the membrane transporters involved in neurotransmitter re-uptake and vesicular storage systems. It seems that MDMA can be transported into the nerve terminals by passive entrance or through a re-uptake transporter, thus acting as inhibitor of the re-uptake of monoamines. Once inside the neuron, MDMA reverses the direction of the membrane transporter facilitating the efflux of 5-HT, NE and DA to the synaptic cleft. In addition, MDMA is a mild inhibitor of the enzyme monoamine oxidase A and B (MAO). Repeated MDMA administration depletes the catecholamines supply producing a decrease in its pharmacological effects (acute tolerance). Finally, MDMA inhibits of the activity of the rate-limiting enzyme tryptophan hydroxylase with consequent decrease in the formation of serotonin (Green et al., 2003).

1.2.4 Pharmacological effects

Similarly to the other amphetamine type compounds (ATS), MDMA is a powerful psychostimulant producing an increased alertness, wakeful, insomnia, energy and self-confidence in association with decreased fatigue and appetite as well as enhanced mood, well-being and euphoria. In addition, MDMA users have consistently reported feelings of euphoria, happiness, increased energy, increased peacefulness, feeling close to others and increased insight, described by some authors as ‘entactogen’. (Nichols, 1986). High doses can

cause convulsions, stereotypic movements and psychosis while it can induce hallucinations at usual recreational doses. Fatigue, anxiety and tiredness can appear when the effects vanished. These negative symptoms ('crash') are more intense when high or repeated doses are administered and depression and lethargy can appear. Chronic MDMA use may be associated with the so-called 'amphetamine psychosis' characterised by psychotic reactions, hallucinations and paranoia. MDMA shows a high abuse potential and can induce dependence, tolerance and withdrawal symptoms.

MDMA given at recreational doses (range tested 50 to 150 mg) to healthy volunteers produces mydriasis and marked increases in systolic and diastolic blood pressure, heart rate, and pupillary diameter. MDMA induced changes on oral temperature. The time course of this observation is biphasic, as a slight decrease at 1 h and a slight increase at 2 and 4 h are observed. MDMA induces a slight dose-dependent impairment on psychomotor performance (Lamers et al., 2003, Brookhuis et al., 2004). MDMA produces a marked rise in plasma cortisol and prolactin concentrations. The elimination half-life of MDMA is about 8-9 h. As drug concentrations increase, a parallel increase in physiologic and hormonal measures is observed. Both peak concentrations and peak effects are obtained between 1 and 2 h and decreased to baseline values 4-6 h after drug administration (de la Torre et al., 2000b; de la Torre et al., 2004b).

1.2.5 Immunological effects

Acute MDMA treatment produces a time-dependent immune dysfunction associated with MDMA plasma concentrations in healthy volunteers. A decrease in $CD4^+$ T-cells and functional responsiveness of lymphocytes to mitogenic stimulation is observed, while percentage of natural killer cells significantly increase (Pacifici et al., 2000). A rise of cortisol

plasma concentrations supported the hypothesis of MDMA-induced release of corticotrophin-releasing factor from the median eminence of the hypothalamus and subsequent HPA-axis and SNS activation. Repeated MDMA administration to healthy MDMA consumers produces a time-dependent immune dysfunction similar to that observed with the ingestion of a single dose, and the first of the two administrations parallels time-course of MDMA-induced cortisol stimulation kinetics and MDMA plasma concentrations. Response to the second dose is either long lasting as compared with the first dose and/or disproportionate and does not show any parallelism with cortisol and MDMA plasma concentrations (Pacifici et al., 2001). Therefore, it was postulated an immune dysfunction mediated by a glucocorticoid-independent mechanism involving directly sympathetic nervous system (SNS) (Young et al., 2000). Alterations in several immunological parameters have been observed also in basal values of lymphocytes subsets in a population of recreational users of MDMA participating in different clinical trials (Pacifici et al., 2002). Absolute number of lymphocytes and in particular T lymphocytes and CD4 T-helper cells subsets showed a trend toward reduced values, although cell counts were within normal limits. By contrast, NK cells in MDMA consumers were reduced to one third of those from healthy individuals. It can be concluded that MDMA induces permanent alterations in immunologic homeostasis, which may result in general health status impairment and subsequent increased susceptibility to infection and immune-related disorders.

1.2.6 Pharmacokinetics

1.2.6.1 Absorption

MDMA is mostly consumed orally, but in contrast to AM and MA, the racemic mixture is usually ingested (Zhao et al., 2001).

After oral ingestion of MDMA, maximum plasma levels appear between 1.5 and 3 hours. It should be noted that after the administration of five different doses of MDMA (50, 75, 100, 125 and 150 mg), $C_{max,}$ and AUC_{24h} increase both according to the dose given, although in case of the 150 mg dose, the increase in MDMA kinetic parameters was not proportional to the dose suggesting non-linear pharmacokinetics (de la Torre et al., 2000a, Mas et al., 1999). This finding is explained by an interaction of MDMA metabolites with key enzymes regulating MDMA metabolic disposition (de la Torre et al., 2004b). In vitro data suggest that MDMA can act as inhibitor of the CYP2D6 enzyme, through mechanisms that include a competitive interaction and/or the formation of a metabolic complex between MDMA and this enzyme (de la Torre et al, 2000b). Pharmacokinetic parameters reported by others authors (Helmlin et al., 1996) using both similar and different MDMA doses are consistent with the findings of de la Torre et al. (2000a). An enantioselective disposition of MDMA has been described in humans. After an oral administration of 40 mg of racemic MDMA, R-(-)MDMA mean C_{max} value was significantly greater than that of the S-(+)MDMA, and the mean enantiomeric ratio (R/S) of the AUC_{0-24} indicated a more rapid elimination of the more pharmacologically active S-(+) enantiomer (Pizarro et al., 2002; Fallon et al., 1999). The short elimination half-life of (S)-MDMA (4.8 h) is consistent with the subjective effects and psychomotor performance reported in subjects exposed to a single dose of 100 mg MDMA, whereas the much longer half-life of the (*R)*-enantiomer (14.8 h)

correlates with mood and cognitive effects experienced on the next days after MDMA use (Pizarro et al., 2004).

1.2.6.2 Distribution

Similarly to classical amphetamines, MDMA shows a low protein binding (c.a. 20%) (de la Torre et al., 2000b; Mas et al., 1999). In practice, this low binding determines that almost the total amount of drug available in plasma may diffuse to the extravascular compartment. Information on the distribution parameters of MDMA in humans after controlled administration is very limited. From the data reported by de la Torre et al., (2000b) a distribution volume of 452 ± 137 L (6.4 L/kg) can be established following oral administration of 100 mg MDMA. With regard to MDMA enantiomers, Fallon et al. indicate after the administration of 40 mg racemic MDMA, the more extensive distribution of the more active S-(+)MDMA enantiomer (595 $\pm$ 204 L vs. 383 $\pm$ 97 L for the R-(-) enantiomer), supporting the hypothesis of enantioselective disposition of MDMA in humans.

1.2.6.3 Metabolism

Principal metabolic pathways of MDMA metabolism include: O-demethylenation of the methylenedioxy group and the N-demethylation of the secondary amine (Ensslin et al., 1996; Maurer, 1996; de la Torre et al., 2000b; Kraemer and Maurer, 2002) (Figure 7). The O-demethylenation of MDMA gives rise to metabolic intermediate with a catechol group: 3,4-dihydroxymethamphetamine- HHMA that is methylated preferentially in position 3 to 4-hydroxy-3 methoxymethamphetamine, HMMA.

On the other hand, the N-demethylation of MDMA gives rise to the 3,4 methylendioxyamphetamine (MDA) which is further metabolized by O-demethylation

likewise MDMA- to 3,4-dihydroxyamphetamine (HHA) and consequently to 4-hydroxy-3-methoxyamphetamine (HMA). Another possibility consists in a de-amination, forming benzoic acid derivatives, followed by an oxidation to glicine derivatives (Maurer, 1996). Finally, whereas HMMA and HMA can form glucuronide/sulphate conjugates; HHMA and HHA are generally found as sulphate conjugates.

Metabolic reactions involved in the first steps of amphetamine derivatives disposition are the most relevant from a toxicological and therapeutic point of view. In vitro studies have shown that phenylethylamines (as a class) are to some extent substrates or inhibitors of cytochrome P_{450} isoenzyme CYP2D6 (de la Torre, 2004). In particular, MDMA O-demethylenation shows a biphasic kinetics with a high affinity component regulated by CYP2D6 and a low affinity component regulated mainly by the isoenzyme CYP1A2 and to a lesser extent by CYP2B6 and CYP3A4.

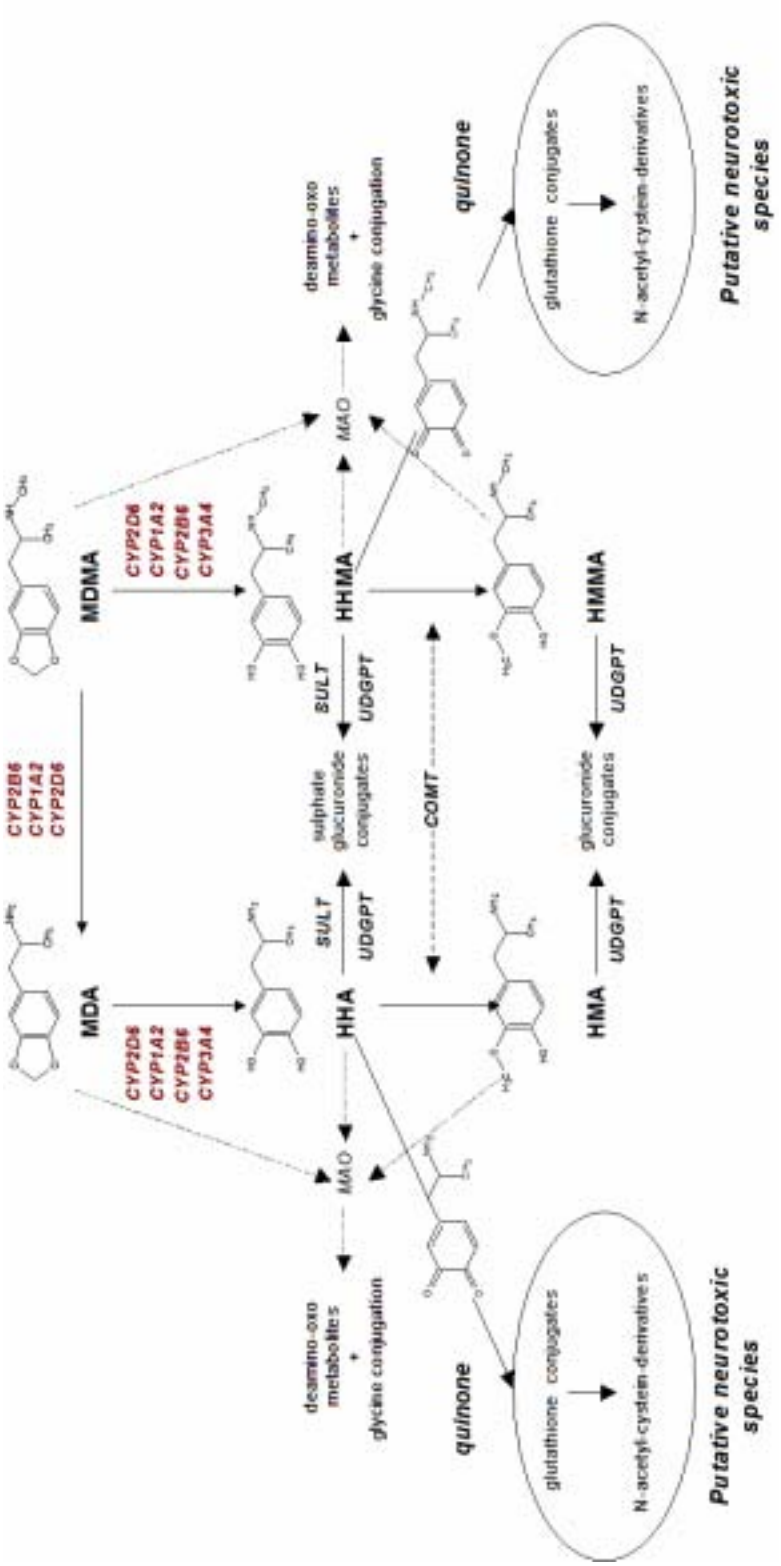


Figure 7: Principal metabolic pathways of MDMA

The CYP2D6 is expressed polymorphically in humans and subjects genotypically classified as poor metabolizers (9% of Caucasian and 1% of Asian populations) were postulated to be more susceptible to acute toxic effects of amphetamine derivatives or to abuse liability (Kraner et al., 2001). In addition, as CYP2D6 regulates the biotransformation of many therapeutic drugs, it may be also the source of a number of drug-amphetamines interactions. However, clinical data seem not to support fully these expectations (Gilhooly and Daly, 2002). In fact, it has been shown that CYP2D6 overall most probably accounts for less than 30% of MDMA (Segura M, 2004 in press) disposition at the metabolic step of O-demethylenation; other isoenzymes involved in this reaction would be CYP1A2 and to a lower extent by CYP2B6 and CYP3A4 (Tucker et al., 1994, Kreth et al., 2000). A CYP-independent mechanism has also been reported for designer drugs (Maurer et al., 2000).

Furthermore, many amphetamines are administered as racemates. CYP2D6 usually displays selectivity for one of the enantiomers, which, in the case of MDMA, is the S(+) (the more active one). The enantiomer that is not CYP2D6 metabolised plus the fraction of the active enantiomer not metabolised through this isoenzyme, underwent metabolic reactions through isozymes of cytochrome P_{450}, as already described. Since several isozymes participate in the same reaction, the impact of CYP2D6 genotype in the pharmacology of these drugs is lessened. A further step of complexity is introduced by the enzymatic reaction itself. The O-demethylenation reaction of MDMA follows the formation of a metabolite enzyme complex that inactivates CYP2D6 (Delaforge et al., 1999) and probably is the basis for non-linear pharmacokinetics described for MDMA (de la Torre et al., 2000a; Delaforge et al., 1999). In practice, methylenedioxy-derivatives once ingested, have the property to convert phenotypically subjects to poor metabolizers for the next dose ingested (within a short period of time), independently of his/her genotype. Even more interestingly, the active enantiomer

does not accumulate disproportionately in the body as the alternative metabolic pathways become more relevant. Most probably, concerns should be more focused on drugs with a narrow therapeutic index, which are substrates of CYP2D6 that may interact with MDMA. In vitro data suggest that homozygous individuals for several functional CYP2D6 allelic variants may have an impaired MDMA disposition capacity. In this respect, the functional allelic variant CYP2D6*10/*10, very common among Asians (75%), and variants CYP2D6 *2 and *17, more prevalent among African populations should be taken into consideration as susceptibility to toxicity risk factors as compared to homozygous individuals for the functional wild type genotype CYP2D6*1/*1 (Ramamoorthy et al., 2001, Ramamoorthy et al., 2002).

N-dealkylation of MDMA is catalysed by CYP2B6 (Kreth et al., 2000). N-dealkylation speed for methylenedioxy-derivatives is nearly of one order of magnitude lower than for O-demethylenation, and it is characterised by apparently monophasic kinetics. Finally, the O-methylation is catalyzed by catecol-O-methyltransferase COMT (Tucker et al., 1994; Kreth et al., 2000).

Oxidative stress, reactive oxygen (ROS), and nitrogen (RNS) species are known to be involved in neurotoxicity induced by amphetamines (Imam et al., 2001; Carvalho et al., 2001; Lin et al., 1992). In the case of MDMA, it has been postulated that oxidative stress may be mediated by active metabolites resulting from adduct formation with glutathione (Monks et al., 2004).

1.2.6.4 Accumulation in the other tissues

Because MDMA is a weak base with a quite low protein binding, this compound is likely to accumulate in biological fluids and matrices with pH values lower than that of the

plasma (pH 7.4) ("ion-trapping phenomena") (Pichini et al., 1996; Kintz and Samyn, 1999). Therefore, given that saliva and sweat are both more acidic than plasma, it is apparent that MDMA maybe readily distributed in these body fluids.

Although a pilot study on sweat testing of MDMA after a controlled administration in two volunteers has been reported (Pacifici et al., 2001), MDMA excretion in fluids and matrices other than plasma and urine after a controlled drug administration was never investigated, nor an interpretation of toxicological data on the basis of observed clinical effects was presented.

Salivary concentrations of MDMA were obtained in consumers with concentrations ranging from hundreds to thousands ng/ml saliva, while levels in sweat collected by cotton wipe varied in the maximum magnitude of hundreds ng/wipe (Kintz and Samyn 1999). Similar range of concentrations was obtained by in MDMA consumers undergoing roadside drug testing (Samyn et al., 1999; Samyn et al., 2000; Samyn et al., 2002; Wood et al., 2003).

Accumulation of MDMA in a keratin matrix (hair and nails) depends on complex mechanisms not fully understood. In the case of hair, hypothesised mechanisms include: transport across the membranes; biotransformation and drug melanin affinity depending on the physicochemical properties of the drug, such as lipid solubility, molecular size and geometry of the drug molecule; concentration gradient; pH gradient; plasma protein binding; and blood flow at hair follicles (Pötsch et al., 1997). It has been extensively shown that MDMA is promptly incorporated in hair matrix, at least after a single dose as shown in animals (Nakahara and Hanajiri, 2000). Also in case of hair, determination of MDMA in this matrix has been performed in non-controlled studies. Therefore, this substance has been easily detected in scalp and body hair of declared consumers with maximum concentrations in range

of tens ng/mg hair and associated to self-reported consumption (Sachs and Kintz, 1992; Kintz et al., 1995; Kintz and Cirimele, 1997).

In contrast to the hair, nails do not contain melanin and this may reduce drug incorporation into this substrate. However, studies with antifungal drugs suggested incorporation from systemic circulation via nail bed and nail matrix (Palmeri et al., 2000). This double mechanism of incorporation does not allow temporal evaluations of drug intake such as those obtained in hair samples (Le Gros and Dudley Buxton, 1938). Nonetheless, due to a lesser growth speed, this matrix was shown to accumulate a higher amount of MDMA with respect to hair from a substance consumer (Cirimele et al., 1995).

1.2.7 Excretion

As previously shown, MDMA undergoes an extensive metabolism and the amount of unaltered drug excreted in urine is usually smaller. The comparison of renal and total clearance of MDMA shows that about 80% of the drug is cleared metabolically through the liver, while about 20% of the dose is excreted unaltered in urine (Mas et al., 1999).

In general terms, as MDMA is a weak basic substance, renal excretion is increased by urinary acidification and decreased by urinary alkalinization (Quinn et al., 1997). This fact leads to a significant variation in the elimination half-life and for this reason, it is told that MDMA consumers frequently ingest large amounts of bicarbonate antacids to prolong drug effects (Quinn et al., 1997).

Urinary clearance of different MDMA doses appeared to be rather constant. On the contrary, non-renal clearance was shown to be dose-dependent. In a controlled clinical trial, the administration 125 mg oral MDMA resulted in 100% reduction of non-renal clearance (38.1 ± 13.3 L/h) as compared to a 75 mg dose (74.0 ± 71.1 L/h), suggesting impairment in

the MDMA hepatic clearance (de la Torre et al., 2000b). After controlled administration of different MDMA doses, the urinary recovery is approximately 60%, independently from the dose given. MDMA itself has been detected in urine from consumers (Tedeschi et al., 1993; Jurado et al., 2000). However, the percentage of unchanged drug accounted for an average of 15%, a similar recovery has been reported for 3,4-dihydroxymethamphetamine (HHMA). Higher recoveries rate have been detected for 4-hydroxy-3-methoxymethamphetamine (HMMA) the main metabolite found in urine (>20%) with less than 2% of the dose excreted as MDA (Helmlin et al., 1996; de la Torre et al., 2000B, Segura M et al., 2001). Studies in controlled settings demonstrated that the elimination half-life of MDMA in the range of 6–9 hours. (Mas et al., 1999).

1.2.8 Side effects and overdose

Ingestion of MDMA can induce several undesirable side effects including loss of appetite, jaw clenching, bruxism, headache, nausea sweating, muscle aches, fatigue and insomnia. (McCann et al., 1996). One important factor in MDMA overdose is hyperthermia that may result from a direct action of the drug on the temperature-regulating centre, from muscular activity associated to dance or tremor and rigidity, high ambient temperatures in crowded places and dehydration. There are a number of reports concerning severe intoxication and death after MDMA abuse. (Henry et al., 1992; Garcia-Repetto et al., 2003). In fact, heat stroke is a severe complication that can cause death, it includes hyperpyrexia, rhabdomyolisis, myoglobinuria, disseminated intravascular coagulation and renal failure. Hyponatremia is an uncommon complication that can be caused by drinking excessive amounts of water and/or by the MDMA-induced secretion of antidiuretic hormone (syndrome

of inappropriate secretion of antidiuretic hormone or SIADH). Fulminant hepatitis has been also reported (Henry et al., 2001).

1.2.9 Neurotoxicity

It has been extensively documented that chronic misuse of amphetamine type stimulants may lead to long lasting impairment of brain function (Ricaurte et al., 1984). Animal studies showing neurochemical and morphological changes in serotonin (5-HT) neurons in response to the administration of methylenedioxy analogues have partially been confirmed with brain imaging studies in humans (i.e. dopamine/serotonin transporters reductions) (Boot et al., 2000; Hegadoren et al., 1999; Ricaurte et al., 2000). Although neurotoxicity to monoaminergic systems seems a class property of amphetamines, MDMA primarily affect 5HT. In addition, MDMA-induced changes in catecholaminergic systems are mild and transient, and long-term deficits of dopamine, norepinephrine and their metabolites or tryptophan hydroxylase activity have been reported (White et al., 1996).

The mechanisms underlying neurotoxic effects are poorly understood, although a pathogenetic role of MDMA-induced hyperthermia and reactive species (free-radicals) formation in brain regions has been postulated (Davidson et al., 2001). Other studies suggest that MDMA-induced acute toxicity and long-term neurotoxicity is dependent on the metabolic disposition of MDMA. Differences in MDMA metabolism among animal species might therefore account for different sensitivities to its neurotoxic effects. The kinetic parameters of enzymes that regulate the formation of neurotoxic metabolites of MDMA differ among species, as does the ability of MDMA to self-inhibit these enzymes and the degree of genetic polymorphisms exhibited by these enzymes. Such features limit allometric scaling across animal models (de la Torre and Farré, 2004).

Neurotoxicity of methylenedioxy-derivatives could be reduced or prevented by the administration of different substances that act at various levels, e.g. acetylsalicylic acid, fluoxetine and citalopram or barbiturates. Hypothermia and drugs that decrease body temperature also reduce neuronal toxicity (Davidson et al., 2001, Seiden and Sabol, 1996).

2. Hypothesis

2. Hypothesis of the study

- It is postulated that MDMA physic-chemical properties: (i) pKa of around 9.9 corresponding to a weak base that facilitates the transfer of MDMA from plasma (pH=7.4) to fluids/matrices with a favourable pH gradient, (ii) high liposolubility with volumes of distribution between 6 and 7 liters per kilogram, (iii) low protein binding, favour its distribution to biological matrices in humans.
- Several non-conventional biological matrices such as hair, sweat and saliva, because of drug accumulation due to its physico-chamical properties, might be of use for the detection of past and recent exposure to MDMA.

3. Aims of the study

3. Aims of the study

The aims of this study were as follows:

1. to investigate the time course of MDMA (and its metabolites) distribution in the following matrices:
    a. saliva,
    b. sweat,
    c. hair.
2. to compare the concentration of MDMA (and its metabolites) in plasma with those observed in saliva after the administration of a single dose of MDMA.
3. to correlate MDMA concentrations in the afore-mentioned biological matrices with MDMA induced pharmacological effects in order to establish the usefulness of MDMA testing in those matrices from a clinical toxicology start point.
4. to develop and validate different analytical approaches (on site test, immunological screening, confirmatory analysis) to identify and quantify MDMA and tentatively its metabolites in saliva, sweat and hair samples.

4. Materials, Subjects and Methods

4. Materials, Subjects and Methods

4.1 Chemicals

Amphetamine (AP), methamphetamine (MA), MDMA, MDA, HMMA, MDEA, [$^2H_5$]MDMA, [$^2H_5$]MDA, [$^2H_5$]AP and [$^2H_8$]MA were supplied by Cerilliant, (Austin, TX, USA). Pholedrine was gently donated by the German School of Sports (Cologne, Germany). MBDB was provided by Lipomed (Cambridge, MA, USA). Drugwipe® "amphetamines", Drugread® hand photometer were supplied by Syen (Gardigiano, Venezia, Italy) and used according to the instruction of the manufacturer (Securetec, Ottobrunn, Germany). Drugread® hand photometer prototype was available only at the time of clinical trial for pharmacokinetics in saliva and it could be applied only for this biological matrix and not for the sweat samples, which were on-site tested just with Drugwipe®. The PharmChek® sweat patches were provided by PharmChem Laboratories (Menlo Park, CA, USA). The patches consisted of a medical grade cellulose blotter paper collection pad, with an area of 15.4 cm^2, covered by a thin layer of polyurethane and acrylate adhesives. Immunoassay for MDMA in sweat was performed by applying the STC Micro-plate EIA kit for Methamphetamine (STC Diagnostics, Bethlehem, PA, USA). C_{18} solid-phase extraction column (SPE) were from Biochemical Diagnostics, Inc., NY, USA and Bond Elut Certify SPE columns from Varian (Harbor City, CA, USA). Potassium dihydrogen phosphate, potassium hydroxide, ethyl acetate, sodium chloride, potassium hydroxide, acetic acid glacial 100%, ammonia solution 25% were provided by Merck. Isotonic solution was prepared adding 0.9 g sodium chloride to 100 mL Milli-Q grade water. N-methyl-

bis(trifluoroacetamide), MBTFA, gas chromatography grade was supplied from Macherey-Nagel (Düren, Germany).

Potassium dihydrogen phosphate, and ammonia solution were from Merck (Darmstadt, Germany). Ultra pure water was obtained using a Milli-Q purification system (Millipore, Molsheim, France). All other reagents were of analytical grade.

4.2 Subjects, study design and applied methodology

4.2.1. Study 1. Pharmacokinetics of MDMA in saliva

Eight male subjects were included in the study. Eligibility criteria required the recreational use of MDMA on at least five occasions. Each participant underwent a general physical examination, routine laboratory tests, urinalysis, and a 12-lead electrocardiogram. The participants had a mean age of 24.4 years, mean weight of 72.7 kg, and mean height of 177.3 cm. All the subjects declared to be MDMA consumers (times of consumption: range 5-100, mean 24). Subjects were phenotyped for CYP2D6 activity using dextromethorphan as a drug-probe. The dextromethorphan/dextrorphan ratio was used to classify subjects as poor or extensive metabolizers (Schmid et al., 1985). All participants were extensive metabolizers. All subjects gave their written informed consent before inclusion in the study and were economically compensated for inconveniences caused by their participation in the study. The study was conducted in accordance with the Declaration of Helsinki, approved by the local Ethical Committee (CEIC-IMAS), and authorised by the "Agencia Española del Medicamento" (reference AEM 98/112) of the Spanish Ministry of Health.

The study design was double blind, randomised, crossover, and controlled. Subjects participated as outpatients in two different randomly assigned 10-hour experimental

sessions in which they were given single doses of 100 mg MDMA, or placebo by the oral route. Volunteers were requested to abstain from consumption of any drug of abuse during the study period, and urine drug testing was performed before each experimental session for opiates, cocaine, cannabinoids and amphetamines. For all four groups of substances tested, all volunteers were negative before each experimental session. MDMA and placebo were prepared by the Pharmacy Service of Hospital del Mar (Barcelona, Spain) as white soft-gelatine capsules (two capsules each time), and administered in a fasting state with 100 mL of tap water.

4.2.1.1 On-site saliva testing for MDMA with Drugwipe®

Saliva was collected with the wiping pad of the test kit for amphetamines for 10 s on the tongue of the subjects at 0 time (pre-dose) and at 1.5, 4, 6, 10, and 24 h after MDMA administration. The other part of the Drugwipe® device, the detection element was an adsorbent pad, which had to be dipped into tap water to initiate the immunochromatographic reaction. In the presence of MDMA or metabolites the detection field of Drugwipe® "amphetamines" had to change its colour from white to pink depending on the amount of drug collected. According to the manufacturer, it was not necessary the coloration of the whole test window, but a line on the edge could be sufficient to qualify a positive result. Coloration remained stable at least for 1 h. Two independent readers classified results as positive or negative. Drugwipe® information sheet reported sensitivity for methamphetamine of 10 ng, understood as the minimum quantity of drug on the collection pad giving rise to a positive immune reaction. Saliva samples were reanalyzed with Drugwipe® test in the analytical laboratory applying a 2 µl volume of saliva on the test pad. At the time of test performing in laboratory, first prototypes of Drugread® hand photometer, were available. Drugread® measures in a

reflectometric mode through a photodiode the absorption of the monochromatic light produced by gold antibody conjugates in the read-out area of the Drugwipe®. Drugread® translates the coloration intensity of the read-out window into a numeric value (arbitrary units) in the range of 300-2500 digits. At the time of the clinical trial, no definitive threshold was established for differentiating samples containing analyte under investigation from samples not containing the substance. Drugread® was contextually used to read the detection field of Drugwipe® once the test was performed.

4.2.1.2.Collection of saliva and blood samples

Samples of blood and mixed saliva (saliva secreted by the different salivary glands obtained without any stimulation over a 5-min period) were collected at 0, 1.5, 4, 6, 10, and 24 h after drug administration. Samples were collected in polypropylene tubes. The salivary pH was recorded at the time of collection, and the samples immediately stored at -20ºC until analysis. Blood samples were centrifuged and the plasma obtained was immediately frozen at –20ºC. Blood and saliva from a placebo group tested negative for the presence of MDMA were used as drug-free blank samples. The collection times selected in the present experiment were based on previous experience from more extensive pharmacokinetic studies (Mas et al., 1999; Camí et al., 2000).

4.2.1.3. Determination of MDMA and its metabolites in plasma and saliva

Frozen saliva and plasma were allowed to thaw at room temperature. Prior to analysis, saliva was centrifuged to discard the mucous part that accumulated at the bottom. One ml of plasma or saliva was spiked with 200 ng of MDMA-[$^2H_5$], MDA-[$^2H_5$] and pholedrine followed by solid-liquid extraction using mixed cationic exchange/hydrophobic interaction columns (Bond-Elut Certify®).

The pH of the samples was adjusted to 6 by adding 1 mL of 0.1 M phosphate buffer, pH 6, and passed through the columns that had been previously conditioned by passing sequentially 2 mL methanol and 2 ml of 0.1 M pH 6 phosphate buffer. Columns were washed consecutively with 1 mL of 1 mol/L acetic acid and 6 mL methanol. MDMA and metabolites were eluted with 2 mL of ethyl acetate containing 2% ammonium hydroxide. After addition of 20 μL MBTFA to prevent drugs losses, the eluates were evaporated to dryness at 40°C under a nitrogen stream. Residues were reconstituted and derivatised with 50 μl of MBTFA at 70ºC for 45 min to obtain trifluoroacetyl derivatives of the analytes.

The concentrations of MDMA and its metabolites in saliva and in plasma were determined by using a HP6890 gas chromatograph coupled to a model HP5973 quadrupole mass spectrometer (Agilent, former Hewlett Packard, Palo Alto, CA, USA). The separation of the compounds was carried out with a cross-linked 5% phenyl-methylsilicone capillary column, 12m X 0.2 mm I.D. and 0.33 μm film thickness (Ultra-2, Hewlett Packard). The samples were injected in splitless mode and helium gas was used as carrier at a flow rate of 1.2 mL/min (measured at 180°C). The mass spectrometer was operated in the electron impact ionisation and selected ion monitoring acquisition mode. Qualifying ions were m/z 154, 162 and 288 for MDMA N-TFA derivative, 162, 134 and 274 for MDA N-TFA derivative, 154, 110 and 260 for HMMA-O-TFA-N-TFA derivative. Ion ratios acceptance criterion was a deviation ≤ 20%. Ions: m/z 154 for MDMA, HMMA and pholedrine, m/z 162 for MDA, m/z 158 for MDMA-[$^2H_5$] and m/z 167 for MDA-[$^2H_5$] were selected for quantification.

This methodology has previously been validated for plasma samples and it was reapplied for saliva and plasma samples in the present study (Mas et al., 1999; de la Torre et al., 2000a).

Calibration curves were prepared in drug-free saliva and plasma by adding appropriate volumes of working methanolic solutions of the analytes under investigation. Peak area ratios between each compound and the internal standard (MDMA-[$^2H_5$] for MDMA, MDA-[$^2H_5$] for MDA, pholedrine for HMMA) were used for calculation. Curves were linear over the 25-400 μg/L concentration range for MDMA and HMMA and over the 2.5-40 μg/L concentration range for MDA. Samples containing concentrations over the working ranges were repeated after appropriate dilution with phosphate buffer.

Analytical recoveries were calculated by comparing the peak areas that were obtained when calibration samples were analysed by adding the reference substances and the internal standards in blank plasma or saliva prior to or after the above-mentioned extraction procedure. Mean analytical recovery of MDMA was 90% both in saliva and in plasma; those of MDA and HMMA were 92% and 74%, respectively.

Four replicate analyses were performed with spiked plasma and saliva containing 25 μg/L MDMA and HMMA and 2.5 μg/L for MDA. Standard deviation of quantitative values was used as a measure of the noise for the calculation of the limit of quantification (10 standard deviations).

Limits of quantification were 5.7 μg/L for MDMA, 1μg/L for MDA and 2.9 μg/L for HMMA in both biological fluids.

Three replicates at three different concentrations of MDMA, MDA and HMMA (25, 100 and 400 μg/L for MDMA and HMMA, and 2.5, 10 and 40 μg/L for MDA) in blank plasma and saliva were analysed for the determination of intra-assay precision and accuracy, while the inter-day precision and accuracy were determined for three different assays of the above-mentioned replicates.

Intra-day precision (expressed as coefficient of variation for specific added target concentrations) and accuracy (expressed as percentage error of concentration found as

compared with added target concentrations) were always lower than 6.5% for all the analytes under investigation. Similarly, inter-day precision and accuracy were lower than 8.5%.

4.2.1.4 pH measurements of salivary samples

The pH of salivary samples from the eight volunteers in the MDMA or placebo groups was measured at all time-intervals with a pH indicator stick (Riedel-de Haën, Hannover, Germany) with pH range 6.4-8 (increments of 0.2 pH units). Two independent observers, who were unaware of treatment conditions, recorded results.

4.2.1.5. Pharmacokinetics and statistical analysis

With regard to saliva and plasma concentrations of MDMA, the following parameters were determined: peak concentration (C_{max}), time taken to reach peak concentration (t_{max}), area under the concentration-time curve from 0 to 24 h (AUC_{0-24}), elimination half-life ($t_{1/2e}$) in plasma and disappearance half life ($t_{1/2d}$) in saliva, elimination constant (k_e) in plasma, and disappearance constant (k_d) in saliva. First order constant kinetics is usually described as elimination constant either for plasma or saliva. As drugs are not properly eliminated from saliva, but rather what is observed is a disappearance rate, it was preferred to use the term disappearance constant to describe this kinetic parameter (O'Neal et al., 1999).

AUC were calculated by the linear trapezoidal rule and the elimination and disappearance constants were calculated by log-linear regression of the three last points with concentration above quantification limit. Correlations between different variables were analyzed by regression analysis. The Wilcoxon's test for nonparametric data was used to assess differences in salivary pH values between treatment and placebo.

Differences associated with *p* values lower than 0.05 were considered statistically significant.

4.2.2. Study 2. Pharmacokinetics of MDMA in sweat patches

Nine volunteers were included in the study. The participants had a mean age of 23.9 years, mean weight of 72.2 kg, and mean height of 177.2 cm. Body surface area of the volunteers calculated using the formula of Dubois and Dubois (Wang and Moss, 1992) showed a mean value of 1.9 m^2, ranging from 1.7 to 2.1 m^2. All the subjects declared to be MDMA consumers. Subjects were phenotyped for CYP2D6 activity using dextromethorphan as drug-probe. All participants were extensive metabolizers. All volunteers gave their written informed consent before inclusion in the study and were economically compensated for inconveniences caused by their participation in the study. The study was conducted in accordance with the Declaration of Helsinki, approved by the Ethical Committee of our institution (CEIC-IMAS), and authorized by the "Agencia Española del Medicamento" number 98/532) of the Spanish Ministry of Health.

The study design was doubled-blind, double-dummy, randomised, crossover, and placebo-controlled. Subjects participated as outpatients in two different randomly assigned 10-hour experimental sessions, one week apart, in which they were given single doses of 100 mg MDMA, or placebo by the oral route. Volunteers were requested to abstain from consumption of any drug of abuse seven days before the start and during the study period, and drug testing in urine was performed before each experimental session for opioids, cocaine, cannabis and methamphetamine. As stated before, the inclusion of subjects in the experimental sessions required a negative urine test by immunoassay. In addition, urine samples collected at predose were analysed as well as

those collected during the clinical trial by GC-MS. None of the urine samples collected at pre-dose tested positive (MDMA and metabolites were absent).

4.2.2.1. On-site sweat testing for MDMA with Drugwipe®

Subjects were swabbed in their armpit for 10 seconds at 0 time (pre-dose) and at 1.5, 4, 6, 10 and 24 h after MDMA administration. Sweat was collected with the wiping pad of the test kit for amphetamines moistened with 20 µl tap water. The other part of the detection element was an adsorbent pad, which had to be dipped into tap water to initiate the immunochromatographic reaction. In the presence of MDMA or metabolites the detection field of Drugwipe® "amphetamines" had to change its colour from white to pink depending on the amount of drug collected. According to the manufacturer, it was not necessary the coloration of the whole test window, but a line on the edge could be sufficient to qualify a positive result. Coloration remained stable at least for 1 h. Two independent readers classified results as positive or negative. Drugwipe® information sheet reported a sensitivity for methamphetamine of 10 ng, understood as the minimum quantity of drug on the collection pad giving rise to a positive immune reaction.

4.2.2.2. Collection of sweat samples by sweat patch

Six patches were applied to the back of each participant, after the skin was cleaned with a 70% isopropyl alcohol swab, and removed at 0 time (pre-dose), and 1.5, 4, 6, 10 and 24 hours post-administration, by pulling and edge from the adhesive layer, taking care not to touch the absorbent pad. After removal, the patch was labelled and stored in plastic bags at -20 °C until analysis. Sweat patches from the placebo condition, which all tested negative for the presence of MDMA by GC-MS, were used as drug-free blank

samples. Furthermore, additional drug free patches were obtained from non-consumers, which wore the sweat patches on the back for a period of 24 hours.

4.2.2.3. Determination MDMA and its metabolites in sweat patches

Sweat patches were allowed to thaw at room temperature and the absorbent pad was removed with clean tweezers. Then, the pad was placed in a testing tube containing 5 mL 0.1M phosphate potassium buffer (pH 6), and shaken for 30 minutes to extract the drug. Buffer was divided in two aliquots, the first (1mL aliquot) for drug screening by immunoassay and the second (4 mL aliquot) for GC-MS analysis. First aliquot of patch buffer extract was passed through a C_{18} solid phase extraction (SPE) column. Columns were preconditioned with 2 mL methanol and 2 mL water. After passing the sample, they were washed with 2 mL water and patch content was eluted using 2 mL methanol. Extraction recoveries obtained with patches worn by control individuals and spiked with analytes under investigation were higher than 95% (for MDMA 10ng/patch, n=5). Methanol eluate was evaporated to dryness and reconstituted with 1mL isotonic solution. Second aliquot, added with 25µl MDMA-[$^2H_5$] (10µg/mL isotonic solution) and 30µl MDA-[$^2H_5$] (1µg/mL isotonic solution), underwent a solid-phase extraction using Bond-Elut Certify Columns. Columns were preconditioned with 2 mL methanol and 2 mL 0.1M phosphate potassium buffer (pH 6), washed with 1 mL 1M acetic acid and 6 mL methanol once the sample had been passed through, and MDMA, and eventual metabolites were eluted with 2 mL of ethylacetate containing 2% ammonia solution. After addition of 20 µL MBTFA to prevent drugs losses, eluates were evaporated to dryness at 40°C under a nitrogen stream. Residues were reconstituted and derivatised with 30 µl of MBTFA at 70ºC for 45 min to obtain trifluoroacetyl derivatives of the analytes.

4.2.2.3.1. Immunoassay for MDMA in sweat

Since at the time of the study no immunoassays were available for screening of MDMA in sweat, STC Micro-plate EIA kit for qualitative/semi-quantitative determination of methamphetamine in serum was used, in accordance with previous experiments on other drugs of abuse screening in sweat (Fay et al., 1996; Fogerson et al., 1997). Cross reactivity of EIA kit for methamphetamine to MDMA (at a concentration of 30 ng/ml) was 1513% and that of MDA (at a concentration of 5000 ng/ml) was 2.8%. Cross-reaction for HMMA was not reported. Cross-reactivity for other sympathomimetic amines was. d-amphetamine: 1.3% (at a concentration of 10000 ng/mL); l-ephedrine: < 1% (at a concentration of 25000 ng/mL); l-amphetamine: 2.5 (at a concentration of 10000 ng/mL); l-methamphetamine: 9.3% (at a concentration of 2500 ng/mL); MDEA: 65.2% (at a concentration of 150 ng/mL); mephentermine: 1.3% (at a concentration of 25000 ng/mL); phenylethylamine: 0.12% (at a concentration of 100000 ng/mL); pseudoephedrine: 5.7 %(at a concentration of 2500 ng/mL). Patch eluate isotonic solution (25 μL) was added to each well along with labelled enzyme and allowed to incubate for 30 minutes at room temperature, in the darkness. Following competition to bind to antibody sites immobilised on the surface of the well, the wells were washed six times with distilled water each and aspirated. The colour produced after a 30 minute incubation was measured spectrophotometrically at 450 nm and at 630 nm. The absorbance was inversely proportional to the quantity of MDMA in the specimen. The calibrators and controls utilized consisted of isotonic solution from sweat pad elution with MDMA added at 0, 0.2, 1, 2, 5, 10, 15, 20 and 25 ng/patch. The limit of detection (LOD) was obtained from the mean absorbance of the drug free samples (A_0). Drug-free patches extracts (n=65) from healthy volunteers (n=11) plus those obtained in the placebo condition of the clinical trial from the nine volunteers at each time-point (n=54)

were used for the mean and standard deviation (SD) calculations of this absorbance. Three times the standard deviation from this mean value was taken as a background noise of the analytical system. The LOD was estimated by interpolating A0 - 3SD in the calibration curve (Fay et al., 1996). A cut-off concentration was also established from the mean zero absorbance (A_0) minus six times the SD as previously calculated.

4.2.2.3.2. Gas Chromatography-Mass Spectrometry analysis of MDMA and its metabolites in sweat

The identification and quantification of MDMA and eventual metabolites in sweat patches was performed using a HP6890 gas chromatograph coupled to a model HP5973 quadrupole mass spectrometer (Agilent, Palo Alto, CA, USA). The separation of trifluoroacetyl derivatives of the analytes was carried out with a cross-linked 5% phenyl-methylsilicone capillary column, 12m X 0.2 mm I.D. and 0.33 μm film thickness (Ultra-2, Hewlett Packard). The samples were injected in splitless mode and helium was used as carrier gas at a flow rate of 1.2 mL/min (measured at 180°C). The mass spectrometer was operated in the electron impact ionization and selected ion monitoring acquisition mode. Qualifying ions were m/z 154, 162 and 288 for MDMA N-TFA derivative, 162, 134 and 274 for MDA N-TFA derivative, 154, 110 and 260 for HMMA-O-TFA-N-TFA derivative. Ion ratios acceptance criterion was a deviation ≤ 20%. Ions: m/z 154 for MDMA N-TFA derivative, and 4-hydroxy-3-methoxymethamphetamine (HMMA-O-TFA-N-TFA) derivative, m/z 162 for 3,4-methylendioxyamphetamine (MDA-N-TFA) derivative, m/z 158 for MDMA-[$^2H_5$]-N-TFA derivative and m/z 167 for MDA-[$^2H_5$]-N-TFA derivative were selected for quantification.

This methodology has previously been validated for plasma and saliva samples as reported above for Study 1 and it was reapplied for sweat patch samples in the present study.

Calibration curves were prepared in drug-free sweat patches by adding appropriate volumes of working isotonic solutions of the analytes under investigation. Drug free patches were from study volunteers administered with placebo (one of the treatment study conditions) and from healthy volunteers wearing patches for 24 hours. Peak area ratios between each compound and their internal standard were used for calculation. Curves were linear over the 10-400 ng/patch concentration range for MDMA and HMMA and over the 2.5-40 ng/patch concentration range for MDA. Samples containing concentrations over the working ranges were repeated after appropriate dilution with phosphate buffer. Analytical recoveries were calculated by comparing the peak areas obtained when calibration samples were analyzed by adding the reference substances and the internal standards in the extract from drug-free sweat patch prior to and after the above-mentioned extraction procedure. Mean analytical recovery, measured from five replicates, was 74% for MDMA, 70% for MDA and 82% for HMMA (20 ng/patch concentration). Four replicate analyses were performed with drug-free sweat patches spiked with low concentrations of analytes (25 ng/patch MDMA and HMMA and 3 ng/patch for MDA). The standard deviation derived from quantitative values (at the afore-mentioned concentrations) was used as a measure of the noise for the calculation of the limit of quantification (LOQ=10 SD). Limits of quantification were 3.2 ng/patch for MDMA, 2.4 ng/patch for MDA and 3.2 ng/patch for HMMA.

Three replicates at three different concentrations of MDMA, HMMA and MDA (12, 160 and 350 ng/patch for MDMA and HMMA, and 3, 16 and 35 ng/patch for MDA) added to drug-free sweat patches were analyzed for the determination of intra-assay

precision and accuracy, while the inter-day precision and accuracy were determined for three independent experimental assays of the aforementioned replicates. Intra-day precision (expressed as coefficient of variation for specific added target concentrations) and accuracy (expressed as percentage error of concentration found as compared with added target concentrations) were always lower than 4.1% for all the analytes under investigation. Similarly, inter-day precision and accuracy were lower than 5.5%.

4.2.2.3.3. Pilot study on skin reabsorption of MDMA from the sweat patch

Preliminary results on MDMA in sweat showed an inflexion in the kinetics at 10 hours post-administration. This observation prompted a pilot study to evaluate the possibility that the skin could reabsorb MDMA already incorporated in patches. A total amount of 200 ng of MDMA in isotonic solution was placed on patches and was applied on the back of two non-consumer subjects, after skin was cleaned with 70 % isopropyl alcohol swabs. Four different patches were applied to each subject and were removed from the skin at 1.5, 4, 10 and 24 hours after application, and then, analyzed by GC-MS.

4.2.3. Study 3. Hair testing for MDMA and its metabolites

Hair samples were collected within the framework of a two-year follow-up study of 40 ecstasy abusers and matched controls. Subjects, with age ranging from 18 to 34 years, were enrolled by word of mouth base. At the first interview, they completed a structured questionnaire which, among other matters, asked about: initial and final age of any drug consumption, last consumption, and total consumption in the previous month and in the last six, and twelve months respectively. Information was provided for several drugs of abuse, among others: 3,4-methylenedioxymethamphetamine or ecstasy, methamphetamine, other psychostimulants like amphetamine and cocaine, cannabis,

and LSD. During the follow-up study, subjects underwent periodical psychophysical screenings, and analysis of urine and hair for principal drugs of abuse. For the purpose of this study, a method was developed and validated to investigate hair samples for the presence of MDMA and its metabolite MDA, as biomarkers of ecstasy consumption (Kintz and Cirimele, 1997; Kintz and Samyn, 1999), and also amphetamine (AP), methamphetamine (MA), MDEA and MBDB to verify self reported declarations and purity of street samples sold as ecstasy.

4.2.3.1 Collection of hair samples

Hair samples (as an entire strand) were cut close to the scalp in the vertex region using a stainless steel scissors. From all the samples collected for the study, thirteen hair samples were used within the method development to assess association between hair analysis and self-reported use of designer drugs in the last month, and in the last six and twelve months. The selection was made according to several patterns of consumption (low: < 2 tablets/month; medium: between 2 and 5 tablets/month; high: more than 5 tablets/month) representative of the population studied, including also individuals who declared no consumption, at least in the last one and six months. Hair strand from the thirteen subjects (all with natural brown hair colour) were divided in three different segments. The first was cut at 1cm from the proximal region, representing hair growth in the last month; the second and the third at 5 and 9 cm to approximately account for the last six and twelve months, respectively. These cuts were decided according to the international literature (Sachs, 1995) and considering the mean length of hair samples from the consumers. All the samples, once washed as described in the next section, were finely cut and a duplicate of 10 mg weight was obtained. Results reported in the present study refer in details the analytical findings regarding MDMA concentration in

the three different segments supposed to be related with information collected in the questionnaire for the last one, six and twelve months consumption. Hair from other 10 ecstasy users was pooled, homogenised and included in each analytical batch as internal quality control of the methodology developed. Drug-free human hair samples obtained from 10 non consumers were reduced in short cuts, analysed during method validation to exclude any source of chromatographic interferences and mixed to obtain a homogeneous pool of blank hair.

4.2.3.2. Determination of MDMA and its metabolites in hair samples

The pooled drug free hair and all the hair samples (10 mg) were washed three times (2 min) with 3 mL dichloromethane in an ultrasonic water-bath and allowed to dry at room temperature.

Then, samples, calibrators, quality control samples (drug free hair spiked with drugs at concentrations and working standard solutions other than those used for calibrators) and the internal quality control positive sample (pooled hair from MDMA users) were fortified with 50 μl of 1μg/ml [$^2H_5$]AP, [$^2H_5$]MDA and [$^2H_5$]MDMA, as internal standards.

The digestion was performed in alkaline conditions and consisted of 1 mL of 1M sodium sulphide (Na_2S) placed in silanized glass tubes with 10 mg hair samples with periodic shaking for 3 h and then kept at room temperature overnight. After digestion, hair samples were ultracentrifuged at 12000 rpm for 10 min and the aqueous layer was transferred in a new silanized glass tube. Analytes were extracted from the aqueous layer with two subsequent portions of 3 mL of tert-butyl methyl ether by rocking mixing for 30 min and centrifuged at 3500 rpm for 5 min. The organic phase was separated and evaporated to dryness under nitrogen stream at 23°C with a c.a.10 psi

pressure. Sample extracts were reconstituted with 1 mL of 0.1M sodium phosphate buffer (pH 6). Reconstituted extracts underwent a solid-liquid extraction (SPE) with Bond Elut Certify columns according to a previously reported method (Ortuño et al., 1999). Eluates from SPE were added with 20 µl of MBTFA to prevent amphetamines losses and evaporated to dryness under nitrogen stream at 40°C (c.a.10 psi pressure). Trifluoroacyl derivatives were formed by reaction with 50 µl of MBTFA as derivatization agent in a dry bath at 70°C during 45 min.

Gas chromatography-mass spectrometry analysis was performed in a Hewlett Packard 6890 gas chromatograph coupled to an HP 5973 quadrupole mass spectrometer detector (Palo Alto, CA). The gas chromatograph was fitted with an HP 7683 auto sampler injector. Samples were injected in splitless mode into a 12m x 0.2mm I.D., 0.33 µm film thickness 5% phenylmethylsilicone column (Ultra 2-Hewlett Packard).

The oven temperature was initially maintained at 70ºC during 2 min and programmed to 160ºC at 30ºC per min, then to 170ºC at 5ºC per min, to 200ºC at 15ºC, and finally to 290ºC at 30ºC per min. The injector and the interface were operated at 280ºC. Helium was used as carrier gas at a flow rate of 1.2 ml/min.

The mass spectrometer was operated in electron impact ionization mode at 70 eV. Qualifying ions selected for analytes under investigation were: m/z 91, 118, 140 for AP-N-TFA, m/z 91, 118, 154 for MA-N-TFA, m/z 154, 162, 288 for MDMA-N-TFA, m/z 135, 162, 274 for MDA-N-TFA, m/z 162, 168, 303 for MDEA-N-TFA, m/z 168, 176, 303 for MBDB-N-TFA, m/z 96, 123, 140 for [$^2H_5$]AP-N-TFA, m/z 92, 113, 161 for [$^2H_8$]MA-N-TFA, m/z 136, 167, 280 for [$^2H_5$]MDA-N-TFA, m/z 158, 164, 294 m/z for [$^2H_5$] MDMA-N-TFA. Ion ratio acceptance criterion was a deviation ≤ 20% of the average of ion ratios of all the calibrators. The ions: m/z 118 for AP-N-TFA, m/z 154 for MA-N-TFA and MDMA-N-TFA, m/z 162 for MDA-N-TFA and MDEA-N-TFA,

m/z 168 for MBDB-N-TFA, m/z 123 for [$^2H_5$]AP-N-TFA, m/z 161 for [$^2H_8$]MA-N-TFA, m/z 136 for [$^2H_5$]MDA-N-TFA, m/z 158 for [$^2H_5$] MDMA-N-TFA used for quantification.

Calibration curves were prepared from 10 mg drug-free hair from blank pool by adding appropriate volumes of metanolic solutions of the analytes under investigation. Peak area ratios between each compound and their internal standard were used for calculation. Calibration curves were linear over the limit of quantification (different for each analyte) to 20 ng/mg hair. Samples containing concentrations over the working ranges were repeated after appropriate dilution.

Recoveries were analyzed at three different concentrations, 0.5, 10 and 20 ng/mg hair, using four replicates for each evaluated concentration and ranged between 75 and 95% for all the analytes under investigation.

Five replicates of blank samples added with 5 ng/mg of ISTDs were used for calculating the limits of detection and quantification. Standard deviation (SD) of the analytical background response was used to determine the detection limit (LOD=3.3 SD) and the quantification limit (LOQ=10 SD). The obtained quantification limits were the following: 0.25 ng/mg for AP, 0.15 ng/mg for MA, MDMA and MDEA, 0.10 ng/mg for MDA, and 0.25 ng/mg for MBDB.

Five replicates at three different concentrations of the analytes (0.6, 7.5 and 17.5 ng/mg hair) spiked in blank hair were used for the determination of intra-assay precision (expressed as coefficient of variation for specific added target concentrations) and accuracy (expressed as percentage error of concentration found as compared with target added concentrations), Intra-day precision and accuracy were always lower than 15% for all the analytes under investigation at the three different concentrations. Similarly,

inter-day precision and accuracy, determined in three different experimental days, were lower than 20%.

Mid-term stability test was performed for hair samples stored at ambient temperature. Hair pool from 10 ecstasy consumers, used as internal quality control, was included in each analytical batch during a three months period. The stability was expressed as a percentage of the initial concentration (first analyzed batch) of the analytes in pooled hair. No relevant degradation was observed in the pooled positive control analysed in a three months period, with differences when compared to the initial concentration lower than 5%. Selectivity tests were performed with 10 hair samples from non-consumers extracted and analysed for assessment of potential interferences from endogenous substances. The apparent response at the retention times of the analytes under investigation was compared to the response of analytes at the limit of quantification. Furthermore, potential interferences from principal drugs of abuse (opiates, cocaine and main metabolites, cannabinoids: delta-9-tetrahydrocannabinol and 11-nor-9-carboxy-tetrahydrocannabinol) were also evaluated spiking 10 mg of blank hair spiked with 10 ng of the aforementioned substances and carried through the entire procedure. Nor endogenous substances extracted from hair matrix, nor of the drugs of abuse other than analytes under investigation carried through the entire procedure interfered with the assay.

5. Results

5. Results

5.1. Study 1. Pharmacokinetics of MDMA in saliva

Figure 8 shows the time-course of MDMA concentrations in saliva and plasma for each of the eight volunteers. At 1.5 h after administration of MDMA, concentrations appeared to be the highest both in saliva (range 1728.9 to 6510.6 μg/L) and plasma (range 134.9 to 223.0 μg/L).

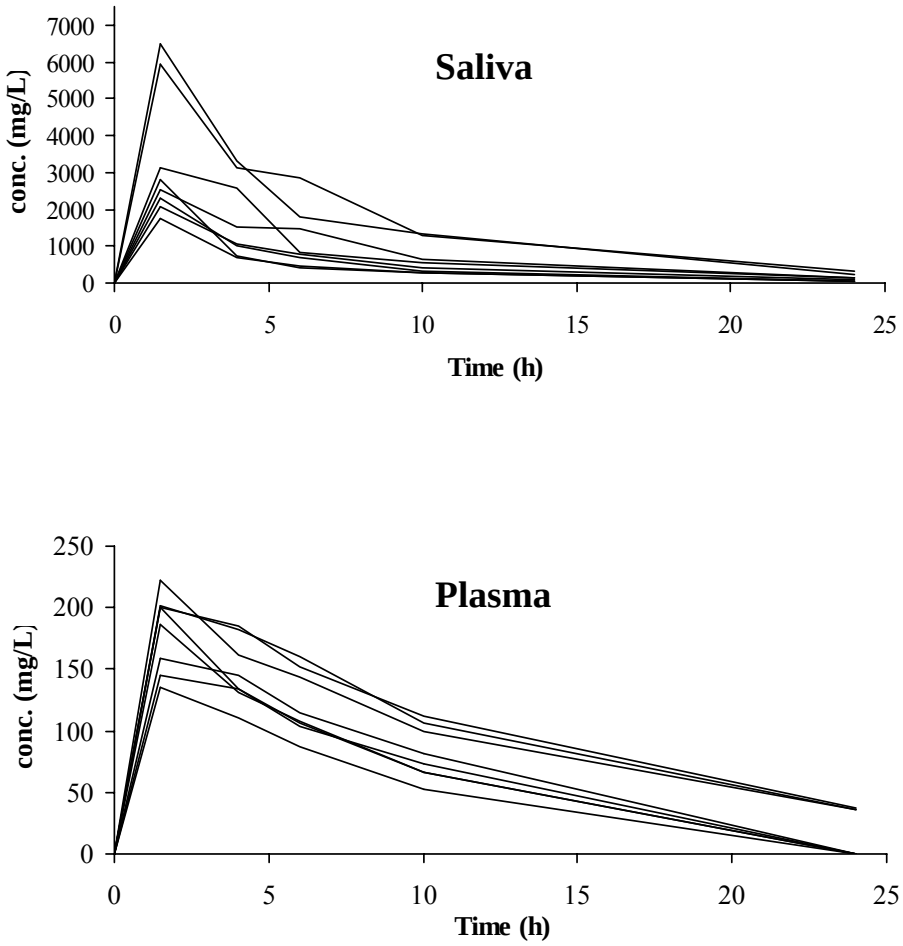


Figure 8. Concentration time-profiles of MDMA in saliva and plasma

It should be noted that two out of the eight volunteers had peak MDMA concentrations in saliva more than twofold higher than the mean peak concentrations in the remaining six subjects. These two individuals were also the ones showing highest plasma peak concentrations. Following the absorption phase, saliva and plasma MDMA levels declined to a mean concentration (standard deviation, SD) at 24 h of 126.2 (101.8) μg/L and 13.5 (18.6) μg/L, respectively. Mean concentration-time curves of MDMA in saliva and plasma are shown in Figure 9. **MDMA concentrations in saliva were one order of magnitude higher than those observed in plasma.**

HMMA, the major metabolite of MDMA, was detected in the non-conjugated form in trace amounts but quantification was not possible. **MDA was also excreted in saliva, with concentrations representing about 4–5 % of the concentration of salivary MDMA (AUC comparisons), as was also observed in case of plasma (**Figure 9).

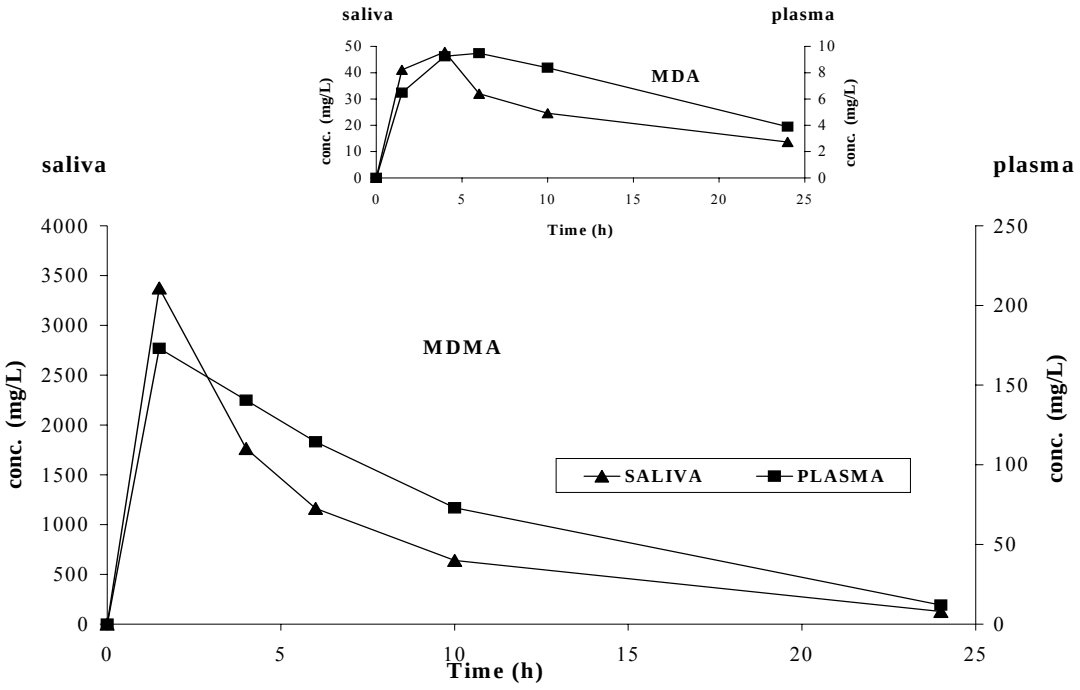


Figure 9. Plasma and salivary concentrations of MDMA and MDA

MDA highest salivary concentrations occurred between 1.5 and 4 h after drug administration, while in case of plasma maximum concentrations were between 4 and 6 h.

MDMA consumption could be detected in saliva, by wiping the Drugwipe® collection pad over the tongue, already at 1.5 hours and as far as 10 hours after drug administration (Fig. 10a). However, although data available from GC/MS showed that volunteers had salivary MDMA concentrations in the range of thousands μg/L in the first hours after drug administration, direct application of the device on the tongue produced one negative result and four negative results at 1.5 h and 4 hours from drug use, respectively. Positivity to the test decreased noticeably at 6 and 10 hours after MDMA administration.

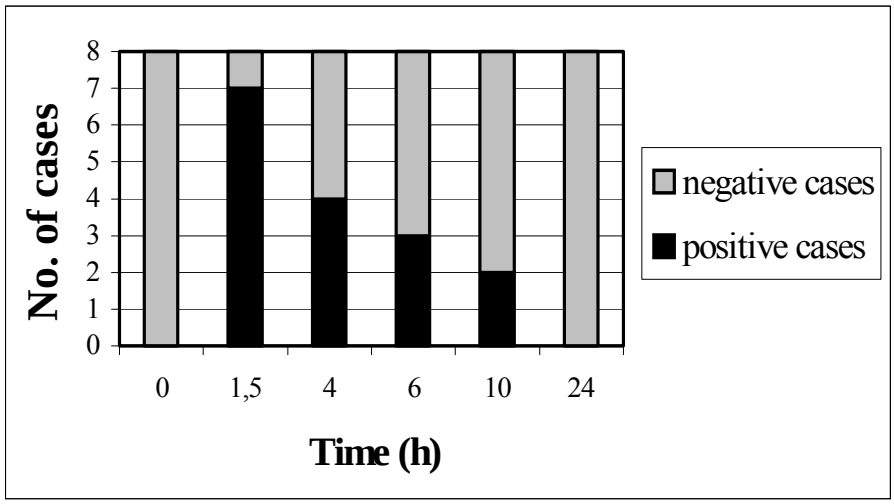


Figure 10a. Drugwipe® on saliva collected with test pad directly on the tongue

The most reasonable explanation for the high number of negative results was the insufficient amount of saliva collected from the tongue of volunteers with the device. In order to verify this hypothesis, the test was repeated in the laboratory setting, applying a pre-selected volume of 2 μl of saliva to the device.

This volume was proved to be the largest volume that was completely absorbed by the test pad of the Drugwipe®. Once runned, test was read visually and then using Drugread® hand photometer. Results obtained with Drugwipe® and Drugread® in this phase of the study were compared with the quantification of MDMA in saliva samples by gas chromatography/mass spectrometry (GC/MS).

Drugwipe® performed better if applied to a pre-selected volume of saliva sample rather than directly wiped on the tongue (Fig. 10b), i.e. all the subjects gave a positive result at 1.5 and 4 hours after drug administration, and at 6 hours after treatment only one out of 8 subjects gave a negative result. This subject showed MDMA salivary concentration of 414.4 μg/L by GC/MS, which was the lowest among all the volunteers at that time. At 10 hours from MDMA treatment, it was still possible to detect consumption in five of the eight subjects. The three subjects who had a negative result, presented MDMA salivary concentration by GC/MS always below 400 μg/L, while the other five showed concentrations always above 450 μg/L. At 24 hours, no positive results were reported, as it was the case of direct application of the device on the tongue. At that time, mean salivary MDMA concentration in the eight subjects was 126.2 μg/L (range from 27.7 to 318.0 μg/L).

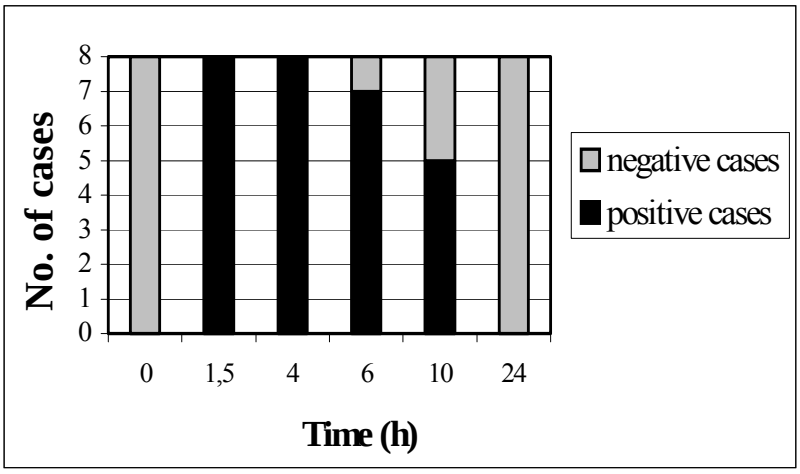


Figure 10b: Drugwipe® on 2 μl of saliva applied to the test pad in the laboratory

On the other hand, when Drugwipe® was applied to salivary samples from the placebo group, a faint color change in the read-out window of the device was seen in some cases. Samples were classified as negative, but interpretation of some results remained difficult.

Drugread® hand photometer proved useful to solve problems of visual interpretation when reading the test window of Drugwipe®.

In fact, Drugread® measurements in digits (mean ± standard deviation, n=8) were significantly different (Student's t test, $p < 0.001$) when results obtained in the placebo group at 1.5, 4 and 6 hours (519.1 ± 116.7 digits, 497.1 ± 118.1 digits, 476.5 ± 65.7 digits, respectively) were compared with those obtained in subjects given MDMA at 1.5 h (1100.0 ± 177.4 digits), 4 h (1083.7 ± 217.4 digits) and 6 h (992.9 ± 205.1 digits) after drug administration. Furthermore, Drugread® mean readings presented a time course profile similar to the mean time-concentration curve of MDMA in saliva measured by GC/MS (Fig.10c). The apparent slower disappearance rate in the Drugread® signal was probably due more to a saturation effect in Drugwipe® test pad coloration (ceiling effect) than to the contribution of MDMA metabolites since **MDMA was reported as the principal analyte which could be detected in saliva, while its principal metabolites were found only in minute amounts**.

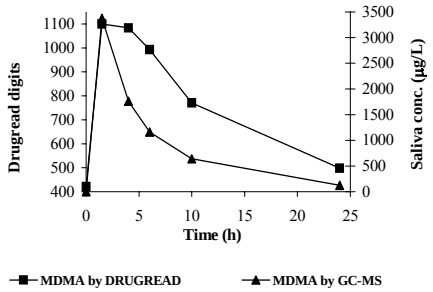


Figure 10c. A comparison of time curves of MDMA in saliva measured by Drugwipe® in combination with Drugread® and GC/MS

Pharmacokinetic parameters for MDMA in saliva and plasma are presented in Table 2. It is important to point out that these are only "apparent" parameters due to the few concentration-time data points for saliva and plasma evaluated in this study. Nonetheless, the apparent pharmacokinetic parameters of MDMA in plasma were in accordance with those reported by our group in a previous study (Ortuño et al., 1999). The t_{max} was attained at 1.5 h in both saliva and plasma. The disappearance half life($t_{1/2d}$) and disappearance constant (k_d) values in saliva were greater than plasma elimination half life and elimination constant values.

Table 2. Apparent Pharmacokinetic Parameters for MDMA in Saliva and Plasma

	Saliva			**Plasma**		
	Mean	SD	CV %	Mean	SD	CV %
$AUC_{0\text{-}24\ h}$ (μg/L·hours)	20843.1	12656.6	60.7	1598.6	733.3	45.9
t_{max} (hours)	1.5			1.5		
C_{max} (μg/L)	3375.6	1812.8	53.7	181.4	31.3	17.2
K_d^a ($hours^{-1}$)	0.1279	0.0231	18.6	0.0988	0.0189	19.1
$T_{1/2\ d}^a$ (hours)	5.6	0.9	16.9	7.2	1.4	19.0

SD: standard deviation; CV: coefficient of variation; $AUC_{0\text{-}24}$= area under the curve from 0 to 24 h; t_{max} = time to peak concentrations; C_{max}: peak concentration; k_d = disappearance constant; $t_{1/2\ d}$ = disappearance half-life. [a]Elimination parameters for plasma

Figure 11 shows the 24 h profile of mean salivary pH in both placebo and MDMA treatment conditions. Salivary pH had a mean value of 7.4 and 7.3 at predose time in MDMA and placebo samples, respectively. At 1.5 h after drug administration, corresponding to MDMA t_{max} , salivary pH in MDMA group showed a mean (SD)

statistical decrease to 6.9 (0.2) as compared with 7.3 (0.2) for placebo (Wilcoxon's test $p < 0.001$). **The decrease in pH values was homogeneous as all subjects treated with MDMA showed lower values than those observed when treated with placebo.**

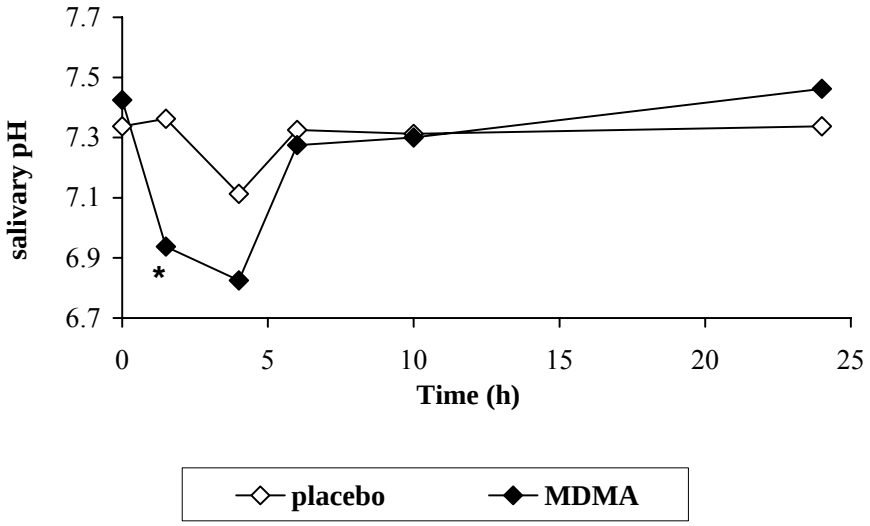


Figure 11. Time profile of salivary pH in each treatment condition

At 4 h after treatment (1 hour after a light meal given to volunteers) minimum mean (SD) values of 6.8 (0.4) and 7.1 (0.3) were obtained in MDMA and placebo samples, respectively. The two mean values were not statistically different. In fact, six of the eight subjects showed the same pattern, in one subject no changes were observed, and in another subject, pH value in the placebo condition was higher than that of MDMA. Finally, pH turned to predose values between 6 and 24 h after treatment administration both in MDMA and placebo groups.

The time-course curve of S/P ratio during the 24 h after drug administration is presented in Figure 12.

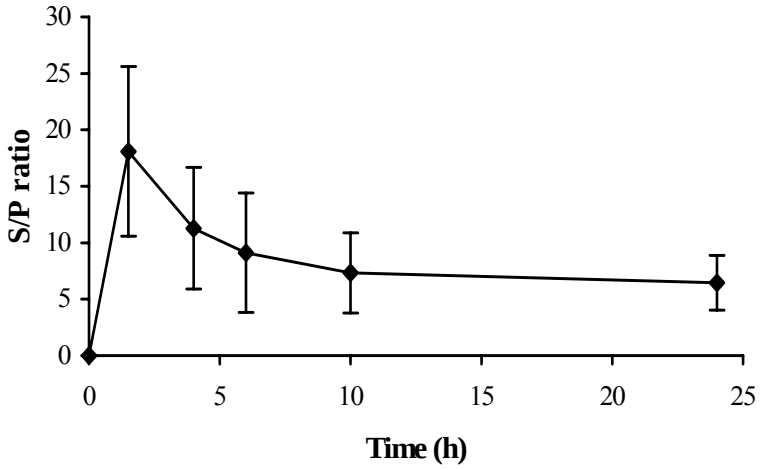
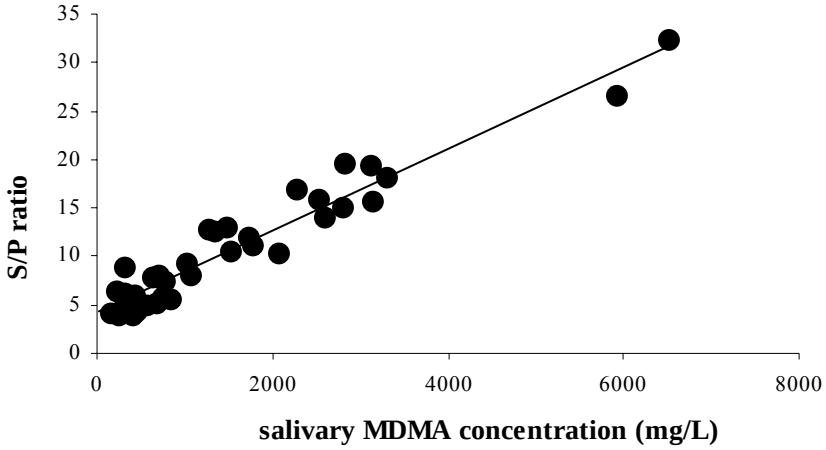


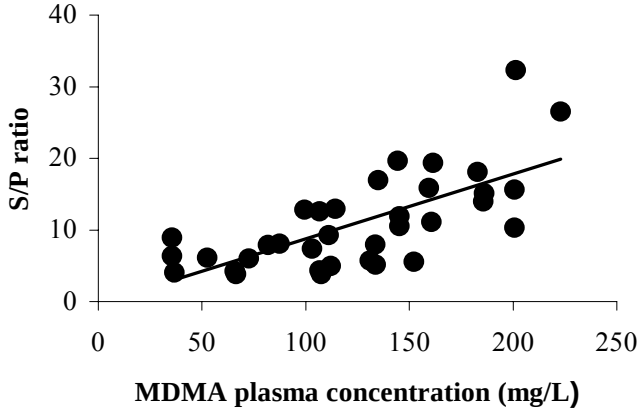
Figure 12. Time profile of MDMA saliva to plasma (S/P) ratio

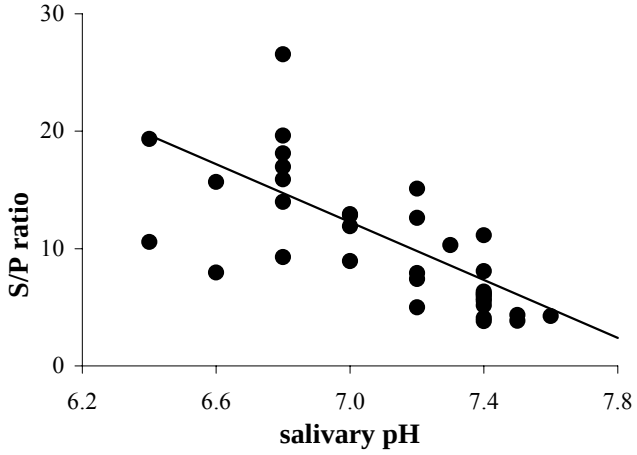
The S/P ratio exhibited a mean (SD) maximum value of 18.1 (7.9) at 1.5 h corresponding to MDMA $t_{max.}$. The high variability observed (coefficient of variation 43%) was due to the two individuals with highest MDMA salivary peak concentrations, who had S/P ratio values of 26.6 and 32.3, while the remaining volunteers showed values in the range between 10.3 and 16.9. In the post-absorption phase, the S/P ratio declined to reach values around 7.3 and 6.4 at 10 and 24 hours after drug administration, respectively. S/P ratios showed a strong correlation with salivary MDMA concentrations ($r = 0.96$, $p< 0.001$) as well as a lower but significant correlation with salivary pH values ($r = 0.62$, $p<0.002$) and plasma MDMA concentrations ($r = 0.69$, $p<0.001$) (Figure 13).

In any case, notwithstanding the variation of S/P ratio during time course of MDMA administration, salivary concentrations were correlated to plasma concentrations (r=0.81, *p*< 0.001).

Figure 13. Correlation between MDMA saliva/plasma (S/P) ratio and MDMA concentrations in saliva, plasma and with mean salivary pH during treatment administration







5.2 Study 2. Pharmacokinetics of MDMA in sweat patches

Table 3 summarizes all the results obtained for immunoassay and GC-MS analysis of sweat patches and Drugwipe® on-site sweat test of the 9 subjects administered orally 100 mg MDMA.

Drugwipe® could detect MDMA consumption in sweat already at 1.5 hours and as far as 24 hours after drug administration (Figure 14).

Although all the subjects gave a positive result at 1.5 hours after drug administration, at 4 hours two out of the nine subjects gave a negative result and at 6 and 10 hours after treatment three subjects gave a negative result. At 24 hours from MDMA treatment, it was still possible to detect consumption in three of the nine subjects. Results were consistently negative in all placebo samples.

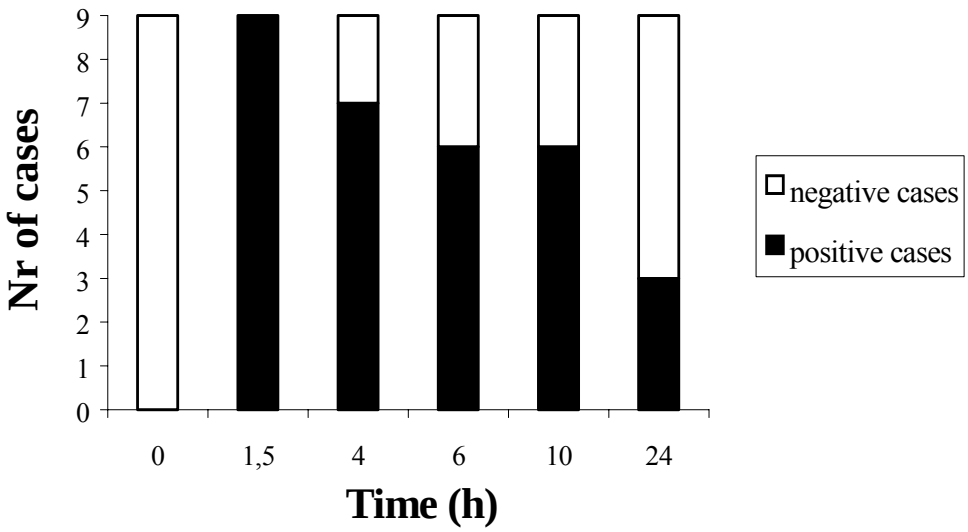


Figure 14. Performance of the Drugwipe ® on-site test directly applied for 10 sec on the armpit of the 9 volunteers administered 100 mg MDMA.

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	Subject 1					Subject 2					Subject 3				
	Pcbo	MDMA	MDMA	Drugwipe		Pcbo	MDMA	MDMA	Drugwipe		Pcbo	MDMA	MDMA	Drugwipe	
Time	EIA-STC		GC/MS	Pcbo	MDMA	EIA-STC		GC/MS	Pcbo	MDMA	EIA-STC		GC/MS	Pcbo	MDMA
(h)	(ng/ml)		(ng/ml)			(ng/ml)		(ng/ml)			(ng/ml)		(ng/ml)		
0	0.0	0.0	0.0	-	-	0.0	0.0	0.0	-	-	0.0	0.0	0.0	-	-
1.5	0.0	9.9	1.5	-	+	1.7	32.8	44.3	-	+	0.3	90.4	175.3	-	+
4	0.0	13.9	12.2	-	+	0.0	170.8	113.7	-	-	0.0	99.3	554.9	-	+
6	0.0	5.2	6.7	-	-	0.2	100.3	84.6	-	-	1.6	441.5	946.7	-	+
10	0.0	37.0	25.8	-	-	0.0	34.2	79.8	-	-	0.7	350.1	845.5	-	+
24	0.0	784.1	906.7	-	-	0.1	215.4	269.7	-	-	0.0	995.6	1326.1	-	-

	Subject 4					Subject 5					Subject 6				
	Pcbo.	MDMA	MDMA	Drugwipe		Pcbo	MDMA	MDMA	Drugwipe		Pcbo	MDMA	MDMA	Drugwipe	
Time	EIA-STC		GC/MS	Pcbo	MDMA	EIA-STC		GC/MS	Pcbo		EIA-STC		GC/MS	Pcbo	MDMA
(h)	(ng/ml)		(ng/ml)	-	-	(ng/ml)		(ng/ml)	-	-	(ng/ml)		(ng/ml)	-	-
0	0.0	0.0	0.0	-	+	0.2	0.0	0.0	-	+	0.0	0.0	0.0	-	+
1.5	0.2	96.3	19.2	-	+	0.7	8.3	4.5	-	+	2.0	9.1	14.5	-	+
4	0.1	436.8	433.4	-	+	0.3	44.0	183.3	-	+	1.6	82.4	86.9	-	+
6	0.6	387.4	625.6	-	+	0.6	58.0	55.4	-	+	0.3	105.7	123.9	-	+
10	0.8	645.0	487.8	-	+	0.0	5.3	6.7	-	+	1.6	17.6	50.2	-	+
24	2.3	319.7	398.6	-	+	0.0	101.5	241.3	-	+	0.0	498.7	377.2	-	-

	Subject 7					Subject 8					Subject 9				
	Pcbo	MDMA	MDMA	Drugwipe		Pcbo	MDMA	MDMA	Drugwipe		Pcbo	MDMA	MDMA	Drugwipe	
Time	EIA-STC		GC/MS	Pcbo	MDMA	EIA-STC		GC/MS	Pcbo	MDMA	EIA-STC		GC/MS	Pcbo	
(h)	(ng/ml)		(ng/ml)			(ng/ml)		(ng/ml)			(ng/ml)		(ng/ml)		
0	0.8	0.0	0.0	-	-	2.0	0.0	0.0	-	-	0.0	0.0	0.0	-	-
1.5	0.0	33.9	1.0	-	+	0.8	34.9	3.2	-	+	0.0	5.2	27.2	-	+
4	0.3	9.4	6.5	-	-	1.4	48.3	29.2	-	+	1.9	61.3	33.6	-	+
6	0.5	28.7	14.9	-	+	0.0	27.8	184.0	-	+	0.1	57.0	21.6	-	-
10	2.81	22.62	9.4	-	+	1.1	152.8	65.2	-	+	0.0	4.8	4.3	-	-
24	0.00	56.43	42.5	-	-	0.0	209.1	1023.8	-	+	0.0	201.5	293.2	-	-

Table 3: Immunoassay and GC-MS analysis of sweat patches and Drugwipe on-site sweat test of the 9 subjects administered orally 100 mg MDMA

With respect to the immunoassay used as a screening test to analyze sweat patches, the limit of detection LOD, was found to be 1.4 ng MDMA/patch. Using this LOD as threshold concentration to differentiate placebo and treatment samples, ten false positive results (according to GC-MS) were obtained in case of placebo samples (10 out of 54 samples). **A cut-off of 4.3 ng MDMA/patch, calculated as aforementioned, was applied as a more conservative positivity criterion, and in this case no false positive results for placebo samples were observed.** All sweat patch samples, from the nine participants administered 100 mg MDMA, presented a negative result before drug administration. **By contrast, positive results were obtained at 1.5 hours and as long as 24 hours after single drug administration in sweat patches from all participants.** All samples, screened by immunoassay irrespective of the positive or negative result, were subsequently analyzed by GC-MS. **A good correlation (r=0.85) was found between values obtained by immunoassay screening and GC-MS confirmation**

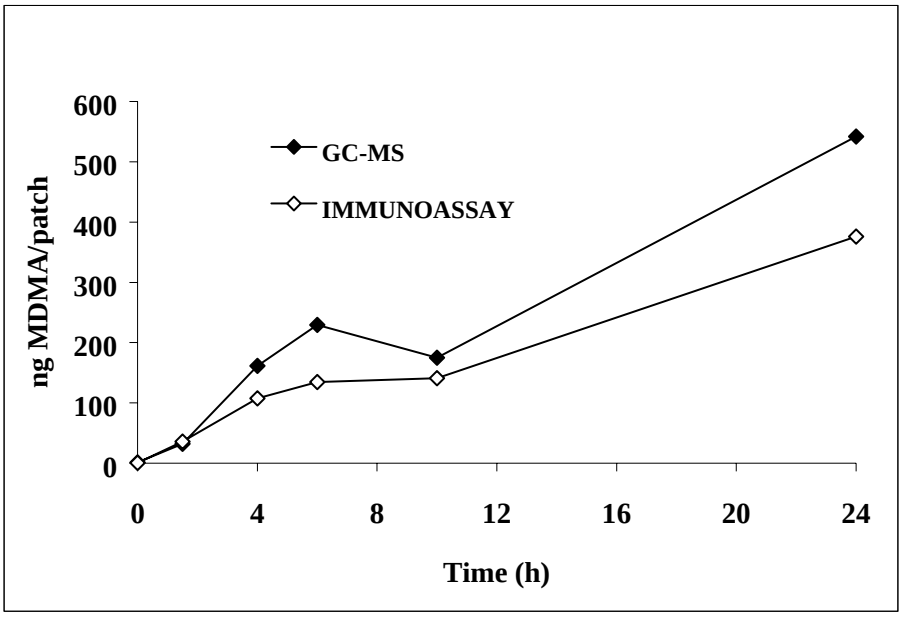


Figure 15. Comparison of time curves of MDMA contained in sweat patches applied to the back of the nine volunteers by immunoassay and GC-MS.

All samples showing results outside the dynamic range of the calibration curve were diluted accordingly with extracts from worn drug free patches

Figure 16 shows the time-course of MDMA concentrations in sweat patches in a 24 hours period for each of the nine volunteers (mean values can be found in Figure 15). MDMA appeared in sweat prior to 1.5 hours after drug administration, (mean = 41.2 ng/patch, range: 3.2-175.3 ng/patch) in seven volunteers, but two, which presented MDMA for the first time in the 4 hours post-administration sweat patch. Four out of nine individuals presented a first peak concentration at 4 h following administration, (range: 12.2-183.3 ng MDMA/patch) while five volunteers presented a peak at 6 h (range: 6.7-946.7 ng MDMA/patch). Following the peak concentration, at 10 h post-administration MDMA levels in sweat patch declined to a mean concentration of 175.0 ng MDMA/patch. (range: 4.3-845.5 ng MDMA/patch). **At 24 h after drug intake, the highest concentrations of MDMA in sweat patches were observed**, with a mean value 542 ng MDMA/patch and a range from 42.5 to 1326.1 ng MDMA/patch. **Inter-subject variability of MDMA concentration in the sweat patches was very large. MDMA concentration variability ranged from a factor of 30 at 24 h to a factor of 140 at 6h.** It should be noted that two of the nine volunteers (volunteers 3 and 4 at Figure 16) had exceedingly high MDMA concentrations. These two individuals also showed high MDMA concentrations in plasma and saliva after being administered 100 mg MDMA (Study 1). Concentrations of MDMA encountered in the 24 h post-administration patches were used to estimate total amount of MDMA excreted in the 24 hours as the ratio between area of the patch and total body surface area of each volunteer.

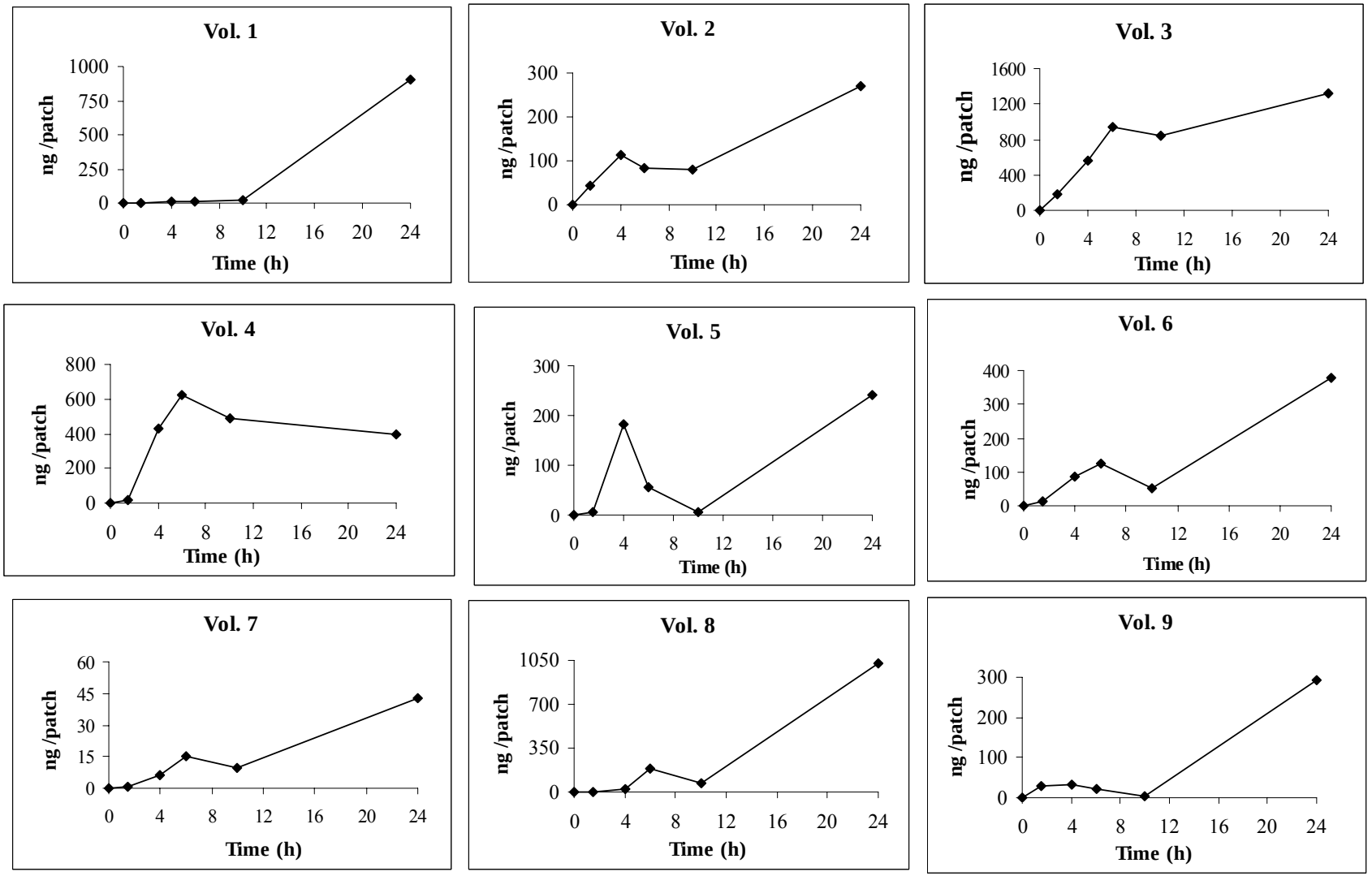


Figure 16. Concentration-time profile of MDMA container in sweat patches applied to the back of the 9 volunteers and removed at different time intervals.

Mean concentration of MDMA excreted in sweat resulted to be 0.6 mg (Confidence Interval 95% 0.28-0.92 mg) **equivalent to about 0.6% of the administered dose**, approximating sweating at different body sites to the excretion obtained at the back of individuals (Figure 17).

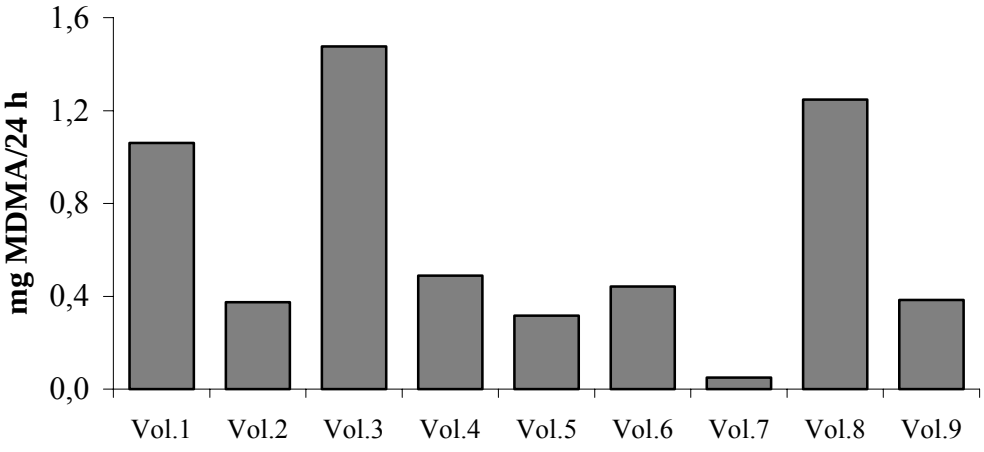


Figure 17. Total amount of MDMA excreted in sweat during the 24 hours post-administration for the nine volunteers.

Sweat patch specimens were also analyzed for MDMA metabolites by GC-MS. **HMMA, the major plasma metabolite, was not detected in sweat**. On the other hand, **MDA, was found only in trace amounts in seven out of 9 participants**, but quantification was not possible. However, **in the two volunteers with highest MDMA concentrations (volunteer 3 and 4), MDA could be quantitated from 4 h to 24 hours post-administration**. Concentrations ranged from 24.8 and 18.1 ng MDA/patch at 4 hours to 23.7 and 8.9 ng MDA/patch at 24 hours post-administration (Figure 18).

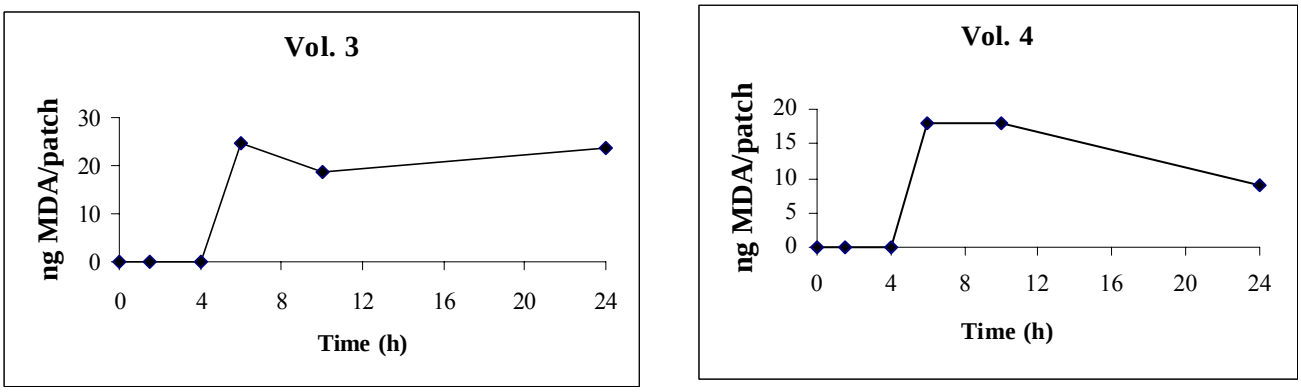


Figure 18. Concentration time-profiles of MDA contained in sweat patches from volunteers 3 and 4 at different time intervals.

Figure 19 shows the results obtained in the experiment concerning skin

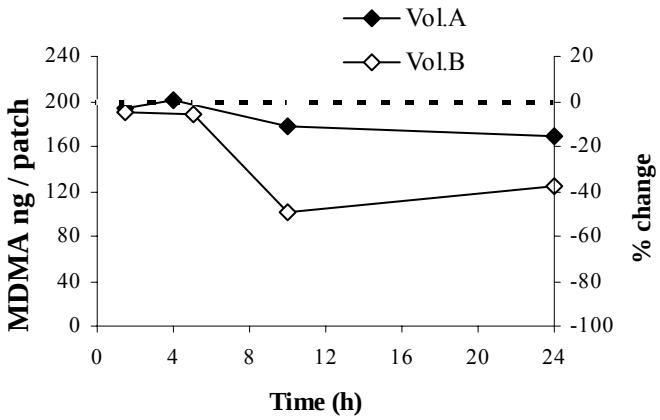


Figure 19. Time profile of skin reabsorption of 200 ng MDMA spiked in sweat patches applied to the back of two control volunteers

reabsorption of MDMA from the sweat patch. At 1.5 and 4 hours after patch application on the back of two volunteers, amounts of MDMA in the sweat patches remained almost unchanged. Conversely, at 10 and 24 h post application, in both volunteers, a decrease in MDMA concentration in the patch was observed. In fact, the 10 h patch, from volunteer A, showed a 12% decrease in the amount of MDMA initially present, and this result was confirmed in the 24 h patch. Volunteer B, exercising in the

gymnasium, showed a 49% reduction of MDMA concentration in the 10 h post-application patch while in the subsequent 24 h patch, some recovery of MDMA concentration (22% over the previous value) but a net decrease (40%) over the initial amount spiked was observed.

5.3 Study 3. Hair testing for MDMA and its metabolites

Table 4 presents the results obtained after applying the developed analytical methodology to proximal 1, 5 and 9 cm hair segments of individuals claiming use of "ecstasy".

Table 4: MDMA concentration in different hair segments from study subjects an self reported MDMA tablet consumption.

Subjects	MDMA tablets last month	MDMA conc. 1 cm hair seg.(ng/mg)	MDMA tablets last 6 months	MDMA conc. 5 cm hair seg. (ng/mg)	MDMA tablets last 12 months	MDMA conc. 9 cm hair seg. (ng/mg)
1	2	3.08	12	1.67	20	0.86
2	2	3.14	12	3.46	16	4.41
3	2	2.35	6	1.03	10	0.63
4	12	4.34	36	3.40	72	2.06
5	1.5	1.70	7.5	0.96	15	0.83
6	0	0.00	0	0.00	0	0.23
7	5	6.98	15	4.37	25	3.50
8	3	2.98	72	2.71	150	1.51
9	4	4.15	24	2.26	96	1.20
10	0	0.00	0	0.00	1	0.00
11	4.5	4.53	27	6.90	54	6.42
12	0	0.00	0	0.48	10	0.26
13	12	4.68	72	2.62	150	2.46

It has to be said that these results were obtained digesting the samples with sodium sulphide solution. This reagent already increased drug recovery from keratin matrix in

case of nicotine and flunitrazepam (Claffey et al., 2000). For this reason, this digestion agent was tested during method development and compared with other digestion approaches commonly used to extract drugs of abuse and in particular amphetamines from hair samples, such as sodium hydroxide, and resulted to be the best compromise between recovery of analytes from hair matrix, clean-up of extracts and absence of chromatographic interferences. Consequently, this procedure was used to examine hair from ecstasy consumers. As shown in the Table, MDMA was always found in subjects declaring its consumption. It has to be said that in those hair samples MDA could be also determined, and when other amphetamines were absent, the metabolite/parent drug (MDA/MDMA) ratio ranged between 0.04 and 0.06 in agreement with other authors (Cooper et al., 2000), which in case of higher ratios claimed the presence of the same MDA in the illegal ecstasy preparation. Furthermore, although outside the aim of this study, it can be added that methamphetamine could be found only in four out of the seven individuals, which declared "speed" consumption without any dose-concentration relationship. Similarly, AP could never be detected in hair samples of subjects claiming methamphetamine consumption while it was found, together with MA, in a hair sample of an individual, which did not declare consumption.

This finding is in agreement with what reported by other authors (Cooper et al., 2000) which affirmed that LOD and LOQ of AP higher than those for the other compounds could decrease the number of potentially positive results. MBDB was never detected in the examined subjects, as this amphetamine derivative seemed not to be present in Spain, nor as at the moment in any other European country. The single case of MDEA also demonstrated the scarce diffusion of this methylenedioxyderivative in Spain.

Figure 20 reports MDMA concentrations as a function of monthly consumed tablets in the last month, last six months and finally last twelve months obtained by dividing the cumulative number of consumed tablets (as reported in Table 4) by the declared average monthly consumption.

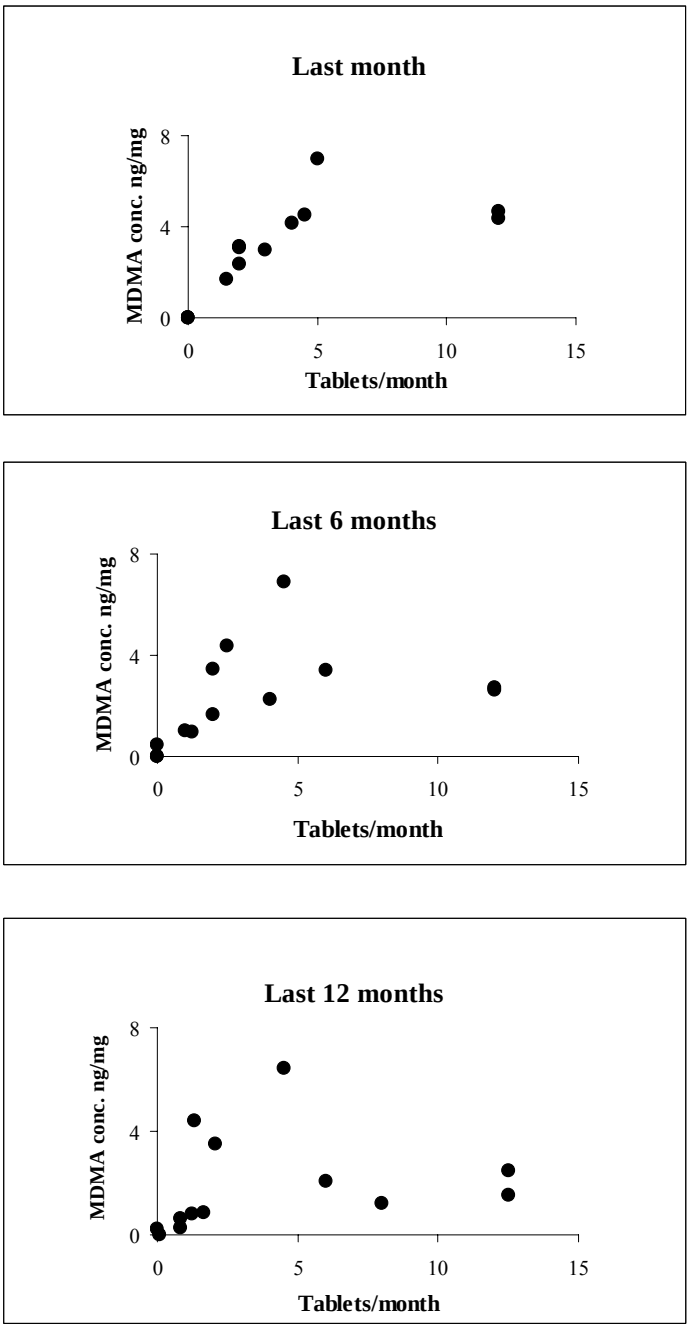
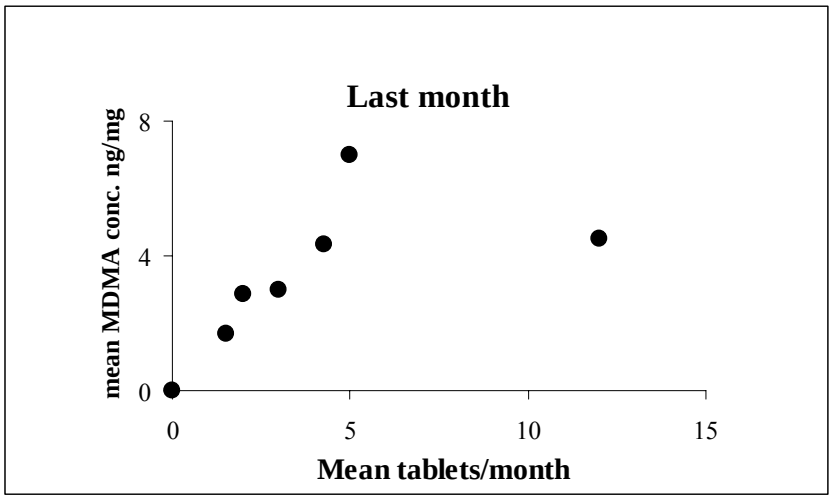
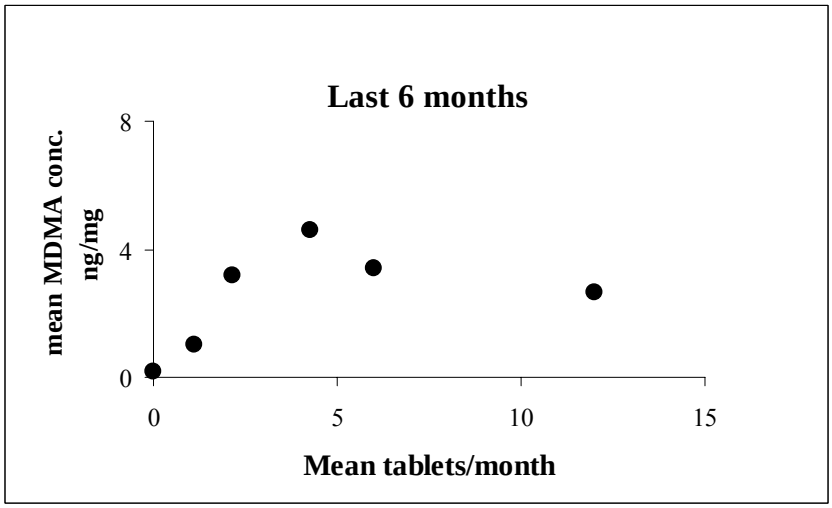


Figure 20: MDMA hair concentrations as a function of monthly consumed tablets.

The calculation of monthly consumed tablets in the last six and twelve months was made to investigate if an eventual concordance between self-reported drug use and MDMA in hair in the recent past (last month) could be also extended to more remote period (up to one year). **Concordance between the self-reported data on last month "ecstasy" use and MDMA hair concentration was quite good (r^2 = 0.92) in all the examined subjects, if excluding the two individuals, which declared a high consumption of the drug** (12 tablets in the last month). The inclusion of MDMA hair concentrations from these two subjects significantly decrease correlation between drug use history and laboratory findings (r^2 = 0.45). When comparing self declaration of monthly consumed tablets within the last six months, concordance with hair MDMA values decreased both excluding (r^2 = 0.56) or including the two heavy ecstasy consumers (r^2 = 0.16). No correlation seemed to exist between number of mean consumed tablets in the last twelve months and MDMA in hair, nor for low and medium consumption (r^2 = 0.11) or including the high one (r^2 = 0.04). Although a limited number of individuals was investigated in this study, it was attempted to generalize the obtained results, grouping subjects with similar declared drug use in the last month, last six months and last twelve months and comparing with the mean of MDMA concentrations found in the corresponding 1 cm, 5 cm and 12 cm hair segments of individuals belonging to the same drug use group.

Figure 21 graphically reports the results obtained. Differently from the what shown when considering single individuals, **an excellent concordance between mean values of self report drug use and mean MDMA hair concentration in hair segments corresponding to the last one, six and twelve months was found in groups of subjects consuming less than 5 tablets of MDMA a month** (r^2 = 0.92, 0.94 and 0.97 for the last one, six and twelve months respectively).





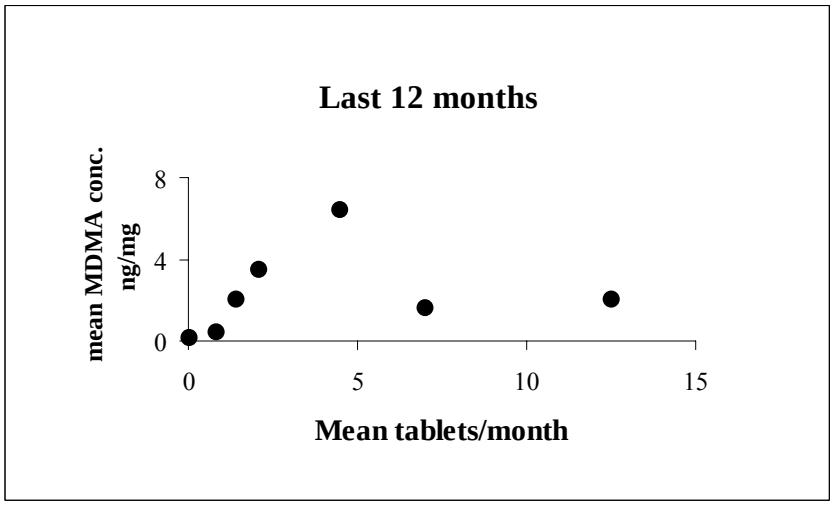


Figure 21: MDMA mean hair concentrations as a function of mean monthly consumed tablets in individuals with similar pattern of drug use.

6. Discussion

6. Discussion

6.1 General considerations

Drug law enforcement authorities have already started showing some interest for drug testing in alternative matrices. It is calculated that the Home Office in United Kingdom is running as much as 250,000 saliva tests per year. The renewal of driver's licenses in Italy, in subjects known as former drug abusers are the object of hair testing to verify abstinence (Montagna et al., 2000). In Germany, approximately 60% of all police forces are using routinely sweat testing at the road side (Steinmeyer et al., 2001). Regarding clinical settings, in the context of drug dependent subjects under treatment or under evaluation for a new treatment, particularly when evaluation of abstinence is indispensable, urine drug testing is progressively being substituted by hair testing (Pepin and Gaillard, 1997). A single hair analysis may reflect past exposure to drugs in the last months, while several urine samples have to be analysed to obtain equivalent information. Therefore, testing in alternative matrices is no longer a scientific theory but is becoming a reality. The procurement of those matrices is less invasive than urine drug testing or blood sampling, and is preferred by law enforcement agencies. Then, the genuine interest on these alternative matrices is there. Consequently, the industry is developing more and more analytical devices to facilitate testing.

Our knowledge on the distribution of drugs and specifically MDMA on these matrices is relatively scarce and heterogeneous depending on the matrix considered. There are some issues that need to be further investigated in more controlled settings. Firstly, to have a better knowledge on the mechanisms involved in the diffusion of drugs in these alternative substrates. Secondly, to establish which are the desired concentrations that should be targeted by analytical devices in each scenario. It is quite apparent that sensitivity needed to perform saliva testing is not the same than that for hair testing. For those matrices (sweat and saliva) whose concentrations better parallel

plasma concentrentrations, it is very likely that, from a law enforcement perspective, there will be an interest on setting up some kind of threshold concentrations. In this forensic context it is relevant to know if a given subject is under the influence of a drug or not. Most of these questions remain open, and the use of these matrices will no be effective until they are answered.

6.2 Study 1. Pharmacokinetics of MDMA in saliva

Overall, the patterns of salivary and plasma MDMA concentration-time profiles from different subjects agreed well, but certain inter-individual variations were evident in case of salivary MDMA levels. Data obtained for plasma samples were more homogeneous and are in agreement with previously reported findings after the administration of the same MDMA dose to eight subjects (de la Torre et al., 2000a).

MDMA appeared in saliva in concentrations remarkably greater than those in plasma (Figure 9). This is not surprising and may be attributable to several causes. Drugs are generally incorporated into saliva by passive diffusion because of a concentration gradient in which only the free fraction of drug (not bound to proteins) diffuses through lipidic membranes from plasma to saliva. It should be noted that saliva has little protein binding capacity compared to plasma. There are not specific data on the fraction of MDMA bound to plasma proteins, but from what is known for amphetamine and methamphetamine (Baselt et al., 1995), it should be around 20%. In practice, such low binding means that MDMA available in plasma may diffuse to saliva. In addition, the passage across cell membranes is favoured for molecules like MDMA with low molecular weight (Gorodischer and Koren, 1992). Furthermore, MDMA is a basic drug with a pKa around 9.9 (data derived from methamphetamine, (Baselt et al., 1995) and since, in normal conditions (absence of salivary flow stimulation) saliva is

more acidic than blood, MDMA turns to its ionized form, which cannot diffuse back into plasma and thus accumulates in saliva (Pichini et al., 1996).

The theoretical S/P ratio for MDMA should be around 3.9 as calculated with the Henderson-Hasselbalch equation (Kidwell et al., 1998). In our study S/P ratio mean values ranged between 18.1 at peak MDMA concentrations and 6.4 at 24 h after drug administration. However, it has to be noted that there were two individuals who presented S/P ratios that were exceedingly high. Mean S/P ratio values raise to 20 times difference between plasma and salivary concentrations at t_{max} when most probably this difference could be closer to 10 (mean value of 6 subjects, excluding outliers). In any case, **it is apparent that excretion of MDMA in saliva occurs to a greater extent than expected**.

Interindividual variations in the MDMA S/P ratio, as well as in salivary MDMA levels, could be partly explained by the fact that saliva was collected without flow stimulation. It is known that stimulation of saliva secretion raises the pH to values approaching plasma pH. In the case of basic drugs, such as MDMA or cocaine, this reduces the salivary drug concentration and the variability in S/P ratios is narrowed (Gorodischer and Koren, 1992; Kato et al., 1993).

In this study collected saliva was non-stimulated in order to measure true saliva pH. This approach allowed the observation of eventual pH changes produced by MDMA. In addition most volunteers administered with MDMA experienced jaw clenching (de la Torre et al., 2000b), and the production of stimulated saliva by conventional methods like chewing seemed inadequate for this drug. **Salivary pH appeared to be affected by MDMA concentration in saliva after drug administration**. In volunteers administered MDMA, salivary pH was lowered, attaining a maximal effect at MDMA $t_{max.}$ Most probably this contributed to the accumulation of the drug in the salivary fluid. In the two cases where S/P ratios were extremely high, a

poor correlation with salivary pH was observed (Fig. 10), therefore other mechanisms might operate in the diffusion to saliva. Changes in salivary pH observed at 4 h after drug administration represented the combined effect of MDMA and the ingestion of a light breakfast at 3 h after drug administration. In the hours following maximum MDMA concentrations, S/P ratios decreased and reached almost constant values (around 7), but still higher than the theoretical figure from the Henderson-Hasselbalch equation. Since MDMA acts on serotonergic neurotransmission, with resulting vasoconstriction and significant changes in haemodynamics (Mas et al., 1999), a reduction in saliva production probably occurs, which concentrates this fluid. In fact, subjects administered MDMA usually refer dry mouth (Liechti et al., 2001). **Presumably MDMA also impairs salivary flow through its sympathomimetic effects, producing a sympathetic constriction of salivary bed** (Gorodischer and Koren, 1992). Consequently, buffering capacity, which is maximal in conditions of flow stimulation, can be reduced and the pH of mixed saliva obtained from the oral cavity (the one measured) may not be the same as the pH at the site of saliva secretion (Gorodischer and Koren, 1992, Arneberg et al., 1989). Hence, a dynamic concentration gradient takes place, which probably produces MDMA S/P ratios higher than those calculated with the Henderson-Hasselbalch equation. Unfortunately, a limitation of this study resided in the fact that it was not possible to measure salivary flow during collection, a variable that could have explained apparent deviations from theoretic values.

Saliva to plasma ratios exceeding theoretical values were also found by Samyn et al. (2000) who measured MDMA in saliva and plasma in subjects who admitted recent drug abuse. Similar results were also reported by Cook et al. (1993) when comparing methamphetamine concentrations in saliva and plasma of volunteers administered the drug by smoking and intravenous routes. Both authors always found

S/P ratios exceeding theoretical values and they attributed the circumstance to buccal contamination by tablets or smoked drugs at least for the first hours after drug administration. However, this is not the case of the present study, where MDMA was administered as capsules. Moreover, if a buccal contamination can be hypothesised for the first two hours after drug administration, this event had to be excluded in the following hours, since the light meal –snack and juice- given to the volunteers at three hours from the start of the treat should have eliminated this occurrence. Indeed, S/P ratios exceeding theoretical values were also found when methamphetamine was administered intravenously (Cook et al., 1993). **MDMA was the main compound detected in saliva.** In fact, MDA was found in the saliva in minute abundance relative to MDMA, as it is in plasma (Mas et al., 1999). Regarding HMMA, only trace amounts could be found in saliva since it is mainly present in plasma in its glucuronoconjugated form (de la Torre et al., 2000), which does not diffuse through the lipid barrier dividing salivary ducts from the systemic circulation. The evidence that MDMA was the major analyte found in saliva was in agreement with results reported by Cone (1993) for cocaine and methamphetamine, as well as with those reported by Kintz (1997) for N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB). **Therefore, it can also be stated for MDMA, that the parent drug is the target compound to be detected in saliva to assess consumption.** Apparent pharmacokinetic parameters calculated for plasma and saliva show that disappearance of MDMA from saliva occurs more rapidly than in plasma. This apparent discordance in the disposition of MDMA in the body may most probably be related to changes in salivary pH discussed previously during the first four hours of MDMA kinetics. Despite a faster disappearance of the drug in saliva, since MDMA concentrations are one order of magnitude higher in saliva compared to those observed in plasma, salivary concentrations showed an average value of around 100 μg/L at 24 h. In contrast, it was not always possible to detect the drug in plasma at

that same time. **Regarding the possible correlation between salivary concentrations of MDMA and subjective effects, results from parallel investigations in our research centre show a good agreement between MDMA concentrations in saliva and increase in blood pressure, heart rate, pupillary diameter** (Mas et al., 1999) **and on the decrease in psychomotor performance accompanied by subjective feelings of intoxication** (Camí et al., 2000; Farré et al., 2004). Indeed, all the afore mentioned pharmacological effects rise and fall with a profile similar to that of salivary MDMA, presenting peak effects, statistically different from placebo treatment, between 1.5 and 2 h after MDMA administration, with return to basal values approximately 4 hours after drug treatment.

The measurement of MDMA in saliva appears to be a suitable alternative to plasma analysis in clinical and toxicological situations, where detection of recent abuse is requested. Despite changes in the S/P ratio during the time-course of MDMA in saliva and plasma, the correlation between MDMA concentrations in the two biological fluids indicates that salivary concentrations of this drug may be a predictor of plasma concentrations. It should be acknowledged, nevertheless, that results have been obtained in controlled conditions where several factors that may modify saliva concentrations and difficult to ascertain in drug users were not considered: fasting state, dehydration, buccal contamination and others.

Due to higher concentrations encountered, saliva exhibits a larger time-window for detection of MDMA consumption. This may help to establish if subjects are under the influence of the drug in a much less invasive way than with plasma and without specific requirements for sample collection, thus facilitating on-site sample collection and drug testing performance.

Indeed, **on-site test in saliva with the Drugwipe® test device gave interesting results with a single MDMA administration.** From the results obtained, it can be

noticed that the procedure for sample collection on the surface of the tongue seemed not to be adequate for the performance of on-site saliva testing of amphetamines. In addition, as reported above, MDMA impairs salivary flow, producing a sympathetic constriction of salivary bed (Mas et al., 1999) and hence making difficult saliva procurement following a surface wiping procedure. Following observations made in the present study, **direct wiping should have been avoided preferring application of an established volume of saliva which could allow delivery of a sufficient quantity to the test pad, which can be easily done "on-site", collecting saliva and applying it to the test pad.** Although a limited number of subjects participated in this study, the present results showed that Drugwipe® in combination with Drugread® detects adequately MDMA in saliva in the first 6 hours after administration. On the other hand, the analytical device gives rise to a negative response in a range of salivary concentrations down to about 450 μg /L (0.9 ng MDMA in two μl of saliva applied to the test pad). These concentrations can be found in individuals around 6 to 10 hours after the administration of 100 mg MDMA, corresponding to a mean range of 80-120 μg /L in plasma and 3-12 mg/L in urine (Mas et al., 1999; de la Torre et al., 2000). Conversely, **the 0-6 h time window is the period of maximal pharmacological effects of MDMA. In this time interval, an individual is at highest risk of psychomotor impairment that may have consequences in some demanding tasks like driving.** Six hours after MDMA ingestion, while drug is still present in several biological fluids, most subjective and physiological effects (i.e. psychomotor performance, cardiovascular function) return towards basal conditions (Mas et al., 1999). Hence, **if the objective of on-site saliva testing is not only to detect the consumption of a given drug but also to determine if an individual is under the effects of the drug, on-site saliva testing with Drugwipe® coupled with Drugread ® fits for that purpose.** Undoubtedly,

appropriate confirmation with a reference chromatographic method in saliva samples has always to be performed.

6.3 Study 2. Pharmacokinetics of MDMA in sweat patches

Drug testing in sweat through patches was originally designed as an approach of detecting drug consumption in subjects wearing them for a 7 days time period (Burns and Baselt, 1995). In the present study, sweat patches have been used for a shorter period of time (24 hours) as a tool to monitor MDMA excretion in sweat and to evaluate the reliability of available on-site testing devices by immunoassay (i.e. Drugwipe®).

The present results show for the first time that MDMA is excreted in sweat after a controlled administration of a single 100 mg dose. Excretion of an amphetamine analogue, L-dimethylamphetamine, in human sweat was first demonstrated in 1972 (Vree et al., 1972) and more recently other amphetamine derivatives have been detected in the sweat of drug users (Fay et al., 1996; Samyn and Van Haeren 2000; Kintz, 1997).

Drugs are generally incorporated into sweat by passive diffusion because of a concentration gradient in which only the free fraction of drug (unbound to proteins) diffuses through lipid membranes from plasma to sweat (Vree et al., 1972; Kidwell et al., 1998). Furthermore, MDMA is a basic drug with a pKa around 9.9 (data derived from methamphetamine, (Baselt et al., 1995)) and since, in normal conditions sweat is more acidic than blood, (mean value of 6.3) and its normal pH is maintained under non-occlusive dressings (Aly et al., 1978), MDMA is converted to its ionized form and should accumulate in sweat. The excretion of a basic drug in sweat can be theoretically calculated by a modification of the Henderson-Hasselbalch equation (Kidwell et al., 1998). In the case of MDMA, the theoretical sweat/plasma ratio should be around 12, indicating a significant accumulation of the drug in the sweat matrix. Unfortunately, this

hypothesis could not be verified in our study as the sweat patch, which loses water content during wear, did not allow the calculation of volume of sweat collected, nor the accurate measurement of sweat pH. In any case, **sweat patch technology permitted the monitoring of MDMA accumulation in sweat during the 24 hours after administration.** This information was used to calculate total amount of MDMA excreted in sweat. This calculation was most probably an overestimation of the real figures as perspiration is not homogeneous in the whole body surface but is a first approach for estimating the amount of drug excreted through this biological fluid. **Estimations suggest that a non-negligible fraction of the dose is excreted through sweat, providing a sensitive basis for sweat testing of MDMA exposure.**

Analytical methodology applied in this study--immunological screening and GC-MS confirmation-- appeared to be adequate for monitoring single drug use within 24 hours.

No immunoassays were available at the time of the study for detection of drugs in sweat; however, a commercial test kit for plasma samples was adapted for this purpose. An extensive validation of the immunoassay for use with alternative specimens would have required calculation of parameters such as diagnostic sensitivity, specificity cross-reactivity with other analytes and analysis of samples from populations encountered in screening situations. Because the lack of access to populations routinely screened for drug testing through sweat patches, a proper validation was not possible. On the other hand, the advantage of a controlled administration study is that the capability of a sweat patch technology for diagnosing the use of even a single dose of MDMA could be clearly defined.

GC-MS analysis was performed in all samples irrespective of EIA results, with a good correlation (r=0.85) between values obtained by immunoassay screening and GC-MS confirmation (Figure 15). Nonetheless, it has to be mentioned that lengthy

extraction procedures had to be applied for a supposedly "simple" biological matrix such as sweat, due to the complexity of many different components contained in the collector, in order to avoid analytical interferences both in screening and confirmation assays and to set up a robust and reproducible chromatographic system. In particular, the extraction used for the immunoassay resulted in several chromatographic interferences in the GC-MS determination of analytes. Another procedure was applied in order to increase method selectivity, although a lower analytical recovery was achieved.

Using this sweat collector, a full investigation of MDMA pharmacokinetics in sweat was not possible. However, cumulative excretion, as reported, and the time-course of the excretion of this drug in sweat could be defined. MDMA was detected in sweat by 1.5 hours after administration, corresponding to its concentration peak in plasma, and at least until 24 hours post-administration. MDMA concentrations tended to progressively increase during the 24 hours, with an inflexion at 10 hours from consumption. **It can be postulated that the decrease in sweat patch content of MDMA was probably due to a reabsorption by the skin.** This phenomenon has also been observed for other drugs, such as cocaine, diazepam or MBDB (Kintz, 1996c; Kintz, 1997; Kidwell and Smith, 2001) and was confirmed in our pilot experiment on skin reabsorption of MDMA from the sweat patch in non-consumer volunteers.

It is known that temperature and physical exercise can change sweat flow and pH. A rise in body temperature or physical exercise could increase sweat flow producing an increase in sweat pH and the volume of sweat in contact with the patch, facilitating drug transfer to the skin (Kidwell and Smith, 2001). Indeed, in our study the subject (volunteer B) that had a higher percentage of skin reabsorption participated in moderate gymnasium exercise during the experiment. This observation may be of relevance considering MDMA consumption patterns (rave parties, overcrowded, with

high ambient temperature and physical exercise associated to dancing). Nevertheless, the impact of this observation in sweat testing performed in 'normal' conditions (i.e. 7 days wearing patch) is unclear. What it can be nevertheless concluded is that the final amount of MDMA collected in the patch is the result of a more dynamic process (excretion vs. reabsorption) than expected by authors and by other research groups.

Different perspiration rates and volumes of sweat between individuals could also explain the large inter-subject variability in sweat excretion of the same administered dose of MDMA. The evidence of inter-subject variability in sweat excretion of same dosage of drug was also reported after controlled administration of 90 mg oral codeine (Kintz et al., 1996d) and 50 and 126 mg intranasal cocaine (Burns and Baselt, 1995). However, a relevant difference between MDMA and the other drugs of abuse administered in controlled settings resides in the total amount of drug excreted in the sweat patch. While in the case of codeine (90 mg/dose), cocaine (126 mg/dose) and MBDB (100 mg/dose), maximum concentrations hardly exceeded one hundred ng/patch, 100 mg of administered MDMA gave rise to sweat patch maximum concentrations in the order of μg/patch, confirming the theoretical calculation of a high sweat/plasma ratio for this drug. The present study only considers a 24 h collection period, but it can be easily foreseen that as drug users have more than a single dose per rave the application of the patch technology for monitoring MDMA consumption for longer periods of patch wearing has to be hypothesized as very successful.

As discussed in the previous page, sweat patch technology did not allow a study of the pharmacokinetics of MDMA in sweat, which could be related to drug-induced subjective effects. Sweat collection by cotton wipe, as proposed by other authors (Samyn et al., 2002), even presenting the disadvantages of a non-validated collection methodology should have better fitted for this purpose. In any case, even though our

study did not explore this possibility, punctual sweat collection with an immediate qualitative result was performed by using Drugwipe® immunochromatographic test. Quite acceptable results were obtained with the application of this on-site test for rapid screening of MDMA in sweat, although highly variable sweat excretion between individuals also induced by MDMA pharmacological activity on body temperature regulation (Camí et al., 2000).

Drugwipe® information sheet reported sensitivity for methamphetamine of 10 ng, understood as the minimum quantity of drug on the collection pad giving rise to a positive immune reaction. For what was known in saliva (Study 1), the device cannot detect concentrations down to 100 ng/mL of MDMA. Securetec reports, 300 ng/mL for d-amphetamine and 250 ng/mL for MDA. Several studies were performed administering single doses of several over the counter phenylalkylamines (i.e. phenylpropanolamine and ephedrine) and no positive reactions with the device were observed (intramural data). **Taking together all these facts, the device seems to be quite adequate for the detection of amphetamines and designer drugs**. Drugwipe® could detect MDMA consumption in sweat as early at 1.5 hours and as far as 24 hours after drug administration. These findings are in agreement with results obtained in a pilot study including two volunteers (Pacifici et al., 2001). However, while all the subjects gave a positive result at 1.5 after drug administration, in the following 4-10 hours two or three subjects produced negative results. These findings are in accordance with those obtained when wiping the Drugwipe® over the tongue of individuals administered MDMA as gelatine capsules for a rapid test in saliva (study 1). Also in that case, positivity to the test decreased noticeably at 6 and 10 hours after MDMA administration. **The most reasonable explanation for the negative results was the insufficient amount of biological matrix (sweat and saliva) that could be collected wiping the armpit or tongue with the test pad of the device.** Indeed, this hypothesis

was confirmed by the fact that when a pre-selected volume of saliva was applied to the device, the test performed better, giving negative results only in individuals which showed the lowest salivary MDMA concentrations. Unfortunately, in the case of sweat it was not possible to verify if negative results were also attributable to a low MDMA recovery on the collection pad.

In any case, if the objective of on-site sweat testing is not only to detect the consumption of a given drug but also to determine if an individual is under the effects of the drug, on-site sweat testing with Drugwipe® appeared to fit for the purpose. Indeed, 1.5 hours corresponds to maximal effects for some physiological and psychomotor variables. Those effects last at least 6 h unless there is a repeated administration (Mas et al., 1999; Camí et al., 2000; Farré et al., 2004). However, to draw any definite conclusion, a study in a large population sample is needed, given that present results were obtained in a controlled setting with a small number of participants.

6.4 Study 3. Hair testing for MDMA and its metabolites

Recent findings show that drug incorporation in hair depends from melanin concentration in hair (Kronstrand et al., 1999). The hair colour of the subjects recruited for this study was quite homogeneous, being all the subjects natural brown-haired, as is the majority of Spanish population. This fact was the first premise to exclude one of the principal sources of inter-subject variability and indeed **a good association between self-reported consumption of MDMA in the last month and drug concentration in the one cm hair segment was found for subjects with a low and medium drug use** (Figure 20, upper). The two individuals with high ecstasy consumption presented MDMA hair values lower than those that could have been expected, considering values from other consumers. Correlation between the amount of drug ingested and the concentrations detected in hair of single subjects decreases when considering self

declarations and hair MDMA in the farther past (Figure 20, central and lower). Problems of recall bias together with individual metabolism variations and types and frequency of hair treatments can be supposed in accordance with other authors (Cooper et al., 2000). In addition, the heavy consumers continued to show outlier values of hair MDMA, which let hypothesize on one hand an overestimation of ingested drug and or the ingestion of tablets, which did not contain the drug; on the other a phenomenon of "saturation" in hair incorporation of MDMA. Unfortunately, the two hypotheses were not supported by analytical evidences or by other authors: hair from the two individuals did not contain any other amphetamine derivative or drug, which could have been present in "ecstasy" preparations, nor international literature showed a "saturation" effect in hair incorporation of MDMA (Nakahara and Kikura, 1997; Nakahara and Hanajiri, 2000). In any case, other authors observed a plateau in mean MDMA hair concentration from low to high frequency of drug use. (Cooper, 2000). In that case, the lack of increase in MDMA in hair was attributed to the high number of false negative results, which contributed to the final low mean value of hair MDMA. However, the same authors admitted that number of false negative increase from low to high drug use, which could be coherent with lack of increase of drug hair incorporation as a consequence of higher consumption.

Looking at Figure 20 and at Table 4, it is worth of notice that MDMA concentration was sensibly higher in the first hair segment with respect to the 5 and 9 cm segments although subjects declared the same monthly amount of consumed drug. In agreement with other authors, the contribution of sweat and sebum in root segment drug concentration has to be acknowledged together with a longer period of hygienic treatments by longer hair strands (Rothe et al., 1997)

Overall, looking at the results obtained in different hair segments corresponding to different past exposures, one could have concluded that a relationship between self-

reported data and laboratory findings was evident only in the nearer past and for low and medium drug consumption. Nonetheless, **when similar declared drug use in the last one, six and twelve months by different subjects were gathered together and compared with the mean of MDMA concentrations found in the corresponding 1 cm, 5 cm and 12 cm hair segments of individuals belonging to the same drug use group, a good correlation was found up to one year of history consumption in case of low and medium consumption** (Figure 21).

Furthermore, regarding the problem of false negative and false positive results, some observations should be made on the three individuals (6, 10 and 12) that declared no ecstasy consumption at least in the last six months. Although no MDMA was found in the 1 cm segment, related to the last month drug use, amounts lower than 0.5 ng/mg hair were found in two cases in the 5 and 9 cm segments. It has to be said that these individuals were in any case drug users, but which did not consume the drug in the near past. Hence, phenomena such as drug migration along the hair shaft, and/or an irregular hair growth together with recall biases should have lead to the obtained results.

Although the afore discussed findings were obtained in a small group of individuals, and as in case of saliva and sweat further investigation in general population is needed, **we can conclude that MDMA concentration in hair can give a quite good approximation of the history of drug consumption, at least in the last month or for extended periods of time when drug consumption is low.** As already assessed by other authors (Kintz, 1996; Cone, 1996) drug analysis in hair extends the information of drug consumption to a wider time-window than that of other non-invasive biological matrices, such as saliva and sweat. As discussed in the previous paragraphs these latter two matrices, which can account for acute pharmacological effects induced by the drug, results from hair testing can be used to assess repeated exposure to drug and eventual association with long term drug induced effects, such as

neurotoxicity and psychological performance in the specific case of MDMA (Rothe et al., 1997).

7. Conclusions

7. Conclusions

1. MDMA is excreted in saliva, after a single dose administration, with concentrations one order of magnitude higher than those observed in plasma and following a time course kinetics which parallels that of plasma and that of subjective effects and psychomotor performance.
   - The measurement of MDMA in saliva is a valuable alternative to determination of plasma drug concentrations both in clinical and toxicological studies,
   - On-site testing is suitable to detect individuals under the influence of drug effects by non-invasive and rapid collection of salivary specimens.
2. MDMA appears in sweat and can be quantified already in the first few hours after a single dose administration, when subjective effects are apparent. This result makes:
   - the sweat patch technology useful for monitoring MDMA accumulation in sweat at least during the 24 hours after a single administration,
   - On-site sweat testing suitable to detect individuals under the influence of drug effects by non-invasive and rapid collection of minute amounts of sweat.
3. MDMA appears in hair from consumers and can be detected in hair segments corresponding to the last one, six and twelve month of repeated drug use. For this reason:
   - Hair analysis of MDMA can be used to evaluate exposure or abstinence to the drug in the last months
   - Hair concentration of MDMA in different hair segment can predict levels of drug use and can be eventually associated to chronic psychophysical effects induced by the repeated drug use.

4. Analytical approaches, developed and validated during the afore-mentioned studies allowed the identification and quantification not only of the parent drug, but also of its principal metabolites (HMMA and MDA in case of saliva, only MDA in case of sweat and hair) in the different matrices.
5. A common characteristic of the three different matrices is that the parent drug MDMA was always the principal, most abundant analyte detected, whose concentration could be associated with drug-induced effects and drug history.

8. References

8. References

American Academy of Pediatrics Committee on Drugs. Transfer of drugs and other chemicals into human milk. Pediatrics. 2001;108:776-89.

Aly R, Shirley C, Cunico B, Mainbach HI. Effect of prolonged occlusion on the microbial flora, pH, carbon dioxyde and transepidermal water loss on human skin. J Invest Dermatol 1978;71:378-81.

Arneberg P, Storhaug K, Sandvik L. Effect of a slow release transcutaneous scopolamine application on salivary flow, pH, buffering action, and salivary levels of streptococcus mutants and lactobacilli. Scand J Dent Res 1989; 97: 408-14.

Atkinson HC, Begg EJ, Darlow BA Drugs in human milk. Clinical pharmacokinetic considerations. Clin Pharmacokinet 1988;14:217-40.

Bailey B, Klein J, Koren G Noninvasive methods for drug measurement in pediatrics. Pediatr Clin North Am 1997;44:15-26.

Balabanova S, Parsche F, Pirsig W. First identification of drugs in Egyptian mummies. Naturwissenschaften 1992;79:358.

Barrett DA, Simpson J, Rutter N, Kurihara-Bergstrom T, Shaw PN, Davis SS. The pharmacokinetics and physiological effects of buprenorphine infusion in premature neonates. Br J Clin Pharmacol 1993;36:215-9.

Barrett DA, Rutter N Transdermal delivery and the premature neonate. Crit. Rev. Ther Drug Carrier Syst 1994;11:1-30.

Barrett C, Good C, Moore C. Comparison of point-of-collection screening of drugs of abuse in oral fluid with a laboratory-based urine screen. Forensic Sci Int 2001;122:163-6.

Baselt RC, Cravey RH, Disposition of Toxic Drugs and Chemicals in Man. 1995, 4th edition. Chemical Toxicology Institute. Foster City, CA, pp 44 and 475.

Bolton JL, Trush MA, Penning TM, Dryhurst G, Monks TJ. Role of quinones in toxicology. Chem Res Toxicol 2000;13:135-60.

Bonati M, Kanto J, Tognoni G Clinical pharmacokinetics in cerebrospinal fluid. Clin Pharmacokinet 1982;7:312-35.

Boot BP, McGregor IS, Hall W. MDMA (Ecstasy) neurotoxicity: assessing and communicating the risks. Lancet 2000;355:1818-21.

Brookhuis KA, de Waard D, Samyn N. Effects of MDMA (ecstasy), and multiple drugs use on (simulated) driving performance and traffic safety. Psychopharmacology (Berl). 2004;173:440-5.

Burns M, Baselt RC. Monitoring drug use with a sweat patch: an experiment with cocaine. J Anal Toxicol 1995;19:41–8.

Caddy B Saliva as a specimen for drug analysis. In R. C. Baselt (Ed.), Advances in analytical toxicology. 1984;1:198-254. Foster City: Biomedical Publications.

Cairns T, Hill V, Schaffer M, Thistle W.Amphetamines in washed hair of demonstrated users and workplace subjects. Forensic Sci Int 2004a;145:137-42.

Cairns T, Hill V, Schaffer M, Thistle W.Levels of cocaine and its metabolites in washed hair of demonstrated cocaine users and workplace subjects. Forensic Sci Int 2004b;145:175-81.

Camí J, Farré M, Mas M, Roset PN, Poudevida S, Mas A, San L, de la Torre R. Human pharmacology of 3,4-methylenedioxymethamphetamine ("ecstasy"): psychomotor performance and subjective effects. J Clin Psychopharmacol 2000;4:455–66.

Caplan YH, Goldberger BA. Alternative specimens for workplace drug testing. J Anal Toxicol 2001;25:396-9.

Carvalho F, Duarte JA, Neuparth MJ, et al. Hydrogen peroxide production in mouse tissues after acute d-amphetamine administration. Influence of monoamine oxidase inhibition. Arch Toxicol 2001;75:465-9.

Chandra P, Brouwer KL.The complexities of hepatic drug transport: current knowledge and emerging concepts. Pharm Res 2004;21:719-35.

Cho AK, Segal DS, editors. Amphetamine and its analogs. San Diego, CA: Academic Press 1994: 3-34.

Chronister CW, Walrath JC, Goldberger BA. Rapid detection of benzoylecgonine in vitreous humor by enzyme immunoassay. J Anal Toxicol 2001;25:621-4.

Cirimele V, Kintz P, Mangin P. Detection of amphetamines in fingernails: an alternative to hair analysis. Arch Toxicol 1995;70:68-9.

Cirimele V, Kintz P, Doray S, Ludes B.Determination of chronic abuse of the anaesthetic agents midazolam and propofol as demonstrated by hair analysis. Int J Legal Med 2002;116:54-7.

Claffey DJ, Stout PR,. Ruth JA. A comparison of sodium hydroxide and sodium sulfide digestion of mouse hair in the recovery of radioactivity following systemic administration of [3H]-nicotine and [3H]-flunitrazepam. J Anal Toxicol 2000;24:54-8.

Clarke's Isolation and identification of drugs. 2nd ed. A.C. London: Moffat Senior Consulting. The pharmaceutical Press, 1986.

Clauwaert KM, Van Bocxlaer JF, De Letter EA, Van Calenbergh S, Lambert WE, De Leenheer AP. Determination of the designer drugs 3, 4-Methylenedioxymethamphetamine, 3,4-methylenedioxyethylamphetamine, and 3,4-methylenedioxyamphetamine with HPLC and fluorescence detection in whole blood, serum, vitreous humor, and urine. Clin Chem 2000;46:1968-77.

Cone EJ. Saliva testing for drugs of abuse. Ann N Y Acad Sci. 1993; 694:91–127.

Cone EJ Mechanisms of drug incorporation into hair. Ther Drug Monit 1996;18:438-43.

Cone EJ, Kato K, Hillsgrove M. Cocaine excretion in the semen of drug users. J Anal Toxicol 1996;20:139-40.

Cone EJ. Legal, workplace, and treatment drug testing with alternate biological matrices on a global scale. Forensic Sci Int 2001;121:7-15.

Cook CE, Jeffcoat AR, Hill JM, Pugh DE, Patetta PK, Sadler BM, et al. Pharmacokinetics of methamphetamine self-administered to human subjects by smoking S-(+) methamphetamine hydrochloride. Drug Metab Dispos 1993;21:717–23.

Cooper TB, Bark N, Simpson GM. Prediction of steady state plasma and saliva levels of desmetylimipramine using a single dose, single time point procedure. Psychopharmacology 1981;74:115-21.

Cooper GA, Allen DL, Scott KS, Oliver JS, Ditton J, Smith ID Hair analysis: self-reported use of "speed" and "ecstasy" compared with laboratory findings. J Forensic Sci 2000;45:400-6.

Dams R, De Letter EA, Mortier KA, Cordonnier JA, Lambert WE, Piette MHA, Van Calenbergh S, De Leenheer AP. Fatality due to combined use of the designer drugs MDMA and PMA: a distribution study. J Anal Toxicol 2003;27:318-22.

Danhof M, Breimer DD. Therapeutic drug monitoring in saliva. Clin Pharmacokinet 1978 ;3:39-57.

Davenport HW. Salivary secretion. In H. W. Davenport (Ed.), Physiology textbook series. Physiology of the digestive tract: an introductory text. 1977;Fourth ed., pp. 85-94. Chicago: Year Book Medical Publishers.

Dawes C, Macpherson LMD. Effects of 9 different chewing-gums and lozenges on salivary flow-rate and pH. Caries Research 1992;26:176-82.

Davidson C, Gow AJ, Lee TH, Ellinwood EH. Methamphetamine neurotoxicity: necrotic and apoptotic mechanisms and relevance to human abuse and treatment. Brain Res Rev 2001;36:1-22.

Delaforge M, Jaouen M, Bouille G. Inhibitory metabolite complex formation of methylenedioxymethamphetamine with rat and human cytochrome P450. Particular involvement of CYP2D6. Environ Pharmacol Toxicol 1999;7:153-8.

de la Torre R, Farré M, Ortuño J, et al. Non-linear pharmacokinetics of MDMA ('ecstasy') in humans. Br J Clin Pharmacol 2000a; 49:104-9.

de la Torre R, Farre M, Roset PN, Hernandez Lopez C, Mas M, Ortuno J, Menoyo E, Pizarro N, Segura J, Cami J. Pharmacology of MDMA in humans. Ann N Y Acad Sci 2000b; 914:225-37.

de la Torre R, Farre M, Navarro M, Pacifici R, Zuccaro P, Pichini S Clinical pharmacokinetics of amfetamine and related substances: monitoring in conventional and non-conventional matrices. Clin Pharmacokinet 2004a;43:157-85.

de la Torre R, Farre M, Roset PN, Pizarro N, Abanades S, Segura M, Segura J, Cami J. Human pharmacology of MDMA,pharmacokinetics, metabolism and disposition. Ther Drug Monit 2004b;26:137-44.

de la Torre R, Farre M. Neurotoxicity of MDMA (ecstasy): the limitations of scaling from animals to humans. Trends Pharmacol Sci 2004;25:505-8.

de la Torre R, Pichini S. Usefulness of Sweat Testing for the Detection of Cannabis Smoke. Clin Chem 2004, 50:1961-1962.

De Letter EA, Clauwaert KM, Lambert WE, Van Bocxlaer JF, De Leenheer AP, Piette MH Distribution study of 3,4-methylenedioxymethamphetamine and 3,4-methylenedioxyamphetamine in a fatal overdose. J Anal Toxicol 2002;26:113-8.

De Letter EA, Bouche MP, Van Bocxlaer JF, Lambert WE, Piette MH.Interpretation of a 3,4-methylenedioxymethamphetamine (MDMA) blood level: discussion by means of a distribution study in two fatalities. Forensic Sci Int 2004;141:85-90.

Dempsey DA, Partridge JC, Jones RT, Rowbotham MC. Cocaine, nicotine, caffeine, and metabolite plasma concentrations in neonates. J Anal Toxicol 1998;22:220-4.

Dickson TJ, Gurudutt V, Nguyen AQ, Kumfer K, Maxted BW, Brown J, Mahomed Y, Sharp T, Aufiero TX, Fineberg N, March KL. Establishment of a clinically correlated human pericardial fluid bank: evaluation of intrapericardial diagnostic potential. Clin Cardiol 1999;22:I-40-2.

Doran D, Terney J, Varano M, Ware S. A study of the pH of perspiration from male and female subjects exercising in the gymnasium. J Chem Educ 1993:70; 412-416.

Drobitch RK, Svensson CK. Therapeutic drug monitoring in saliva. An update. Clin Pharmacokin 1992;23:365-79.

Drummer OH. Postmortem toxicology of drugs of abuse. Forensic Sci Int 2004;142:101-13.

Ebling FJ. The biology of hair. Dermatol Clin 1987;5:467-81.

Ehrlich M, Daschner FD, Kummerer K. Rapid antibiotic drug monitoring: meropenem and ceftazidime determination in serum and bronchial secretions by high-performance liquid chromatography-integrated sample preparation. J Chromatogr B Biomed Sci Appl 2001;751:357-63.

Ensslin HK, Maurer HH, Gouzoulis E, Hermle L, Kovar KA. Metabolism of 3,4-methylenedioxyethyamphetamine. Drug Metab Dispos 1996;24:813-20.

Evans N, Guy RH, Hadgraft J. Transdermal drug delivery to neonates Int J Pharm 1985;24:259-265.

Fallon JK, Kicman AT, Henry JA, et al. Stereospecific analysis and enantiomeric disposition of 3,4-methylenedioxymethamphetamine (Ecstasy) in humans. Clin Chem 1999;45:1058-69.

Farre M, de la Torre R, Mathuna BO, Roset PN, Peiro AM, Torrens M, Ortuno J, Pujadas M, Cami J. Repeated doses administration of MDMA in humans: pharmacological effects and pharmacokinetics. Psychopharmacology (Berl). 2004;173:364-75.

Fay J, Fogerson R., Schoeendofer D., Niedbala RS, Spiehler V. Detection of methamfetamine in sweat by EIA and GC-MS. J Anal Toxicol 1996;20:398-403.

Ferguson, DB., Fort A. Circadian variations in human resting submandibular saliva flow rate and composition. Arch Oral Biology 1974;19: 47-55.

Ferrara SD, Tedeschi L, Frison G, Rossi A. Fatality due to gamma-hydroxybutyric acid (GHB) and heroin intoxication. J Forensic Sci 1995;40:501-4.

Fogerson R, Schoendorfer D, Fay J, Spiehler V. Qualitative detection of opiates in sweat by EIA and CG-MS J Anal. Toxicol 1997;21:451–7.

Friedler G. Effects of limited paternal exposure to xenobiotic agents on the development of progeny. Neurobehav Toxicol Teratol 1985;7:739-43.

Frison G, Favretto D, Tedeschi L, Ferrara SD. Detection of thiopental and pentobarbital in head and pubic hair in a case of drug-facilitated sexual assault. Forensic Sci Int 2003;133:171-4.

García-Algar O, Vall O, Alameda F, Puig C, Pellegrini M, Pacifici R, Pichini S. Prenatal exposure to arecoline (Areca Nut alkaloid) and birth outcomes. Arch Dis Child 2004 in press.

Garcia-Repetto R, Moreno E, Soriano T, Jurado C, Gimenez MP, Menendez M. Tissue concentrations of MDMA and its metabolite MDA in three fatal cases of overdose. Forensic Sci Int 2003;135:110-4.

Gibson AT, Segal MB. A study of the composition of pericardial fluid, with special reference to the probable mechanism of fluid formation. J Physiol 1978;277:367-77.

Gilhooly TC, Daly AK. CYP2D6 deficiency, a factor in ecstasy related deaths? Br J Clin Pharmacol 2002;54:69-70

Gygi SP, Joseph RE Jr, Cone EJ, Wilkins DG, Rollins DE. Incorporation of codeine and metabolites into hair. Role of pigmentation. Drug Metab Dispos 1996;24:495-501.

Gilman AG, Rall TW, Nies AS, et al. editors. The pharmacological bases of therapeutics. 8th ed. Elmsford: Goodman and Gilman's. 1990.

Gorodischer R, Koren G. Salivary excretion of drugs in children: theoretical and practical issues in therapeutic drug monitoring. Dev Pharmacol Ther 1992;19:161-77.

Green AR, Mechan AO, Elliot JM, O'Shea E, Colado MI. The pharmacology and clinical pharmacology of 3,4 methylenedioxymethamphetamine (MDMA, "ecstasy"). Pharmacol Rev 2003;55:463-508.

Haeckel, R. Relationship between intraindividual variation of the saliva/plasma- and of the arteriovenous concentration ratio as demonstrated by the administration of caffeine. J Clin Chem Clin Biochem 1990;28:279-284.

Haeckel R, Hanecke P. The application of saliva, sweat and tear fluid for diagnostic purposes. Ann Biol Clin 1993;51:903-910.

Harkey MR. Anatomy and physiology of hair. Forensic Sci Int 1993;63:9-18.

Henry JA, Jeffreys KJ, Dawling S. 1992. Toxicity and deaths from 3,4-methylenedioxymethamphetamine ("ecstasy"). Lancet 1992;340:384-7.

Hegadoren KM, Baker GB, Bourin M. 3,4-Methylenedioxymentamphetamine analogues in humans: defining the risks to humans. Neuroscien Biobehav Rev 1999;23:539-53.

Helmlin HJ, Bracher K, Bourquin D, Vonlanthen D, Brenneisen R. Analysis of 3,4-methylenedioxymethamphetamine (MDMA) and its metabolites in plasma and urine by HPLC-DAD and GC-MS. J Anal Toxicol 1996; 20: 432-40.

Hepler BR, Isenschmid DS. *Drug AbuseHandbook*, S.B. Karch, Ed. CRC Press, Boca Raton, FL, 1998, pp 873–889.

Hermle L, Grön G, Spitzer M, Franke B, Walter H, Buechler J, Wunderlich AP, Schwab M, Kovar KA, Enantio-selective cognitive and brain activation effects of N-ethyl-3,4-methylenedioxyamphetamine in humans. Neuropharmacology 2001;41:263-71.

Henry J. Medical risks associated with MDMA use. In: Ecstasy: the complete guide. Holland J, ed. Rochester: Park Street Press 2001: 71-86.

Hofman LF. Human saliva as a diagnostic specimen. J Nutr 2001;131:1621S-5S.

Höld KM, de Boer D, Zuidema J, Maes RA. Saliva as an analytical tool in toxicology 5. Int J Drug testing.1995a www.criminology.fsu.edu/journal

Höld, KM, De Boer D, Zuidema J, Maes RA. Evaluation of the Salivette as sampling device for monitoring b-adrenoceptor blocking drugs in saliva. J Chromatogr B Biomed Appl 1995b; 663:103-110

Huestis MA, Cone EJ. Alternative testing matrices. Drug Abuse Handbook 1998; 11:799- 814.

Huestis MA, Cone EJ, Wong CJ, Umbricht A, Preston KL Monitoring opiate use in substance abuse treatment patients with sweat and urine drug testing. J Anal Toxicol 2000;24:509-21.

Imam SZ, el-Yazal J, Newport GD, Itzhak Y, Cadet JL, Slikker W Jr, Ali SF. Methamphetamine-induced dopaminergic neurotoxicity: role of peroxynitrite and neuroprotective role of antioxidants and peroxynitrite decomposition catalysts. Ann N Y Acad Sci 2001;939:366-80

Jauniaux E, Gulbis B, Acharya G, Thiry P, Rodeck C. Maternal tobacco exposure and cotinine levels in fetal fluids in the first half of pregnancy. Obstet Gynecol 1999;93:25-9.

Joseph RE Jr, Su TP, Cone EJ. In vitro binding studies of drugs to hair: influence of melanin and lipids on cocaine binding to Caucasoid and Africoid hair. J Anal Toxicol. 1996 ;20:338-44.

Jurado C, Kintz P, Menendez M, Repetto M. Influence of the cosmetic treatment of hair on drug testing. Int J Legal Med 1997;110:159-63.

Jurado C, Gimenez MP, Soriano T, Menendez M, Repetto M. Rapid analysis of amphetamine, methamphetamine, MDA, and MDMA in urine using solid-phase microextraction, direct on-fiber derivatization, and analysis by GC-MS. J Anal Toxicol 2000;24:11-6.

Kalia YN, Merino V, Guy RH. Transdermal drug delivery. Clinical aspects. Dermatol Clin 1998;16:289-99.

Kato K, Hillsgrove M, Weinhold L, Gorelick DA, Darwin WD, Cone EJ. Cocaine and metabolite excretion in saliva under stimulated and nonstimulated conditions. J Anal Toxicol 1993;17:338-41.

Karachalios G, Charalabopoulos K. Biliary excretion of antimicrobial drugs. Chemotherapy 2002;48:280-97.

Kidwell DA, Blank DL. Mechanisms of incorporation of drugs into hair and the interpretation of hair analysis data in NIDA Research Monograph: Hair Testing for Drugs of abuse: International Research on Standards and Technology 1995: pp. 19-90.

Kidwell DA, Holland JC, Athanaselis S. Testing for drugs of abuse in saliva and sweat. J Chromatogr B Biomed Sci Appl 1998;713:111-35.

Kidwell A, Smith FP. Susceptibility of PharmCheck^{TM} drugs of abuse patch to environmental contamination. Forensic Sci Int 2001;116: 89-106.

Kidwell DA, Kidwell JD, Shinohara F, Harper C, Roarty K, Bernadt K, McCaulley RA, Smith FP. Comparison of daily urine, sweat, and skin swabs among cocaine users. Forensic Sci Int 2003:23;133:63-78.

Kikura R, Nakahara Y, Mieczkowski T, Tagliaro F. Hair analysis for drug abuse. XV. Disposition of 3, 4-methylenedioxymethamphetamine (MDMA) and its related

compounds into rat hair and application to hair analysis for MDMA abuse. Forensic Sci Int 1997;84:165-77.

Kintz P, Cirimele V, Tracqui A, Mangin P. Simultaneous determination of amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine in human hair by gas chromatography- mass spectrometry. J Chromat B Biomed Appl 1995;670:162-6.

Kintz P. Drug testing in addicts: a comparison between urine, sweat, and hair. Ther Drug Monit 1996a;18:450-5.

Kintz P ed. Drug testing in hair.CRC Press, Boca Raton, Florida, 1996.

Kintz P, Tracqui A, Mangin P, Edel Y. Sweat testing in opioid users with a sweat patch. J Anal Toxicol 1996b;20:393-7.

Kintz P, Tracqui A, Mangin P. Sweat testing for benzodiazepines. J Forensic Sci 1996c;41: 851-4.

Kintz P, Tracqui A, Jamey C, Mangin P. Detection of codeine and phenobarbital in sweat collected with a sweat patch. J Anal Toxicol 1996d;20:197–201.

Kintz P. Excretion of MBDB and BDB in urine, saliva and sweat following single oral administration. J Anal Toxicol 1997;21:570–5.

Kintz P, Cirimele V. Interlaboratory comparison of quantitative determination of amphetamine and related compounds in hair samples. Forensic Sci Int 1997;84:151-6.

Kintz P, Cirimele V, Ludes B. Codeine testing in sweat and saliva with the Drugwipe. Int J Legal Med 1998a;111:82-4.

Kintz P, Bundeli P, Brenneisen R, Ludes B. Dose-concentration relationships in hair from subjects in a controlled heroin-maintenance program. J Anal Toxicol 1998b;22:231-6.

Kintz P, Samyn N. Determination of “Ecstasy” components in alternative biologic specimens J Chromatogr B Biomed Sci Appl 1999;733:137–43.

Kintz P, Samyn N. Use of alternative specimens: drugs of abuse in saliva and doping agents in hair. Ther Drug Monit 2002;24:239-46.

Koren G, Klein J, Forman R, Graham K.Hair analysis of cocaine: differentiation between systemic exposure and external contamination. J Clin Pharmacol 1992 ;32:671-5.

Koren G, Chan D, Klein J, Karaskov T. Estimation of fetal exposure to drugs of abuse, environmental tobacco smoke, and ethanol. Ther Drug Monit 2002;24:23-5.

Kraemer, T, Maurer, H.H. Toxicokinetics of amphetamines: metabolism and toxicokinetic data of designer drugs, amphetamine, methamphetamine, and their N-alkyl derivatives. Ther. Drug Monitor 2002;24:277-89.

Kraner JC, McCoy DJ, Evans MA, Evans LE, Sweeney BJ. Fatalities caused by the MDMA-drug related paramethoxyamphetamine (PMA). J Anal Toxicol 2001;25:645-8.

Kreth K, Kovar K, Schwab M, Zanger UM. Identification of the human cytochrome P450 involved in the oxidative metabolism of "Ecstasy" related designer drugs. Biochem Pharmacol 2000;59:1563-71.

Kronstrand R, Forstberg-Peterson S, Kagedal B, Ahlner J, Larson G. Codeine concentration in hair after oral administration is dependent on melanin content Clin Chem 1999;45:1485-94.

Lamers CT, Ramaekers JG, Muntjewerff ND, Sikkema KL, Samyn N, Read NL, Brookhuis KA, Riedel WJ Dissociable effects of a single dose of ecstasy (MDMA) on psychomotor skills and attentional performance. J Psychopharmacol. 2003;17:379-87.

LeGros Clark WE, Dudley Buxton LH. Studies in nail growth. Br J Dermatol 1938; 50:221–35

Lentner, C. (Ed.). (1981). Geigy scientific tabels (Eighth ed., Vol. 1). Basel: Ciba Geigy.

Liechti ME, Gamma A, Vollenweider FX. Gender differences in the subjective effects of MDMA. Psychopharmacology (Berl.) 2001;154:161-8.

Lin LY, Kumagai Y, Cho AK. Enzymatic and chemical demethylenation of (methylenedioxy)amphetamine and (methylenedioxy)methamphetamine by rat brain microsomes. Chem Res Toxicol 1992;5:401-6.

Mackey-Bojack S, Kloss J, Apple F Cocaine, cocaine metabolite, and ethanol concentrations in postmortem blood and vitreous humor. J Anal Toxicol. 2000;24:59-65.

Mandel ID. Relation of saliva and plaque to caries. J Dent Res 1974;53:246-266.

Mangin P, Kintz P.Variability of opiates concentrations in human hair according to their anatomical origin: head, axillary and pubic regions. Forensic Sci Int. 1993;63:77-83.

Martinez-Madrigal F, Micheau C. Histology of the major salivary glands. Am J Surg Pathol. 1989;13:879-99.

Mas M, Farre M, de la Torre R, Roset PN, Ortuno J, Segura J, Cami J. Cardiovascular and neuroendocrine effects and pharmacokinetics of 3,4-methylenedioxymethamphetamine in humans. J Pharmacol Exp Ther 1999;290:136–45

Maurer HH. On the metabolism and the toxicological analysis of methylenedioxyphenylalkylamine designer drugs by gas chromatography-mass spectrometry. Ther. Drug Monit.1996;18: 465-70.

Maurer HH. Liquid chromatography-mass spectrometry in forensic and clinical toxicology. J Chromatogr B Biomed Sci Appl. 1998; 713 :3-25.

Maurer HH, Bickeboeller-Friedrich J, Kraemer T, Peters FT. Toxicokinetics and analytical toxicology of of amphetamine-derived designer drugs (Ecstasy). Toxicol lett 2000;112-113:133-142.

McCann U, Slate SO, Ricaurte GA. Adverse reactions with 3,4-methylenedioxymethamphetamine (MDMA; "Ecstasy"). Drug Saf 1996;15:107-15.

Mizuno A, Uematsu T, Oshima A, Nakamura M, Nakashima M. Analysis of nicotine content of hair for assessing individual cigarette-smoking behavior. Ther Drug Monit 1993;15:99-104.

Monks TJ, Jones DC, Bai F, Lau SS. The role of metabolism in 3,4-(+)-methylenedioxyamphetamine and 3,4-(+)-methylenedioxymethamphetamine (ecstasy) toxicity. Ther Drug Monit 2004;26:132-6.

Montagna M, Stramesi C, Vignali C, Groppi A, Polettini A. Simultaneous hair testing for opiates, cocaine, and metabolites by GC-MS: a survey of applicants for driving licenses with a history of drug use. Forensic Sci Int 2000;107:157-67.

Moolchan ET, Cone EJ, Wstadik A, Huestis MA, Preston KL. Cocaine and metabolite elimination patterns in chronic cocaine users during cessation: plasma and saliva analysis. J Anal Toxicol 2000;24:458–76.

Moore C, Negrusz A, Lewis D. Determination of drugs of abuse in meconium. J Chromatogr B Biomed Sci Appl 1998; 713:137-46.

Moriya F, Hashimoto Y. Pericardial fluid as an alternative specimen to blood for postmortem toxicological analyses. Leg Med (Tokyo) 1999; 1: 86-94.

Moriya F, Hashimoto Y. Criteria for judging whether postmortem blood drug concentrations can be used for toxicologic evaluation. Leg Med (Tokyo) 2000; 2: 143-51.

Murphy MG, Peck CC, Conner DP, Zamani K, Merenstein GB, Rodden D Transcutaneous theophylline collection in preterm infants. Clin Pharmacol Ther. 1990;47:427-34.

Nakahara Y, Takahashi K, Konuma K.Hair analysis for drugs of abuse. VI. The excretion of methoxyphenamine and methamphetamine into beards of human subjects. Forensic Sci Int 1993;63:109-19.

Nakahara Y.Detection and diagnostic interpretation of amphetamines in hair. Forensic Sci Int 1995;70:135-53.

Nakahara Y, Kikura R, Yasuhara M, Mukai T. Hair analysis for drug abuse. XIV. Identification of substances causing acute poisoning using hair root. I. Methamphetamine. Forensic Sci Int 1997;84:157-64.

Nakahara Y. Hair analysis for abused and therapeutic drugs. J Chromatogr B Biomed Sci Appl 1999;733:161-80.

Nakahara Y, Hanajiri R. Hair analysis for drugs of abuse XXI. Effect of para-substituents on benzene ring of methamphetamine on drug incorporation into rat hair. Life Sci 2000;66:563-74

Nave R, Bethke TD, van Marle SP, Zech K. Pharmacokinetics of [14C]ciclesonide after oral and intravenous administration to healthy subjects. Clin Pharmacokinet 2004;43:479-86.

Niedbala RS, Feindt H, Kardos K, Vail T, Burton J, Bielska B, Li S, Milunic D, Bourdelle P, Vallejo R. Detection of analytes by immunoassay using up-converting phosphor technology. Anal Biochem 2001a;293:22-30.

Niedbala RS, Kardos KW, Fritch DF, Kardos S, Fries T, Waga J, Robb J, Cone EJ. Detection of marijuana use by oral fluid and urine analysis following single-dose administration of smoked and oral marijuana.: J Anal Toxicol 2001b;25:289-303.

Nielsen P, Rasmussen F. Relationships between molecular structure and excretion of drugs. Life Sci 1975;17:1495-512.

Nichols DE. Differences between the mechanism of action of MDMA, MBDB, and the classic hallucinogens. Identification of a new therapeutic class: entactogens. J Psychoactive Drugs 1986;18:305-13.

Okamoto M, Yakawa R, Mamada A, Inoue S, Nagase S, Shibuichi S, Kariya E, Satoh N. Influence of internal structures of hair fiber on hair appearance. III. Generation of

light-scattering factors in hair cuticles and the influence on hair shine. J Cosmet Sci 2003;54:353-66.

O'Neal CL, Crouch DJ, Rollins DE, Fatah A, Cheever ML. Correlation of saliva codeine concentrations with plasma concentrations after oral codeine administration. J Anal Toxicol 1999;23:452-9.

Ortuño J, Pizarro N, Farré M, Mas M, Segura J, Camí J, Brenneisen R, de la Torre. R. Quantification of 3,4-methylenedioxymetamphetamine and its metabolites in plasma and urine by gas chromatography with nitrogen-phosphorus detection. J Chromatogr. B Biomed Sci Appl 1999;723:21-32.

Ostrea EM Jr, Parks PM, Brady MJ. Rapid isolation and detection of drugs in meconium of infants of drug-dependent mothers. Clin Chem 1988;34;2372-3.

Pacifici GM, Nottoli R. Placental transfer of drugs administered to the mother. Clin Pharmacokinet 1995; 28:235-69.

Pacifici R, Altieri I, Gandini L, Lenzi A, Pichini S, Rosa M, Zuccaro P, Dondero F. Nicotine, cotinine, and trans-3-hydroxycotinine levels in seminal plasma of smokers: effects on sperm parameters. Ther Drug Monit 1993;15:358-63.

Pacifici R, Altieri I, Gandini L, Lenzi A, Passa AR, Pichini S, Rosa M, Zuccaro P, Dondero F. Environmental tobacco smoke: nicotine and cotinine concentration in semen. Environ Res 1995;68:69-72.

Pacifici R, Zuccaro P, Farré M, Pichini S, di Carlo S, Roset PN, Hernández-López C, Ortuño J, Segura J, Camí J, de la Torre R. Immunomodulating activity of MDMA. Ann N Y Acad Sci 2000; 914: 215-24.

Pacifici R, Farré M, Pichini S, Ortuño J, Roset PN, Zuccaro P, Segura J, de la Torre R. Sweat testing of MDMA with the Drugwipe analytical device: a controlled study with two volunteers. J Anal Toxicol 2001;25: 144-6.

Pacifici R, Zuccaro P, Farré M, Pichini S, Di Carlo S, Roset PN, Ortuño J, Pujadas M, Bacosi A, Menoyo E, Segura J, de la Torre R. Effects of repeated doses of MDMA ("Ecstasy") on cell-mediated immune response in humans. Life Sci 2001;69:2931-41.

Pacifici R, Zuccaro P, Farré M, Pichini S, Di Carlo S, Roset PN, Palmi I, Ortuño J, Menoyo E, Segura J, de la Torre R. Cell-mediated Immune Response in MDMA users after repeated doses administration: studies in controlled *vs* non controlled settings. Ann N Y Acad Sci 2002;;965:421-34.

Palmeri A, Pichini S, Pacifici R, Zuccaro P, Lopez A. Drugs in nails: physiology, pharmacokinetics and forensic toxicology. Clin Pharmacokinet 2000; 38:95-110.

Parrott AC. Human psychopharmacology of Ecstasy (MDMA): a review of 15 years of empirical research. Hum Psychopharmacol 2001;16:557-77.

Pepin G, Gaillard Y Concordance between self-reported drug use and findings in hair about cocaine and heroin. Forensic Sci Int 1997;84:37-41.

Perez-Reyes M, Wall ME. Presence of delta-9-tetrahydrocannabinol in human milk. N Engl J Med 1982;307:819-20.

Peroutka SJ, Newman H, Harris H. Subjective effects of 3,4-methylenedioxy-methamphetamine in recreational users. Neuropsychopharmacology 1988;1:273-7.

Phillips M. An improved adhesive patch for long-term collection of sweat. Biomater Med Devices Artif Organs 1980;8:13-21.

Phillips M. Sweat-patch testing detects inaccurate self-reports of alcohol consumption. Alcohol Clin Exp Res 1984;8:51-3.

Pichini S, Altieri I, Bacosi A, Di Carlo S, Zuccaro P, Iannetti P, Pacifici R. High-performance liquid chromatographic-mass spectrometric assay of busulfan in serum and cerebrospinal fluid. J Chromatogr 1992;581:143-6.

Pichini S, Zuccaro P, Pacifici R. Drugs in semen. Clin Pharmacokinet 1994;26:356-73

Pichini S., Altieri I., Zuccaro P, Pacifici R. Meconium as a matrix. Clin Pharmacokinet 1996;31:81

Pichini S, Altieri I, Zuccaro P, Pacifici R. Drug monitoring in non-conventional biological fluids and matrices. Clin Pharmacokinet 1996;30:211–28.

Pichini S, Basagaña X, Pacifici R, Garcia O, Puig C, Vall O, Harris J, Zuccaro P , Segura J, Sunyer J. Cord serum cotinine as a biomarker of fetal exposure to cigarette smoke at the end of pregnancy. Environ. Health Perspect 2000; 108:1079-83

Pichini S, Pellegrini M, Pacifici R, Marchei E, Murillo J, Puig C, Vall O, García-Algar O. Quantification of arecoline (Areca nut alkaloid) in neonatal biological matrices by high-performance liquid chromatography/electrospray quadrupole mass spectrometry.Rapid Commun Mass Spectrom 2003a;17:1958-64.

Pichini S, Pacifici R, Pellegrini M, Marchei E, Pérez-Alarcón E, Puig C, Vall O, García-Algar O. Development and validation of a high performance liquid chromatography-mass spectrometry assay for the determination of opiates and cocaine in meconium. J Chromat B Analyt Technol Biomed Life Sci 2003b:794:281-92.

Pichini S, Garcia-Algar O, Munoz L, Vall O, Pacifici R, Figueroa C, Pascual JA, Diaz D, Sunyer Assessment of chronic exposure to cigarette smoke and its change during pregnancy by segmental analysis of maternal hair nicotine. J Expo Anal Environ Epidemiol 2003;13:144-51.

Pichini S, Pacifici R, Pellegrini M, Marchei E, Lozano J, Murillo J, Vall O, García-Algar O. Development and validation of a high performance liquid chromatography-mass spectrometry assay for determination of amphetamine, methamphetamine and methylenedioxy-derivatives in meconium. Anal Chem 2004; 76:2124-32.

Pizarro N, Ortuño J, Farré M, Hernandez-Lopez C, Pujadas M, Llebaria A, Joglar J, Roset PN, Mas M, Segura J, Cami J, de la Torre R. Determination of MDMA and its

metabolites in blood and urine by gas chromatography-mass spectrometry and analysis of enantiomers by capillary electrophoresis. J Anal Toxicol 2002;26:157-65.

Pizarro N, Llebaria A, Cano S, Joglar J, Farre M, Segura J, de la Torre R Stereochemical analysis of 3,4-methylenedioxymethamphetamine and its main metabolites by gas chromatography/mass spectrometry. Rapid Commun Mass Spectrom 2003;17:330-6.

Pizarro N, Farré M, Pujadas M, Peiró AM, Roset PN, Joglar J, de la Torre R. Stereochemical analysis of 3,4-methylenedioxymethamphetamine and its main metabolites in human samples including the catechol-type metabolite (3,4-dihydroxymethamphetamine). Drug Metab Dispos 2004;32:1001-7.

Pötsch L, Skopp G, Moeller M. Biochemical approach on the conservation of drug molecules during hair fiber formation. Forensic Sci Int 1997; 84: 25-35

Pons G, Rey E, Matheson I. Excretion of psychoactive drugs into breast milk. Pharmacokinetics principles and recommendations. Clin Pharmacokinet 1994; 27:270-89.

Quinn DI, Wodak A, Day RO. Pharmacokinetic and pharmacodynamic principles of illicit drug use and treatment of illicit drug users. Clin Pharmacokinet 1997;33:340-400.

Ramamoorthy Y, Tyndale RF, Sellers EM. Cytochrome P450 2D6.1 and cytochrome P450 2D6.10 differ in catalytic activity for multiple substrates, Pharmacogenetics 2001; 11: 477-87

Ramamoorthy Y, Yu AM, Suh N, Haining RL, Tyndale RF, Sellers EM. Reduced (+/-)-3,4-methylenedioxymethamphetamine ("Ecstasy") metabolism with cytochrome P450 2D6 inhibitors and pharmacogenetic variants in vitro. Biochem Pharmacol 2002;63:2111-19.

Randall WC The physiology of sweating. Am J Phys Med 1953;32:292-318.

Ricaurte GA, Schuster CR, Seiden LS. Further evidence that amphetamines produce long-lasting dopamine neurochemical deficits by destroying dopamine nerve fibber. Brain Res 1984; 303: 359-64.

Ricaurte GA, Yuan J, McCann UD. (+/-) 3,4-methylenedioxymethamphetamine ('Ecstasy')-induced serotonin neurotoxicity: studies in animals. Neuropsychobiol 2000; 42: 5-10.

Ripple MG, Goldberger BA, Caplan YH, Blitzer MG, Schwartz S. Detection of cocaine and its metabolites in human amniotic fluid. J Anal Toxicol 1992;16:328-31.

Ritschel, WA, Thompson, GA. Monitoring of drug concentrations in saliva: a non-invasive pharmacokinetic procedure. Methods Find Exp Clin Pharmacol 1983;5:511-25.

Robinson S, Robinson AH. Chemical composition of sweat. Psychol Rev 1954;34:202-20.

Rodieck, R. W. (1988) The Primate Retina, Comparative Primate Biology, Volume 4.

Rollins DE, Klaassen CD. Biliary excretion of drugs in man. Clin Pharmacokinet 1979; 4:368-79.

Robieux I, Koren G, Vandenbergh H, Schneiderman J Morphine excretion in breast milk and resultant exposure of a nursing infant. J Toxicol Clin Toxicol. 1990;28:365-70

Rowland M, Tozer TN. Clinical Pharmacokinetics: concepts and applications. 2nd ed. Philadelphia: Lee and Febiger 1989.

Rothe M, Pragst F, Spiegel K, Harrach T, Fischer K, Kunkel J.Hair concentrations and self-reported abuse history of 20 amphetamine and ecstasy users. Forensic Sci Int 1997;89:111-28.

Sachs H Theoretical limits of the evaluation of drug concentrations in hair due to irregular hair growth. Forensic Sci Int 1995;70:53-61.

Sachs H, Kintz P. Testing for drugs in hair. Critical review of chromatographic procedures since 1992. J Chromatogr Biomed Appl 1998;713:147-61

Samyn N, Verstraete A,Van Haeren C, Kintz P. Analysis of drugs of abuse in saliva. Forensic Sci Rev 1999;11:1-19.

Samyn N, Van Haeren C. On-site testing of saliva and sweat with Drugwipe and determination of concentrations of drugs of abuse in saliva, plasma and urine of suspected users. Int J Legal Med 2000;113:150-4.

Samyn N, De Boeck G, Verstraete AG The use of oral fluid and sweat wipes for the detection of drugs of abuse in drivers. J Forensic Sci 2002;47:1380-7.

Sandborn WJ, Hanauer SB. Systematic review: the pharmacokinetic profiles of oral mesalazine formulations and mesalazine pro-drugs used in the management of ulcerative colitis. Aliment Pharmacol Ther 2003 ;17:29-42.

Scott KS, Oliver JS. Vitreous humor as an alternative sample to blood for the supercritical fluid extraction of morphine and 6-monoacetylmorphine. Med Sci Law 1999;39:77-81.

Segura J, Ventura R, Jurado C. Derivatization procedures for gas chromatographic-mass spectrometric determination of xenobiotics in biological samples, with special attention to drugs of abuse and doping agents. J Chromatogr B Biomed Sci Appl 1998;713:61-90.

Segura M, Ortuño J, Farré M, McLure JA, Pujadas M, Pizarro N, Llebaria A, Joglar J, Roset PN, Segura J, de La Torre R. 3,4 dihydroxymethamphetamine (HHMA). A major in vivo 3,4-methylenedioxymethamphetamine (MDMA) metabolite in humans. Chem Res Toxicol 2001;14:1203-8.

Segura M, Farré M, Pichini S, Peiró AM, Roset PN, Ramírez A, Ortuño J, Pacifici R, Zuccaro P, Segura J, de la Torre R. Contribution of CYP2D6 to 3,4-methylenedioxymethamphetamine (MDMA) disposition in humans: use of paroxetine as a metabolic inhibitor probe. Clin Pharmacokinet 2004, in press.

Seiden LS, Sabol KE. Methamphetamine and methylenedioxymethamphetamine neurotoxicity: possible mechanisms of cell destruction. NIDA Res Monogr 1996;163:251-76.

Schaffer MI, Wang WL, Irving J An evaluation of two wash procedures for the differentiation of external contamination versus ingestion in the analysis of human hair samples for cocaine. J Anal Toxicol 2002;26:485-8.

Schmid B, Bircher J, Preisig R and Küpfer A. Polymorphic dextromethorphan metabolism: co-segregation of oxidative O-demethylation with debrisoquin hydroxylation. Clin Pharmacol Ther 1985;38:618–24.

Shannon IL, Suddick RP. Effects of light and darkness on human parotid salivary flow rate and chemical composition. Arch Oral Biol 1973;18: 601-08.

Skopp G, Potsch L, Moeller MR. On cosmetically treated hair--aspects and pitfalls of interpretation. Forensic Sci Int 1997;84:43-52.

Smith FP. Detection of amphetamine in bloodstains, semen, seminal stains, saliva, and saliva stains. Forensic Sci Int 1981;17:225-8.

Spitzer M, Franke B, Walter H, Buechler J, Wunderlich AP, Schwab M, Kovar KA, Hermle L, Gron G. Enantio-selective cognitive and brain activation effects of N-ethyl-3,4-methylenedioxyamphetamine in humans. Neuropharmacology. 2001;41:263-71.

Steiner E, Villen T, Hallberg M, Rane A. Amphetamine secretion in breast milk. Eur J Clin Pharmacol 1984;27:123-4.

Steinmeyer S, Ohr H, Maurer HJ, Moeller MR Practical aspects of roadside tests for administrative traffic offences in Germany. Forensic Sci Int 2001;121:33-6.

Szeto HH Kinetics of drug transfer to the fetus. Clin Obstet Gynecol. 1993;36:246-54.

Suzuki O, Hattori H, Asano M. Nails as useful materials for detection of methamphetamine or amphetamine abuse. Forensic Sci Int 1984;24:9-16.

Suzuki S, Inoue T. Analysis of methamphetamine in hair, nail, sweat, and saliva by mass fragmentography. J. Anal Toxicol 1989;13:176-8.

Tedeschi L, Frison G, Castagna F, Giorgetti R, Ferrara SD. Simultaneous identification of amphetamine and its derivatives in urine using HPLC-UV. Int J Legal Med 1993;105(5):265-9.

Tucker GT, Lennard MS, Ellis SW, Woods HF, Cho AK, Lin LY, Hiratsuka A, Schmitz DA, Chu TY. The demethylenation of methylenedioxymethamphetamine ("ecstasy") by debrisoquine hydroxylase (CYP2D6). Biochem Pharmacol 1994;47:1151-56.

Valente D, Cassani M, Pigliapochi M, Vansetti G Hair as the sample in assessing morphine and cocaine addiction. Clin Chem 1981;27:1952-3.

Van Dam JG, Van Loenen AC. De aanwezigheid van geneesmiddelen in speeksel. Pharmaceutisch Weekblad1978;113: 65-74.

Van Haeringen NJ. Secretion of drugs in tears. Curr Eye Res 1985;4: 485-8.

Vining R F, McGinley RA. Hormones in saliva. Crit Rev Clin Lab Sci 1986;23:95-146.

Vree TB, Muskens AT, Van Rossum JM. Excretion of amphetamines in human sweat. Arch Int Pharmacodyn Ther 1972;199: 311-7.

Zhao H, Brenneisen R, Scholer A, McNally AJ, ElSohly MA, Murphy TP, Salamone SJ. Profiles of urine samples taken from Ecstasy users at rave parties: analysis by immunoassays, HPLC, and GC-MS. J Anal Toxicol 2001;25:258-69.

Young AJ, Marston WL, Dudler L. Subset-specific regulation of the lymphatic exit of recirculating lymphocytes in vivo. J. Immunol 2000;165:3168-74.

Wan SH, Matin SB, Azarnoff MD. Kinetics, salivary excretion of amphetamine isomers, and effect or urinary pH. Clin Pharmacol Ther 1978;23:585–90.

Wang Y, Moss J, Thisted R. Predictors of body surface area. J Clin Anesth 1992;4:4-10.

Wang WL, Cone EJ Testing human hair for drugs of abuse. IV. Environmental cocaine contamination and washing effects. Forensic Sci Int 1995;70:39-51.

Welp EA, Bosman I, Langendam MW, Totte M, Maes RA, van Ameijden EJ. Amount of self-reported illicit drug use compared to quantitative hair test results in community-recruited young drug users in Amsterdam. Addiction 2003;98:987-94.

Wennig R. Potential problems with the interpretation of hair analysis results. Forensic Sci Int 2000;107:5-12.

Winecker RE, Goldberger BA, Tebbett I, Behnke M, Eyler FD, Conlon M, Wobie K, Karlix J, Bertholf RL. Detection of cocaine and its metabolites in amniotic fluid and umbilical cord tissue. J Anal Toxicol 1997;21:97-104.

Winecker RE, Goldberger BA, Tebbett IR, Behnke M, Eyler FD, Karlix JL, Wobie K, Conlon M, Phillips D, Bertholf RL. Detection of cocaine and its metabolites in breast milk. J Forensic Sci 2001;46:1221-3.

White SR, Obradovic T, Imel KM, Wheaton MJ. The effects of methylenedioxymethamphetamine (MDMA, "Ecstasy") on monoaminergic neurotransmission in the central nervous system. Prog Neurobiol 1996;49:455-79

Wolff K, Farrell M, Marsden J, Monteiro MG, Ali R,Welch S, Strang J. A review of biologic indicators of illicit drug use, practical considerations and clinical usefulness. Addiction 1999;94:1279–98.

Wood M, De Boeck G, Samyn N, Morris M, Cooper DP, Maes RA, De Bruijn EA Development of a rapid and sensitive method for the quantitation of amphetamines in human plasma and oral fluid by LC-MS-MS. J Anal Toxicol 2003;27:78-87.

Wright JD, Pearl L. Knowledge and experience of young people regarding drug misuse. BMJ 1995;310:20-4.

Wu D, Otton SV, Inaba T, et al. Interactions of amphetamine analogs with human liver CYP2D6. Biochem Pharmacol 1997;53:1605-12.

Wyman J, Bultman S.Postmortem distribution of heroin metabolites in femoral blood, liver, cerebrospinal fluid, and vitreous humor. J Anal Toxicol 2004;28:260-3.

9. Appendices

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Drug Monitoring and Toxicology

Usefulness of Saliva for Measurement of 3,4-Methylenedioxymethamphetamine and Its Metabolites: Correlation with Plasma Drug Concentrations and Effect of Salivary pH

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Background: **Saliva is an alternative biologic matrix for drugs-of-abuse testing that offers the advantages of noninvasive, rapid, and easy sampling. We studied the excretion profile of 3,4-methylenedioxymethamphetamine (MDMA) and its metabolites in both saliva and plasma, as well the effect of the drug on salivary pH.**

Methods: **Saliva and plasma samples were obtained from eight healthy MDMA consumers after ingestion of a single 100-mg dose of the drug. Concentrations of MDMA and its main metabolites, 3,4-methylenedioxyamphetamine (MDA) and 4-hydroxy-3-methoxymethamphetamine (HMMA), in saliva and plasma were measured by gas chromatography–mass spectrometry. Apparent pharmacokinetic parameters for MDMA in saliva were estimated, and the saliva-to-plasma ratio at each time interval was calculated and correlated with salivary pH.**

Results: **MDMA, MDA, and HMMA were detected in saliva. Salivary concentrations of MDMA were 1728.9–6510.6 μg/L and peaked at 1.5 h after drug intake. This was followed by a progressive decrease, with a mean concentration of 126.2 μg/L at 24 h. The saliva-to-plasma ratio was 32.3–1.2, with a peak of 18.1 at 1.5 h after drug administration. Salivary pH seemed to be affected by MDMA administration; pH values decreased by 0.6 units (mean pH values of 6.9 and 6.8 at 1.5 and 4 h after drug administration vs predose pH of 7.4).**

Conclusions: **Measurement of MDMA in saliva is a valuable alternative to determination of plasma drug concentrations in both clinical and toxicologic studies. On-site testing is also facilitated by noninvasive and rapid collection of salivary specimens.**

Alternative biologic matrices to urine and plasma have recently been introduced for drug monitoring *(1, 2)*. Analysis of certain matrices, such as tears, cerebrospinal fluid, and bronchial secretions, may reveal the presence of a drug at the site of action, whereas others, such as amniotic fluid, cord blood, or breast milk, are useful for determining fetal and perinatal exposure to drugs *(3–7)*. An individual's past history of medication, compliance, or drug abuse can be obtained from drug testing of hair and nails *(8–11)*, whereas data on the current status of drug use might be also provided by sweat and saliva analysis *(12)*.

In fact, saliva is the only fluid that has successfully been used as an alternative to blood in several pharmacokinetic and pharmacotoxicologic studies *(13–16)*, and there is evidence that when a given drug is detected in salivary specimens, there is a high likelihood that the individual tested is under the pharmacologic effects of the drug. Previous studies on drugs in saliva *(17)* have shown that weak bases, such as cocaine, opiates, benzodiazepines, or nicotine, tend to concentrate in saliva because its pH is slightly acidic compared with that of plasma *(12, 17–19)*. Although some metabolites have been detected, the parent drug is usually the main analyte found in saliva *(2)*. Saliva has the advantage of noninvasive sampling, which is particularly convenient in any situation in which samples must be collected with minimum

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discomfort to the patient *(1–14)*. Saliva flow can be stimulated to ensure adequate sample volume *(18)*, and saliva offers fewer possibilities of adulteration compared with urine specimens *(20)*. Some disadvantages, however, are related to oral contamination from certain routes of administration (smoking, snorting, oral ingestion) and to the method of sample collection, which may influence salivary drug concentrations as a result of changes in pH and flow rate *(12, 18)*.

3,4-Methylenedioxymethamphetamine (MDMA;[5] "ecstasy") is an amphetamine derivative that seems to exert mixed stimulant and hallucinogenic effects *(21)*. Recreational use of MDMA, either alone or in combination with other drugs such as alcohol and cannabis, has become increasingly popular among young people *(22)*. Several cases of acute intoxication have been reported, and law enforcement agencies have expressed a strong interest in on-site roadside testing of potentially intoxicated drivers *(23)*.

Recently, analytical devices based on immunochromatographic methods developed for the detection of drugs of abuse on surfaces have been applied for on-site drug testing with saliva as the biologic fluid *(20)*. These assays on saliva were sufficiently sensitive for the detection of recent cocaine and amphetamine abuse in drug users *(23)*. Until now, however, few reports have presented systematic studies on the excretion profile of amphetamines and related compounds in saliva after controlled administration *(24–26)*.

The aims of this study were as follows: (*a*) to investigate the presence and the concentration of MDMA and its main metabolites, 3,4-methylenedioxyamphetamine (MDA) and 4-hydroxy-3-methoxymethamphetamine (HMMA), in saliva; (*b*) to assess the eventual correlation between salivary and plasma MDMA concentrations; and (*c*) to determine the effect of salivary pH on the MDMA saliva-to-plasma (S/P) ratio. Saliva and plasma samples were obtained from individuals participating in a clinical trial involving the controlled administration of 100 mg of MDMA.

Materials and Methods

PARTICIPANTS AND STUDY DESIGN

Eight males were included in the study. Eligibility criteria required the recreational use of MDMA on at least five occasions. Each participant underwent a general physical examination, routine laboratory tests, urinalysis, and a 12-lead electrocardiogram. The mean age of the participants was 24.4 years (range, 21–33 years), the mean weight was 72.7 kg (range, 60.6–86.5 kg), and the mean height was 177.3 cm (range, 167.5–190 cm). All participants were declared MDMA consumers (mean number of times drug had been consumed, 24; range, 5–100). We phenotyped participants for CYP2D6 activity, using dextromethorphan as a drug probe. The dextromethorphan/dextrorphan ratio was used to classify participants as poor or extensive metabolizers *(27)*. All participants were extensive metabolizers.

All participants gave written informed consent before inclusion in the study and received financial compensation for inconveniences caused by their participation in the study. The study was conducted in accordance with the Declaration of Helsinki, approved by the local Ethical Committee (CEIC-IMAS), and authorized by the Agencia Española del Medicamento (reference AEM 98/532) of the Spanish Ministry of Health.

The study design was double blind, randomized, crossover, and controlled. The volunteers participated as outpatients in two different randomly assigned 10-h experimental sessions in which they were given single 100-mg doses of MDMA or placebo by the oral route. The volunteers were requested to abstain from consumption of any drug of abuse during the study period, and urine drug testing was performed before each experimental session for opiates, cocaine, cannabinoids, and amphetamines. For all four groups of substances tested, all volunteers were negative before each experimental session. MDMA and placebo were prepared by the Pharmacy Service of Hospital del Mar (Barcelona, Spain) as white soft-gelatin capsules (two capsules each time) and administered in a fasting state with 100 mL of tap water.

CHEMICALS

MDMA, MDA, HMMA, and the internal standards MDMA-d_5, MDA-d_5, and pholedrine were purchased from Cerilliant (formerly Radian Analytical Products). *N*-Methylbis(trifluoroacetamide) (MBTFA; gas chromatography grade) was supplied by Macherey-Nagel. Potassium dihydrogen phosphate, potassium hydroxide, ethyl acetate, and ammonia solution were from Merck. Ultrapure water was obtained using a MilliQ purification system (Millipore). All other reagents were of analytical grade.

COLLECTION OF SALIVA AND BLOOD SAMPLES

Samples of mixed saliva (saliva secreted by the different salivary glands) *(12)* were obtained without any stimulation over a 5-min period at 0, 1.5, 4, 6, 10, and 24 h after drug administration. Samples were collected in polypropylene tubes. The salivary pH was recorded at the time of collection, and the samples were immediately stored at −20 °C until analysis. Blood samples were centrifuged, and the plasma obtained was immediately frozen at −20 °C. Blood and saliva from a placebo group that tested negative for the presence of MDMA were used as drug-free blank samples.

The collection times selected in the present experiment

[5] Nonstandard abbreviations: MDMA, 3,4-methylenedioxymethamphetamine; MDA, 3,4-methylenedioxyamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; S/P, salivary-to-plasma; MBTFA, *N*-methylbis(trifluoroacetamide); c_{max}, peak concentration; t_{max}, time to peak concentration; and AUC, area under the curve.

were based on previous experience from more extensive pharmacokinetic studies *(28, 29)*.

determination of salivary and plasma mdma, mda, and hmma

Frozen saliva and plasma were allowed to thaw at room temperature. Before analysis, saliva was centrifuged to remove the mucous part that accumulated at the bottom. To 1 mL of plasma or saliva, we added 200 ng each of MDMA-d_5, MDA-d_5, and pholedrine; we then extracted the drugs from the samples by solid-liquid extraction using mixed cation-exchange/hydrophobic interaction columns (Bond-Elut Certify®) as follows.

We adjusted the pH of the samples to 6 by adding 1 mL of 0.1 mol/L phosphate buffer, pH 6, and passed the samples through the columns, which had previously been conditioned by sequential passage of 2 mL of methanol and 2 mL of 0.1 mol/L phosphate buffer, pH 6. Columns were washed consecutively with 1 mL of 1 mol/L acetic acid and 6 mL of methanol. MDMA and its metabolites were eluted with 2 mL of ethyl acetate containing 20 g/L ammonium hydroxide. After the addition of 20 μL of MBTFA to prevent drugs losses, the eluates were evaporated to dryness at 40 °C under a nitrogen stream. Residues were reconstituted and derivatized with 50 μL of MBTFA at 70 °C for 45 min to obtain trifluoroacetyl derivatives of the analytes *(30)*.

The MDMA concentration in saliva was determined on a HP6890 gas chromatograph coupled to a Model HP5973 quadrupole mass spectrometer (Agilent, formerly Hewlett Packard). The compounds were separated on a cross-linked 5% phenyl-methylsilicone capillary column [Ultra-2; 12 m × 0.2 mm (i.d.); 0.33-μm film thickness; Hewlett Packard]. The samples were injected in splitless mode, and helium gas was used as carrier at a flow rate of 1.2 mL/min (measured at 180 °C). The mass spectrometer was operated in the electron impact ionization and selected-ion monitoring acquisition mode. The ions *m/z* 154 for MDMA, HMMA, and pholedrine; *m/z* 162 for MDA; *m/z* 158 for MDMA-d_5; and *m/z* 167 for MDA-d_5 were selected for quantification *(31)*. This method has previously been validated for plasma samples, and it was reapplied for saliva and plasma samples in this present study *(28, 31)*.

Calibration curves were prepared in drug-free saliva and plasma by adding appropriate volumes of working methanolic solutions of the analytes under investigation. Peak-area ratios between each compound and the internal standard (MDMA-d_5 for MDMA, MDA-d_5 for MDA, pholedrine for HMMA) were used for calculations. Curves were linear at 25–400 μg/L for MDMA and HMMA and 2.5–40 μg/L for MDA. Samples containing concentrations above the working ranges were reanalyzed after appropriate dilution with phosphate buffer.

Analytical recoveries were calculated by comparing the peak areas obtained for calibration samples, prepared by adding the reference substances and the internal standards to blank plasma or saliva, before or after the above-mentioned extraction procedure. The mean analytical recovery for MDMA was 90% in both saliva and plasma; recoveries for MDA and HMMA were 92% and 74%, respectively.

Four replicate analyses were performed with a plasma and saliva supplemented with 25 μg/L MDMA and HMMA and 2.5 μg/L MDA. The standard deviation of the quantitative values was used as a measure of the noise to calculate the limit of quantification (10 SD). The limits of quantification were 5.7 μg/L for MDMA, 1 μg/L for MDA, and 2.9 μg/L for HMMA in both biologic fluids.

To determine intraassay imprecision and accuracy, we analyzed three replicates each of blank plasma and saliva containing three different concentrations of MDMA, MDA, and HMMA (25, 100, and 400 μg/L for MDMA and HMMA; 2.5, 10, and 40 μg/L for MDA); for interday imprecision and accuracy, we measured the above-mentioned replicates on three different days.

Intraday imprecision (expressed as CV for specific added target concentrations) and accuracy (expressed as percentage error of concentration found compared with added target concentrations) were always <6.5% for all analytes under investigation. Similarly, the interday CV and error were <8.5%.

pH measurements of salivary samples

The pH of salivary samples from the eight volunteers in the MDMA or placebo groups was measured at all time intervals with a pH indicator stick (Riedel-de Haën) with a pH range of 6.4–8 (increments of 0.2 pH units). Results were recorded by two independent observers, who were unaware of treatment conditions.

pharmacokinetics and statistical analysis

With regard to saliva and plasma concentrations of MDMA, the following parameters were determined: peak concentration (c_{max}); time taken to reach c_{max} (t_{max}); area under the concentration–time curve from 0 to 24 h (AUC_{0-24}); elimination half-life ($t_{1/2e}$) in plasma and disappearance half-life ($t_{1/2d}$) in saliva; elimination constant (k_e) in plasma; and disappearance constant (k_d) in saliva. First-order constant kinetics are usually described as elimination constants for either plasma or saliva. Because drugs are not properly eliminated from saliva, but rather what is observed is a disappearance rate, we preferred to use the term disappearance constant to describe this kinetic parameter *(32)*.

The AUC for each drug was calculated by the linear trapezoidal rule, and the elimination and disappearance constants were calculated by log-linear regression of the three last points with concentrations above the quantification limit. Correlations between different variables were analyzed by regression analysis. The Wilcoxon test for nonparametric data was used to assess differences in salivary pH values between treatment and placebo. Differences associated with P values <0.05 were considered statistically significant.

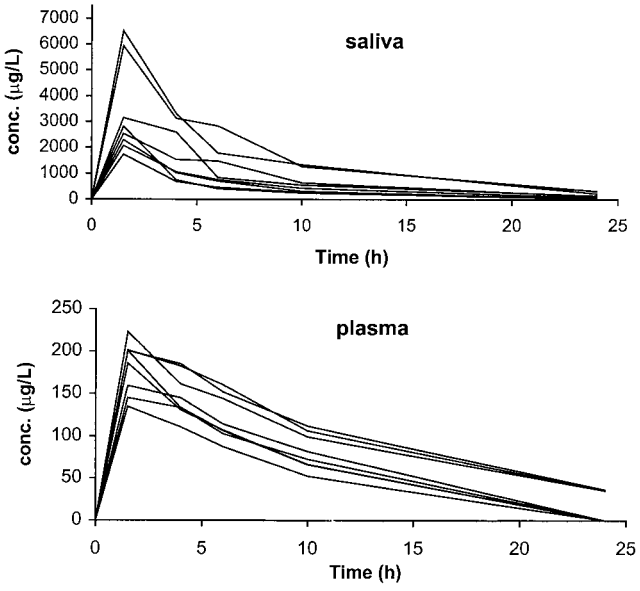

Fig. 1. Concentration–time profiles of MDMA in saliva (*top*) and plasma (*bottom*) for the eight volunteers.

Results

CONCENTRATION–TIME PROFILES AND PHARMACOKINETICS OF MDMA IN SALIVA AND PLASMA

The time courses of the MDMA concentrations in saliva and plasma for each of the eight volunteers are shown in Fig. 1. At 1.5 h after administration of MDMA, concentrations appeared to be the highest in both saliva (range, 1728.9–6510.6 μg/L) and plasma (range, 134.9–223.0 μg/L). It should be noted that two of the eight volunteers had peak MDMA concentrations in saliva more than twofold higher than the mean c_{max} in the remaining six participants. These two individuals were also the ones showing the highest plasma c_{max}. After the absorption phase, saliva and plasma MDMA concentrations decreased to mean concentrations (SD) at 24 h of 126.2 (101.8) μg/L and 13.5 (18.6) μg/L, respectively. The mean concentration–time curves for MDMA in saliva and plasma are shown in Fig. 2. The MDMA concentrations in saliva were one order of magnitude higher than those observed in plasma. The pharmacokinetic parameters for MDMA in saliva and plasma are presented in Table 1. It is important to point out that these are only "apparent" parameters because of the few concentration–time data points for saliva and plasma evaluated in this study. Nonetheless, the apparent pharmacokinetic parameters for MDMA in plasma were in accordance with those reported by our group in a previous study *(30)*. The t_{max} was attained at 1.5 h in both saliva and plasma. The $t_{1/2d}$ and k_d values for saliva were greater than the $t_{1/2e}$ and k_e values for plasma.

MDA AND HMMA IN SALIVA

HMMA, the major metabolite of MDMA, was detected in the nonconjugated form in trace amounts, but quantification was not possible. MDA was also excreted in saliva, with concentrations representing ~4–5% of the concentration of salivary MDMA (AUC comparisons), as was also observed in plasma (Fig. 2). The highest salivary concentrations of MDA occurred between 1.5 and 4 h after drug administration, whereas in plasma, the highest concentrations were between 4 and 6 h.

MEASUREMENTS OF pH IN SALIVARY SAMPLES

The 24-h profiles for mean salivary pH in both the placebo group and MDMA samples are shown in Fig. 3. The mean predose salivary pH was 7.4 and 7.3 in the MDMA and placebo samples, respectively. At 1.5 h after drug administration, which corresponds to the MDMA t_{max}, the salivary pH in the MDMA group showed a mean (SD)

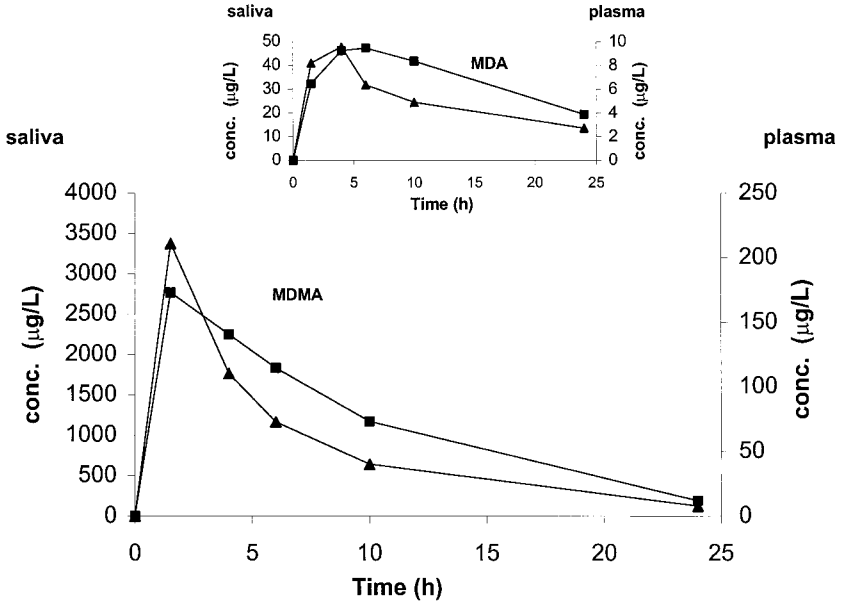

Fig. 2. Comparison of time curves for MDMA and MDA in saliva (▲) and plasma (■).

Table 1. Apparent pharmacokinetic parameters for MDMA in saliva and plasma.

	Saliva			Plasma		
	Mean	SD	CV, %	Mean	SD	CV, %
$AUC_{0-24\ h}$, μg/L · h	20 843.1	12 656.6	61	1598.6	733.3	46
t_{max}, h	1.5			1.5		
c_{max}, μg/L	3375.6	1812.8	54	181.4	31.3	17
k_d,[a] h^{-1}	0.1279	0.0231	19	0.0988	0.0189	19
$t_{1/2d}$,[a] h	5.6	0.9	17	7.2	1.4	19

[a] For plasma, k_e and $t_{1/2e}$.

statistical decrease to 6.9 (0.2) compared with 7.3 (0.2) for the placebo group (Wilcoxon test, $P < 0.001$). The decrease in pH values was homogeneous; all participants treated with MDMA showed lower values than those observed for the placebo group. At 4 h after treatment (1 h after a light meal given to participants), minimum mean (SD) pH values of 6.8 (0.4) and 7.1 (0.3) were obtained in the MDMA and placebo samples, respectively. The two mean values were not statistically different. In fact, six of the eight participants showed the same pattern; in one participant, no changes were observed, and in another, the pH value in the placebo condition was higher than that during MDMA treatment. Finally, pH returned to predose values between 6 and 24 h after treatment in both the MDMA and placebo groups.

S/P ratio for MDMA

The time-course curve for the S/P ratio during the 24 h after drug administration is presented in Fig. 4. The S/P ratio exhibited a mean (SD) maximum value of 18.1 (7.9) at 1.5 h, corresponding to the MDMA t_{max}. The high variability observed (CV = 43%) was attributable to the two individuals with the highest MDMA salivary c_{max}, who had S/P ratios of 26.6 and 32.3, whereas the remaining volunteers had values between 10.3 and 16.9. In the postabsorption phase, the S/P ratio decreased to reach values of ~7.3 and 6.4 at 10 and 24 h after drug administration, respectively. S/P ratios showed a strong correlation with salivary MDMA concentrations ($r = 0.96$; $P < 0.001$) as well as a lower but significant correlation with salivary pH values ($r = 0.62$; $P < 0.002$) and plasma MDMA concentrations ($r = 0.69$; $P < 0.001$; Fig. 5). In any case, regardless of the variation of S/P ratio during the time course of MDMA administration, salivary concentrations were correlated to plasma concentrations ($r = 0.81$; $P < 0.001$).

Discussion

Overall, the patterns of salivary and plasma MDMA concentration–time profiles for different participants agreed well, but certain interindividual variations were evident for salivary MDMA concentrations. Data obtained for plasma samples were more homogeneous and were in agreement with previously reported findings after the administration of the same MDMA dose to eight different individuals *(31)*.

MDMA appeared in saliva in concentrations remarkably higher than those in plasma (Fig. 2). This is not surprising and may be attributable to several causes. Drugs are generally incorporated into saliva by passive diffusion because of a concentration gradient in which only the free fraction of the drug (not bound to proteins) diffuses through lipidic membranes from plasma to saliva. It should be noted that saliva has little protein-binding capacity compared with plasma. There are no specific data on the fraction of MDMA bound to plasma proteins, but from what is known for amphetamine and methamphetamine *(33)*, it should be ~20%. In practice, such low binding means that the MDMA available in plasma may diffuse into the saliva. In addition, the passage across cells membranes is favored for low-molec-

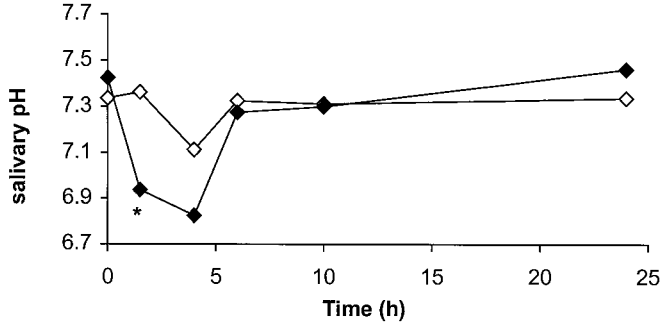

Fig. 3. Time profile of salivary pH during treatment administration. ◇, placebo; ◆, MDMA. *, $P < 0.001$.

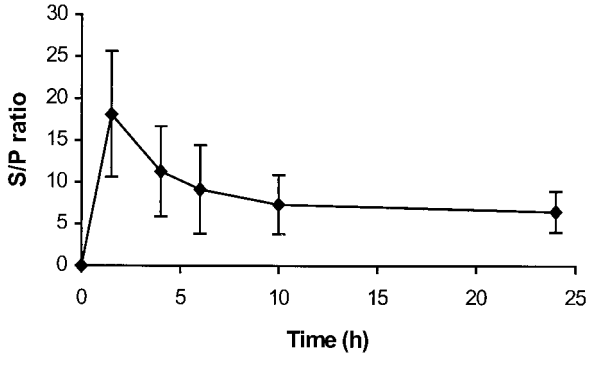

Fig. 4. Time profile of MDMA S/P ratio.

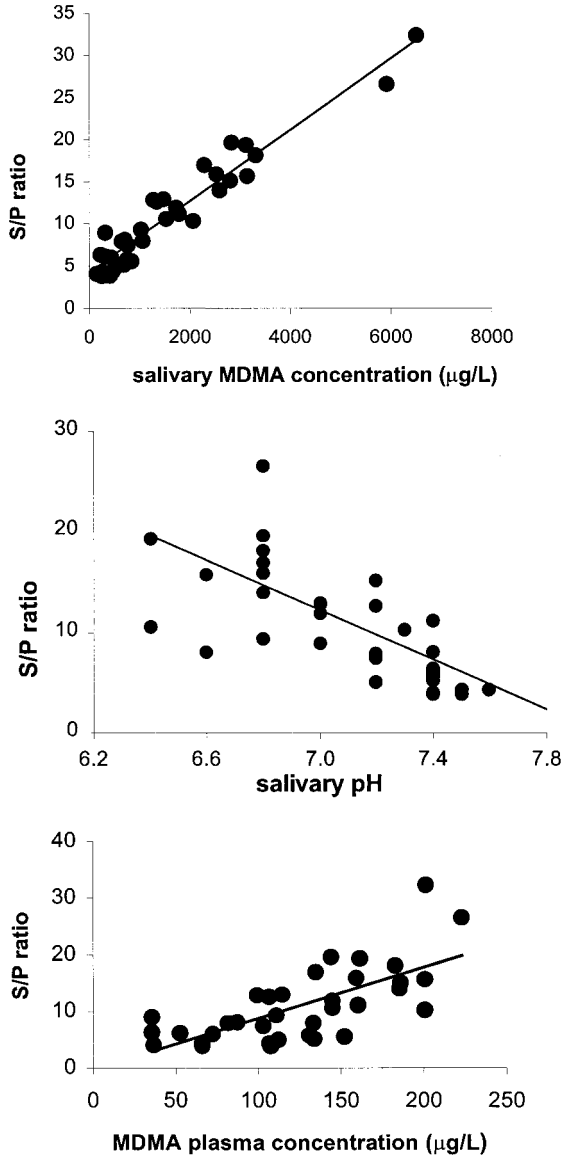

Fig. 5. Correlation between MDMA S/P ratio and MDMA concentrations in saliva (*top*) and plasma (*bottom*) and with mean salivary pH (*middle*) during treatment.

ular weight molecules, such as MDMA *(13, 14)*. Furthermore, MDMA is a basic drug with a pK_a ~9.9 [data derived from methamphetamine *(33)*], and because under typical conditions (absence of salivary flow stimulation) saliva is more acidic than blood, MDMA is converted its ionized form, which cannot diffuse back into plasma and thus accumulates in saliva *(1)*.

The theoretical S/P ratio for MDMA should be ~3.9 as calculated with the Henderson–Hasselbalch equation *(12)*. In our study, the mean S/P ratio was 18.1 at peak MDMA concentrations and 6.4 at 24 h after drug administration. However, it must be noted that there were two individuals who had S/P ratios that were exceedingly high. Mean S/P ratios increased to a 20-fold difference between plasma and salivary concentrations at t_{max}, when most probably this difference may be closer to 10-fold (mean value for six participants, excluding outliers). In any case, it is apparent that excretion of MDMA in saliva occurs to a greater extent than expected. Interindividual variations in the MDMA S/P ratio, as well as in salivary MDMA concentrations, could be partly explained by the fact that the saliva was collected without flow stimulation. It is known that stimulation of saliva secretion increases the pH to values approaching plasma pH. In the case of basic drugs, such as MDMA or cocaine, this reduces the salivary drug concentration, and the variability in S/P ratios is narrowed *(14, 34)*.

In this study, the saliva collected was nonstimulated to measure true saliva pH. This approach allowed the observation of eventual pH changes produced by MDMA. In addition, most volunteers receiving MDMA experienced jaw clenching *(31)*, and the production of stimulated saliva by conventional methods, such as chewing, seemed inadequate for this drug. Salivary pH appeared to be affected by MDMA concentration in saliva after drug administration. In volunteers receiving MDMA, the salivary pH was lowered, attaining a maximal effect at MDMA $t_{max.}$ Most probably this contributed to the accumulation of the drug in the salivary fluid. In the two cases where S/P ratios were extremely high, a poor correlation with salivary pH was observed (Fig. 4), so that other mechanisms may operate in the diffusion to saliva. Changes in salivary pH observed at 4 h after drug administration represented the combined effect of MDMA and the ingestion of a light breakfast 3 h after drug administration. In the hours following maximum MDMA concentrations, S/P ratios decreased and reached almost constant values (~7), but were still higher than the theoretical value from the Henderson–Hasselbalch equation. Because MDMA acts on serotonergic neurotransmission, with resulting vasoconstriction and significant changes in hemodynamics *(28)*, a reduction in saliva production probably occurs, which concentrates this fluid. In fact, individuals receiving MDMA usually exhibit dry mouth *(35)*. Presumably MDMA also impairs salivary flow through its sympathomimetic effects, producing a sympathetic constriction of the salivary bed *(14)*. Consequently, buffering capacity, which is maximal in conditions of flow stimulation, can be reduced, and the pH of mixed saliva obtained from the oral cavity (the one measured) may not be the same as the pH at the site of saliva secretion *(14, 36)*. Hence, a dynamic concentration gradient takes place, which probably produces MDMA S/P ratios higher than those calculated with the Henderson–Hasselbalch equation. Unfortunately, one limitation of this study was that it was not possible to measure salivary flow during collection, a variable that could have explained apparent deviations from theoretic values.

S/P ratios exceeding theoretical values were also found by Samyn and Van Haeren *(23)*, who measured MDMA in saliva and plasma in individuals who admitted recent drug abuse. Similar results were also reported by

Cook et al. *(37)*, who compared methamphetamine concentrations in the saliva and plasma of volunteers receiving the drug by smoking and intravenous routes. Both authors always found S/P ratios exceeding theoretical values and attributed the circumstance to buccal contamination by tablets or smoked drugs, at least for the first hours after drug administration. However, this is not the case of the present study, where MDMA was administered as capsules. Moreover, if buccal contamination can be hypothesized for the first 2 h after drug administration, this event had to be excluded in the following hours because the light meal (snack and juice) given to the volunteers 3 h after the start of treatment should have eliminated this contamination. Indeed, S/P ratios exceeding theoretical values were also found when methamphetamine was administered intravenously *(37)*.

MDMA was the principal analyte detected in saliva. In fact, MDA was found in the saliva in minute abundance relative to MDMA, as it is in plasma *(28)*. Regarding HMMA, only trace amounts could be found in saliva because it is mainly present in plasma in its glucuronoconjugated form *(38)*, which does not diffuse through the lipid barrier dividing salivary ducts from the systemic circulation. The evidence that MDMA was the major analyte found in saliva was in agreement with results reported by Cone *(17)* for cocaine and methamphetamine, as well as with those reported by Kintz *(26)* for *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine. Therefore, it can also be stated for MDMA that the parent drug is the target compound to be detected in saliva to assess consumption.

The apparent pharmacokinetic parameters calculated for plasma and saliva showed that MDMA disappears more rapidly from saliva than from plasma. This apparent discordance in the disposition of MDMA in the body may most probably be related to the changes in salivary pH discussed above during the first 4 h of MDMA kinetics. Despite the faster disappearance of the drug from saliva, because MDMA concentrations are one order of magnitude higher in saliva than those observed in plasma, salivary concentrations showed an average value of ~100 μg/L at 24 h. In contrast, it was not always possible to detect the drug in plasma at that same time.

Regarding the possible correlation between salivary concentrations of MDMA and cardiovascular effects, results from previous investigations showed a good agreement between MDMA concentrations in saliva and changes in blood pressure, heart rate, and pupillary diameter *(28)*. Indeed, the pharmacologic effects rose and fell with a profile similar to that of salivary MDMA, presenting peak effects statistically different from placebo treatment between 1 and 2 h after MDMA administration. Similar results may be obtained when comparing the time course of MDMA in saliva and subjective feelings of intoxication *(29)*. Subjective effects reach their maximum between 1.5 and 2 h and return to basal values ~4 h after drug treatment.

In conclusion, the measurement of MDMA in saliva appears to be a suitable alternative to plasma analysis in clinical and toxicologic situations where detection of recent abuse is requested. Despite changes in the S/P ratio during the time course for MDMA in saliva and plasma, the correlation between MDMA concentration in the two biologic fluids indicates that salivary concentrations of this drug may be a predictor of plasma concentrations. It should be acknowledged, nevertheless, that the results were obtained under controlled conditions where several factors that may modify saliva concentrations and that are difficult to ascertain in drug users were not considered: fasting state, dehydration, buccal contamination, and others. Because of the higher concentrations encountered, saliva exhibits a larger time window for detection of MDMA consumption. This may help establish whether individuals are under the influence of the drug in a much less invasive way than with plasma and without specific requirements for sample collection, thus facilitating on-site sample collection and drug testing.

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References

1. Pichini S, Altieri I, Zuccaro P, Pacifici R. Drug monitoring in non-conventional biologic fluids and matrices. Clin Pharmacokinet 1996;30:211–28.
2. Wolff K, Farrell M, Marsden J, Monteiro MG, Ali R, Welch S, et al. A review of biologic indicators of illicit drug use, practical considerations and clinical usefulness. Addiction 1999;94:1279–98.
3. Van Haeringen NJ. Secretion of drugs in tears. Curr Eye Res 1985;4:485–8.
4. Bonati M, Kanto J, Tognoni G. Clinical pharmacokinetics of cerebrospinal fluid. Clin Pharmacokinet 1982;7:312–5.
5. Wong GA, Pierce TH, Goldstein E, Hoeprich PD. Penetration of antimicrobial agents into bronchial secretion. Am J Med 1975;9: 219–23.
6. Pacifici GM, Nottoli R. Placental transfer of drugs administered to the mother. Clin Pharmacokinet 1995;28:235–69.
7. Pichini S, Basagaña X, Pacifici R, Garcia O, Puig C, Vall O, et al. Cord serum cotinine as a biomarker of fetal exposure to cigarette smoke at the end of pregnancy. Environ Health Perspect 2000; 108:1079–83.
8. Uematsu T. Therapeutic drug monitoring in hair samples. Clin Pharmacokinet 1993;25:83–7.
9. Pichini S, Pacifici R, Altieri I, Pellegrini M, Zuccaro P. Determination of opiates and cocaine in hair as trimethylsilyl derivatives using gas chromatography-tandem mass spectrometry. J Anal Toxicol 1999;5:343–8.
10. Segura J, Stramesi C, Redón A, Ventura M, Sánchez CJ, González G, et al. Immunological screening of drugs of abuse and gas chromatographic-mass spectrometric confirmation of opiates and cocaine in hair. J Chromatogr B 1999;724:9–21.
11. Palmeri A, Pichini S, Pacifici R, Zuccaro P, Lopez A. Drugs in nails: physiology, pharmacokinetics and forensic toxicology. Clin Pharmacokinet 2000;38:95–110.

12. Kidwell D, Holland J, Athanaselis S. Testing for drugs of abuse in saliva and sweat. J Chromatogr B 1998;713:111–35.

13. Mucklow JC, Bending MR, Kahn GC, Dollery CT. Drug concentration in saliva. Clin Pharmacol Ther 1978;24:563–637.

14. Gorodischer R, Koren G. Salivary excretion of drugs in children: theoretical and practical issues in therapeutic drug monitoring. Dev Pharmacol Ther 1992;19:161–77.

15. Schramm W, Smith RH, Craig PA, Kidwell DA. Drugs of abuse in saliva: a review. J Anal Toxicol 1992;16:1–9.

16. Drobitch RK, Svensson CK. Therapeutic drug monitoring in saliva: an update. Clin Pharmacokinet 1978;3:365–79.

17. Cone EJ. Saliva testing for drugs of abuse. Ann N Y Acad Sci 1993;694:91–127.

18. Jenkins AJ, Oyler JM, Cone EJ. Comparison of heroin and cocaine concentrations in saliva with concentrations in blood and plasma. J Anal Toxicol 1995;19:359–74.

19. Moolchan ET, Cone EJ, Wstadik A, Huestis MA, Preston KL. Cocaine and metabolite elimination patterns in chronic cocaine users during cessation: plasma and saliva analysis. J Anal Toxicol 2000;24:458–67.

20. Kintz P, Cirimele V, Ludes B. Codeine testing in sweat and saliva with the drugwipe. Int J Legal Med 1998;111:81–4.

21. Peroutka SJ, Newman H, Harris H. Subjective effects of 3,4-methylenedioxymethamphetamine in recreational users. Neuropsycopharmacology 1988;1:273–7.

22. Wright JD, Pearl L. Knowledge and experience of young people regarding drug misuse, 1969–94. BMJ 1995;310:20–4.

23. Samyn N, Van Haeren C. On-site testing of saliva and sweat with drugwipe and determination of concentration of drugs of abuse in saliva, plasma and urine of suspected users. Int J Legal Med 2000;113:150–4.

24. Wan SH, Matin SB, Azarnoff MD. Kinetics, salivary excretion of amphetamine isomers, and effect or urinary pH. Clin Pharmacol Ther 1978;23:585–90.

25. Kintz P, Samyn N. Determination of "Ecstasy" components in alternative biologic specimens. J Chromatogr B 1999;733:137–43.

26. Kintz P. Excretion of MBDB and BDB in urine, saliva and sweat following single oral administration. J Anal Toxicol 1997;21:570–5.

27. Schmid B, Bircher J, Preisig R, Küpfer A. Polymorphic dextromethorphan metabolism: co-segregation of oxidative o-demethylation with debrisoquin hydroxylation. Clin Pharmacol Ther 1985;38:618–24.

28. Mas M, Farré M, de la Torre R, Roset PN, Ortuño J, Segura J, et al. Cardiovascular and neuroendocrine effects and pharmacokinetics of 3,4-methylenedioxymethamphetamine in humans. J Pharmacol Exp Ther 1999;290:136–45.

29. Camí J, Farré M, Mas M, Roset PN, Poudevida S, Mas A, et al. Human pharmacology of 3,4-methylenedioxymethamphetamine ("ecstasy"): psychomotor performance and subjective effects. J Clin Psychopharmacol 2000;4:455–66.

30. Ortuño J, Pizarro N, Farré M, Mas M, Segura J, Camí J, et al. Quantification of 3,4-methylenedioxymethamphetamine and its metabolites in plasma and urine by gas chromatography with nitrogen-phosphorus detection. J Chromatogr B 1999;723:221–32.

31. de la Torre R, Farré M, Roset PN. Hernández López C, Mas M, Ortuño J, et al. Pharmacology of MDMA in humans. Ann N Y Acad Sci 2000;914:225–37.

32. O'Neal CL, Crouch DJ, Rollins DE, Fatah A, Cheever L. Correlation of saliva codeine concentrations with plasma concentrations after oral codeine administration. J Anal Toxicol 1999;23:452–9.

33. Baselt RC, Cravey RH. Disposition of toxic drugs and chemicals in man, 4th ed. Foster City, CA: Chemical Toxicology Institute, 1995:44, 475.

34. Kato K, Hillsgrove M, Weinhold L, Gorelick DA, Darwin WD, Cone EJ. Cocaine and metabolite excretion in saliva under stimulated and nonstimulated conditions. J Anal Toxicol 1993;17:338–41.

35. Liechti ME, Gamma A, Vollenweider FX. Gender differences in the subjective effects of MDMA. Psychopharmacology 2001;154:161–8.

36. Arneberg P, Storhaug K, Sandvik L. Effect of a slow release transcutaneous scopolamine application on salivary flow, pH, buffering action, and salivary levels of streptococcus mutants and lactobacilli. Scand J Dent Res 1989;97:408–14.

37. Cook CE, Jeffcoat AR, Hill JM, Pugh DE, Patetta PK, Sadler BM, et al. Pharmacokinetics of methamphetamine self-administered to human subjects by smoking *S*-(+)methamphetamine hydrochloride. Drug Metab Dispos 1993;21:717–23.

38. de la Torre R, Farré M, Ortuño J, Mas M, Brenneisen R, Roset PN, et al. Non-linear pharmacokinetics of MDMA ('ecstasy') in humans. Br J Clin Pharmacol 2000;49:104–9.

On-Site Testing of 3,4-Methylenedioxymethamphetamine (Ecstasy) in Saliva with Drugwipe and Drugread: A Controlled Study in Recreational Users, *Simona Pichini,[1,2] Mònica Navarro,[2] Magí Farré,[2,3] Jordi Ortuño,[2] Pere Nolasc Roset,[2] Roberta Pacifici,[1] Piergiorgio Zuccaro,[1] Jordi Segura,[2,4] and Rafael de la Torre[2,4*]* [1] Clinical Biochemistry Department, Istituto Superiore di Sanità, 00161 Rome, Italy; [2] Department of Pharmacology, Institut Municipal d'Investigació Mèdica (IMIM), E-08003 Barcelona, Spain; [3] Universitat Autònoma de Barcelona, E-08193 Barcelona, Spain; and [4] Universitat Pompeu Fabra, E-08003 Barcelona, Spain; * address correspondence to this author at: Drug Research Unit, Department of Pharmacology, Institut Municipal d'investigació Mèdica (IMIM), C/Doctor Aiguader 80, E-08003 Barcelona, Spain; fax 34-93-2213237, e-mail rtorre@imim.es)

Saliva is an alternative biologic specimen for drugs-of-abuse testing with several advantages over conventional matrices such as blood and urine *(1, 2)*, e.g., weak bases tend to concentrate in saliva because its pH is usually more acidic than the pH of plasma. The most important advantage of saliva is the ease of sample collection. Specimens can be obtained in a matter of minutes under direct observation and without embarrassment to the donor. Special devices have recently been introduced that allow analysis of saliva at the site of specimen collection for on-site screening tests. Commercially available on-site devices include two multitest electronic readers, the Cozart Rapidscan® (Abingdon) and the Avitar OralScreen® (Avitar Inc.), and one single-test visual device, the Drugwipe® (Securetec). Drugwipe is the only on-site test on saliva for which results of clinical studies have been published *(3, 4)*. Briefly, Drugwipe is an immunochromatographic test strip, based on the Frontline urine test strip from Boehringer Mannheim (F. Hoffmann-La Roche) *(5)*. A pink color in the test window indicates the presence of the analyte to which the test is specifically addressed, and different devices are needed for detection of each class of drugs of abuse. Although Drugwipe was designed to be read visually, evaluation of color intensity may be highly subjective, and easy read-out of the resulting coloration may be hindered by poor light conditions. For this reason, a Drugread® hand photometer has recently been developed. Drugread measures, in a reflectometric mode through a photodiode, the absorbance of the monochromatic light produced by gold antibody conjugates in the read-out area of the Drugwipe. Drugread translates the color intensity in the read-out window into a numeric value (arbitrary units) in the range of 300-2500. To date, no definitive threshold has been established for differentiating samples containing an analyte under investigation from samples not containing the substance.

Recreational use of 3,4-methylenedioxymethamphetamine (MDMA; "ecstasy"), either alone or in combination with other drugs, such as alcohol and cannabis, has become increasingly popular among young people *(6)*. Several cases of acute intoxication leading to death have been reported, and law enforcement agencies are increasingly interested in roadside on-site testing of potentially intoxicated drivers *(4)*. We evaluated the suitability of saliva testing of MDMA with the Drugwipe "amphetamines" and Drugread in individuals administered a single oral dose of 100 mg of MDMA.

Eight healthy volunteers who were recreational MDMA users gave their written informed consent to participate in a randomized, double-blind, crossover balanced with placebo clinical trial. The volunteers participated as outpatients in two experimental sessions, separated by a 1-week washout period, held in a controlled indoor setting. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board of our hospital and authorized by the Spanish Health Authorities (AEM 98/532).

Each participant was given a single dose of 100 mg of MDMA or placebo by the oral route, as white soft-gelatin capsules. Saliva drug testing with the Drugwipe analytic device was performed on site, in the clinical trials unit. The device was wiped for 10 s on the tongue of each participant at 0 time (predose) and at 1.5, 4, 6, 10, and 24 h after MDMA administration. At the same time points, participants provided a sample of saliva (1–2 mL) by spitting into a plastic tube; samples were stored immediately at −20 °C. Samples were analyzed for MDMA and its metabolites by gas chromatography–mass spectrometry (GC-MS) as described previously *(7)*. Because a preliminary evaluation of results showed quite relevant discrepancies between the two analytic approaches, saliva samples were reanalyzed with the Drugwipe test in the laboratory by application of a volume of saliva on the test pad. At the time of testing in the laboratory, the first prototypes of the Drugread hand photometer were available. Thus, the Drugread was used contextually to read the detection field for the Drugwipe once the test was performed. Laboratory personnel who read tests with the Drugwipe were unaware of the GC-MS results.

MDMA consumption could already be detected in saliva, by wiping the Drugwipe over the tongue, at 1.5 h and as long as 10 h after drug administration (Fig. 1A). However, although the GC-MS data showed that volunteers had salivary MDMA concentrations in the range of thousands of micrograms per liter in the first hours after drug administration (mean values, 3375.6 and 1762.7 μg/L at 1.5 and 4 h, respectively) *(7)*, direct application of the device on the tongue produced one negative result and four negative results at 1.5 and 4 h after drug use, respectively. The positivity of the test decreased noticeably at 6 and 10 h after MDMA administration.

The most reasonable explanation for the high number of negative results was the insufficient amount of saliva collected from the tongues of volunteers by the device. To verify this hypothesis, we repeated the test in the laboratory, applying a preselected volume of 2 μL of saliva to the device. This volume was the largest volume that could

be completely absorbed by the Drugwipe test pad. Once run, the test was read visually and then with the Drugread hand photometer. Results obtained with Drugwipe and Drugread in this phase of the study were compared with the MDMA concentrations measured by GC-MS in saliva samples *(7)*.

The Drugwipe performed better when a preselected volume of saliva sample was applied to the pad than when the pad was wiped directly on the tongue (Fig. 1B): all participants gave a positive result at 1.5 and 4 h after drug administration, and at 6 h after treatment only one of eight participants gave a negative result. This individual had a salivary MDMA concentration of 414.4 μg/L by GC-MS, which was the lowest among all of the volunteers at that time.

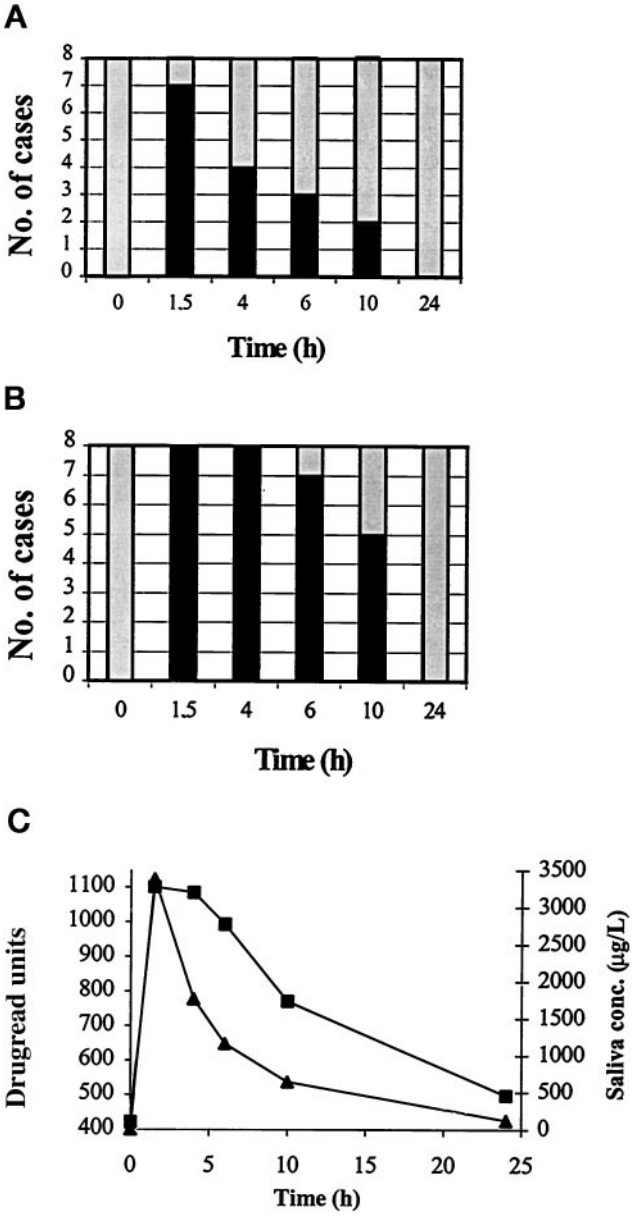

Fig. 1. Performance of the Drugwipe test.

(*A*), Drugwipe directly applied on the tongues of eight volunteers who had received 100 mg of MDMA. (*B*), Drugwipe used in a laboratory setting; 2 μL of saliva were applied to the device. For *A* and *B*, ■, positive cases; ▨, negative cases. (*C*), Drugwipe used in combination with the Drugread hand photometer. A comparison of time curves of MDMA in saliva measured by the Drugwipe in combination with the Drugread (■) and by GC-MS (▲) is also shown.

At 10 h after administration of MDMA, it was still possible to detect consumption in five of the eight volunteers. The three individuals who had a negative result always had salivary MDMA concentrations <400 μg/L by GC-MS, whereas the other five always had concentrations >450 μg/L. At 24 h, no positive results were reported, as was the case with direct application of the device on the tongue. At that time, the mean salivary MDMA concentration in the eight volunteers was 126.2 μg/L (range, 27.7–318.0 μg/L).

On the other hand, when the Drugwipe was applied to salivary samples from the placebo group, a faint color change in the read-out window of the device was seen in some cases. The same problem had occurred in a previous use of the Drugwipe with sweat samples of individuals treated with MDMA or placebo *(8)* and was attributed to the previously described presence of endogenous amines that may interfere with the Drugwipe test for amphetamines *(9)*.

On the basis of this previous experience, samples were classified as negative, but interpretation of some results remained difficult. The Drugread hand photometer was useful for solving problems of visual interpretation when reading the test window of Drugwipe. In fact, Drugread measurements in arbitrary units (mean ± SD; n = 8) were significantly different (Student *t*-test, $P < 0.001$) when results obtained in the placebo group at 1.5, 4, and 6 h (519.1 ± 116.7, 497.1 ± 118.1, and 476.5 ± 65.7 arbitrary units, respectively) were compared with those obtained in individuals given MDMA at 1.5 h (1100.0 ± 177.4 units), 4 h (1083.7 ± 217.4 units), and 6 h (992.9 ± 205.1 units) after drug administration. Furthermore, mean Drugread readings presented a time course profile similar to the mean time–concentration curve for MDMA in saliva measured by GC-MS (Fig. 1C). The apparent slower disappearance rate in the Drugread signal was probably attributable more to a saturation effect in Drugwipe test pad coloration (ceiling effect) than to the contribution of MDMA metabolites. In fact, MDMA was reported as the principal analyte that could be detected in saliva, whereas its principal metabolites were found only in minute amounts *(7)*.

The Drugwipe was initially developed for detecting drugs of abuse on surfaces and was subsequently applied to sweat and only recently to saliva *(3, 4)*. Nevertheless, the procedure for sample collection on the surface of the tongue does not appear to be adequate for the performance of on-site saliva testing of amphetamines and may need to be redefined. In addition to potential technical problems in the design of the device, the pharmacologic properties of amphetamines may hinder the test. MDMA impairs salivary flow, producing a sympathetic constriction of the salivary bed *(7)* and making saliva procurement difficult after a surface wiping procedure.

On the basis of observations made in the present study, we recommend that direct wiping be avoided, preferring application of an established volume of saliva that could allow delivery of a sufficient quantity to the test pad, which can be easily done "on site" by collecting saliva and applying it to the test pad. In the future, manufacturers of Drugwipe may want to standardize batch-to-batch devices to preselected concentrations for amphetamine-related drugs, taking into account the differences in doses and routes of administration currently in use as well as the time window to be covered by the analytic device in relation to peak effects of the drug. In fact, although a limited number of individuals participated in this study, the present results show that the Drugwipe in combination with the Drugread adequately detected MDMA in saliva in the first 6 h after administration. On the other hand, the analytic device gives a negative response in a range of salivary concentrations down to ~450 μg/L (0.9 ng of MDMA in 2 μL of saliva applied to the test pad). These concentrations can be found in individuals ~6–10 h after the administration of 100 mg of MDMA, corresponding to a mean range of 80–120 μg/L in plasma and 3–12 mg/L in urine *(10, 11)*. Conversely, the 0–6 h time window is the period of maximal pharmacologic effects of MDMA. In this time interval, an individual is at highest risk of psychomotor impairment that may have consequences in some demanding tasks, such as driving. Six hours after MDMA ingestion, although the drug is still present in several biologic fluids, most subjective and physiologic effects (i.e., cardiovascular function) return to basal conditions *(10)*. Hence, if the objective of on-site saliva testing is not only to detect the consumption of a given drug but also to determine whether an individual is under the effects of the drug, on-site saliva testing with the Drugwipe coupled with the Drugread fits that purpose. Ultimately, appropriate confirmation with a reference chromatographic method for saliva samples should be performed.

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References

1. Pichini S, Altieri I, Zuccaro P, Pacifici R. Drug monitoring in non conventional biological fluids and matrices. Clin Pharmacokinet 1996;30:211–28.
2. Kidwell D, Holland J, Athanaselis S. Testing for drugs of abuse in saliva and sweat. J Chromatogr B 1998;713:111–35.
3. Kintz P, Cirimele V, Ludes B. Codeine testing in sweat and saliva with the Drugwipe. Int J Legal Med 1998;111:81–4.
4. Samyn N, Van Haeren C. On-site testing of saliva and sweat with Drugwipe and determination of concentration of drugs of abuse in saliva, plasma and urine of suspected users. Int J Legal Med 2000;113:150–4.
5. Wennig R, Moeller MR, Haguenoer JM, Marocchi A, Zoppi F, Smith BL, et al. Development and evaluation of immunochromatographic rapid tests for screening of cannabinoids, cocaine, and opiates in urine. J Anal Toxicol 1998;22:148–55.
6. Wright JD, Pearl L. Knowledge and experience of young people regarding drug misuse, 1969–94. BMJ 1995;310:20–4.
7. Navarro M, Pichini S, Farré M, Ortuño J, Roset PN, Segura J, de la Torre R. Usefulness of saliva for measurement of 3,4-methylenedioxymethamphetamine and its metabolites: correlation with plasma drug concentrations and effect of salivary pH. Clin Chem 2001;47:1788–95.
8. Pacifici R, Farré M, Pichini S, Ortuño J, Roset PN, Zuccaro P, et al. Sweat testing of MDMA with the Drugwipe analytical device: a controlled study with two volunteers. J Anal Toxicol 2001;25:144–6.
9. Rörich J, Schmidt K, Bratzke H. Application of the novel immunoassay TRIAGE-TM to a rapid detection of antemortem drug abuse. J Anal Toxicol 1994;18:407–14.
10. Mas M, Farré M, de la Torre R, Roset PN, Ortuño J, Segura J, Camí J. Cardiovascular and neuroendocrine effects and pharmacokinetics of 3,4-methylenedioxymethamphetamine in humans. J Pharmacol Exp Ther 1999;290:136–45.
11. de la Torre R, Farré M, Roset PN. Hernández López C, Mas M, Ortuño J, et al. Pharmacology of MDMA in humans. Ann NY Acad Sci 2000;914:225–37.

Addition of Quantitative 3-Hydroxy-Octadecanoic Acid to the Stable Isotope Gas Chromatography–Mass Spectrometry Method for Measuring 3-Hydroxy Fatty Acids,

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Mitochondrial fatty acid oxidation (FAO) is a catabolic pathway that supplies energy for the normal physiologic functioning of many tissues when glucose is unavailable, and it also supplies energy for some tissues even when glucose is available *(1, 2)*. The FAO pathway is complex and not fully understood. Quantitative measurement of the concentrations of 3-hydroxy-fatty acids (3-OHFAs) in plasma or serum samples from individuals who are suspected of having a deficiency in FAO, especially in the enzyme step involving the L-3-hydroxyacyl-CoA-dehydrogenases, is a useful tool to aid in diagnosis *(3, 4)*. This study adds the quantitative measurement of 3-hydroxy-octadecanoic acid (3-OH-C18) to the previously reported assay *(4)* that measures the six shorter chain-length FAO intermediates, 3-hydroxy-hexanoic acid (3-OH-C6), 3-hydroxy-octanoic acid (3-OH-C8), 3-hydroxy-decanoic acid (3-OH-C10), 3-hydroxy-dodecanoic (3-OH-C12), 3-hydroxy-tetradecanoic acid (3-OH-C14), and 3-hydroxy-hexadecanoic acid (3-OH-C16).

3-OH-C18 was synthesized by the method of Jones et al. *(4)*, with the following changes. The precursor for 3-OH-C18 was not commercially available; thus the 3-OH-C18 precursor, hexadecanal, was synthesized first by the method of Landini et al. *(5)*. A saturated solution of potassium chromate (0.55 mol/L) in 300 mL/L aqueous sulfuric acid was reacted with 0.01 mol of 1-hexadecanal dissolved in 60 mL of methylene chloride in the presence of 0.001 mol of tetrabutylammonium hydrogen sulfate as

Usefulness of Sweat Testing for the Detection of MDMA after a Single-Dose Administration*

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Abstract

Nine healthy male subjects and recreational users of 3,4-methylenedioxymethamphetamine (MDMA) participated in a study aimed to assess the usefulness of sweat testing for the detection of MDMA after a single 100-mg dose. Sweat was collected for up to 24 h with the PharmChek sweat patches from which drugs were eluted and then analyzed by immunoassay and gas chromatography–mass spectrometry using deuterated internal standards. The usefulness of a rapid onsite test, the Drugwipe immunochemical strip test, was also assessed. In the sweat patches, MDMA was detected as early as 1.5 h after consumption and peaked at 24 h. Intersubject variability was large; peak MDMA concentrations for the same dose varied in magnitude 30-fold. MDMA concentrations ranged between 3.2 and 1326.1 ng/patch. Only traces of the minor metabolite 3,4-methylenedioxyamphetamine were detected. In all subjects, the onsite test with the Drugwipe was positive at 1.5 h (peak time of MDMA plasma concentration). However, few false-negative results (18%) appeared in the first 6 h after administration. Both sweat patch testing and the onsite sweat strip test may find useful application for noninvasive monitoring of MDMA abuse in sweat.

Introduction

Recreational use of 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy"), either alone or in combination with other drugs such as alcohol and cannabis, has become increasingly popular among young people at night-time dance parties (1). Several cases of acute intoxication resulting in death (2) and law enforcement agencies, interested in roadside onsite testing of potentially intoxicated drivers (3) made MDMA detection in biological fluids an important issue for monitoring whether individuals have consumed and/or have been acting under the effect of the drug (1,4–6). Although blood and urine sampling have been consistently used for this purpose, drawbacks associated with these bodily fluids (invasive procedures, privacy issues, availability of techniques to adulterate positive results) have limited their utility (7).

Sweat and saliva are alternative biological substrates for the analysis of drugs of abuse (8). In fact, saliva is the only fluid that has successfully been used as an alternative to blood in several pharmacokinetic and pharmacotoxicological studies (9,10). Recently, saliva has been applied to measurement of MDMA and its metabolites in a controlled-administration study (5) in which it was demonstrated that the likelihood for a subject being under the pharmacologic effects of MDMA is high when the drug is detected in salivary specimens in amounts greater than 1000 ng/mL.

Sweat is another alternative matrix for drugs-of-abuse testing that may provide an additional tool for monitoring drug use (11). Although the use of sweat for drug testing has been hampered by difficulties in sample collection and sensitivity of analytical methods (12), successful sweat testing for several drugs of abuse (7,11,13–17) has been accomplished because of substantial advances facilitating sample collection and improving the accuracy of diagnostic techniques. These include development of the sweat patch technology by PharmChem Laboratories (Menlo Park, CA) (16) and availability of onsite testing devices, such as Drugwipe from Securetec (Ottobrunn, Germany) (18,19). Sweat patches are applied to the skin for a number of hours or days. The patch design allows oxygen, carbon dioxide, and water vapor to escape while the nonvolatile components, including drugs of abuse, are retained in the absorbent pad (10). Because the patches can be worn for up to one week and no drug degradation seems to occur during this time interval for amphetamines (15), the window of drug detection can be longer than that provided by urine testing (16). Further extraction of drugs from the patch is required, but analyses are relatively easy, given the simple chemical composition of sweat compared to blood or urine. On the other hand, Drugwipe immunochromatographic test strip has been recently applied to

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onsite sweat analysis for drugs of abuse at the roadside (19).

Although a variety of drugs of abuse, and among them amphetamines and MDMA, have been detected in sweat (7,11,13–17,20–23), little information is available on the excretion profile of amphetamines and related compounds in sweat after controlled administration (20,22,24).

We investigated the presence and the concentration of MDMA and its main metabolites [3-methoxy-4-hydroxymethamphetamine (HMMA) and 3,4-methylenedioxyamphetamine MDA] in sweat by gas chromatography–mass spectrometry (GC–MS), the time course and the interindividual variability of MDMA excretion, the suitability of immunoassay screening and the feasibility of a rapid onsite test to detect MDMA in sweat. Sweat samples were obtained from individuals participating in a clinical trial involving the controlled administration of 100 mg MDMA.

Experimental

Chemicals and materials

Drugwipe "amphetamines" was supplied by Syen (Gardigiano, Venezia, Italy) and used according to the instructions of the manufacturer (Securetec, Ottobrunn, Germany). The PharmChek sweat patches were provided by PharmChem Laboratories. The patches consisted of a medical-grade cellulose blotter paper collection pad with an area of 15.4 cm^2, covered by a thin layer of polyurethane and acrylate adhesives. Immunoassay was performed by applying the STC Micro-plate EIA kit for Methamphetamine (STC Diagnostics, Bethlehem, PA). Methanol was analytical grade (Merck, Darmstadt, Germany), and water was Milli-Q grade (Millipore, Molsheim, France). C_{18} solid-phase extraction columns (SPE) were from Biochemical Diagnostics, Inc. (NY) and Bond Elut Certify SPE column from Varian (Harbor City, CA). Potassium dihydrogen phosphate, sodium chloride, potassium hydroxide, ethylacetate, acetic acid glacial 100%, ammonia solution 25% were provided by Merk. Isotonic solution was prepared adding 0.9 g sodium chloride to 100 mL Milli-Q grade water. *N*-Methylbis(trifluoroacetamide), GC-grade (MBTFA) was supplied by Macherey-Nagel (Düren, Germany). All drugs and deuterated standards were purchased from Radian (now Cerilliant, Austin, TX).

Subjects

Male subjects were recruited by "word of mouth". Eligibility criteria required the recreational use of MDMA on at least five occasions. Each participant underwent a general physical examination, routine laboratory tests, urine analysis, and a 12-lead electrocardiogram. Their drug use habits were recorded in an interview with a standardized questionnaire.

Nine subjects were included in the study. The participants had a mean age of 23.9 years, mean weight of 72.2 kg, and mean height of 177.2 cm. Body surface area of the subjects, calculated using the formula of Dubois and Dubois (25), showed a mean value of 1.9 m^2, ranging from 1.7 to 2.1 m^2.

All the subjects declared use of MDMA. Subjects were phenotyped for CYP2D6 activity using dextromethorphan as drug-probe. The dextromethorphan/dextrorphan ratio was used to classify subjects as poor or extensive metabolizers (26). All participants were extensive metabolizers.

All subjects gave their written informed consent before inclusion in the study and were economically compensated for inconveniences caused by their participation in the study. The study was conducted in accordance with the Declaration of Helsinki, approved by the Ethical Committee of our institution (CEIC-IMAS), and authorized by the 'Agencia Española del Medicamento' number 98/532) of the Spanish Ministry of Health.

The study design was double-blind, randomized, cross-over, and placebo-controlled. Subjects participated as outpatients in two different randomly assigned 10-h experimental sessions, one week apart, in which they were given single doses of 100 mg MDMA or placebo by the oral route. Subjects were requested to abstain from consumption of any drug of abuse seven days before the start and during the study period, and drug testing in urine (Abbott Axsym System, Abbott Laboratories, North Chicago, IL) was performed before each experimental session for opioids, cocaine, cannabis, and methamphetamine Subjects were also tested 24 h after the administration of MDMA or placebo to exclude self-administration of the drug in the evening when subjects were not under medical supervision. At the time that the study was performed, the Abbott test for methamphetamine was the most reliable for the detection of MDMA consumption. As stated before, the inclusion of subjects in the experimental sessions, required a negative urine test by immunoassay. In addition, urine samples collected at pre-dose were analysed as well as those collected during the clinical trial by GC–MS. None of the urine samples collected at pre-dose tested positive (MDMA and metabolites were absent).

Pharmaceutical-grade MDMA and placebo (containing only lactose) were prepared by the Pharmacy of Hospital del Mar (Barcelona, Spain) as white soft-gelatin capsules and administered in the fasting state with 120 mL of tap water (two capsules each time).

Onsite sweat testing with Drugwipe

Subjects were swabbed in their armpit for 10 s at time 0 (pre-dose) and at 1.5, 4, 6, 10, and 24 h after MDMA administration. Sweat was collected with the wiping pad of the test kit for amphetamines moistened with 20 mL tap water. The other part of the detection element was an adsorbent pad, which had to be dipped into tap water to initiate the immunochromatographic reaction. In the presence of MDMA or metabolites, the detection field of Drugwipe "amphetamines" changed its color from white to pink, depending on the amount of drug collected. According to the manufacturer, the coloration of the whole test window was not necessary, but a line on the edge could be sufficient to qualify a positive result. Coloration remained stable for at least 1 h. Results were classified as positive or negative by two independent readers. The Drugwipe information sheet reported a sensitivity for methamphetamine of 10 ng, as the minimum quantity of drug on the collection pad giving rise to a positive immune reaction. The following sub-

stances are reported to generate a positive test result if present in the specified concentrations: *d*-amphetamine (300 ng/mL), *d*-methamphetamine (300 ng/mL), MDA (250 ng/mL), MDMA (250 ng/mL), *l*-amphetamine (100,000 ng/mL), and *l*-methamphetamine (25,000 ng/mL). Additional cross-reactivity information can be found in the information sheet provided by the manufacturer.

Sweat collection

Six patches were applied to the back of each participant after the skin was cleaned with a 70% isopropyl alcohol swab and removed at time 0 (pre-dose), and 1.5, 4, 6, 10, and 24 h postadministration by pulling an edge from the adhesive layer, taking care not to touch the absorbent pad. After removal, the patch was labeled and stored in plastic bags at –20°C until analysis. Sweat patches from the placebo condition, which all tested negative for the presence of MDMA by GC–MS, were used as drug-free blank samples. Furthermore, additional drug-free patches were obtained from non-consumers who wore the sweat patches on their backs for a period of 24 h.

Extraction of analytes from sweat patches

Sweat patches were allowed to thaw at room temperature, and the absorbent pad was removed with clean tweezers. Then the pad was placed in a testing tube containing 5 mL 0.1M phosphate potassium buffer (pH 6) and shaken for 30 min to extract the drug.

Buffer was divided in two aliquots, the first (1-mL aliquot) for drug screening by immunoassay and the second (4-mL aliquot) for GC–MS analysis.

The first aliquot of patch buffer extract was passed through a C_{18} SPE column. Columns were preconditioned with 2 mL methanol and 2 mL water. After passing the sample, they were washed with 2 mL water and the patch content was eluted using 2 mL methanol. Extraction recoveries obtained with patches worn by control individuals and spiked with analytes under investigation were higher than 95% (for MDMA 10 ng/patch, $n = 5$). Methanol eluate was evaporated to dryness and reconstituted with 1 mL isotonic solution.

The second aliquot, was spiked with 25 µL MDMA-d_5 (10 µg/mL isotonic solution) and 30 µL MDA-d_5 (1 µg/mL isotonic solution) and underwent a solid-phase extraction using Bond-Elut Certify Columns. Columns were preconditioned with 2 mL methanol and 2 mL 0.1M phosphate potassium buffer (pH 6), washed with 1 mL 1M acetic acid and 6 mL methanol once the sample had been passed through, and MDMA, and metabolites were eluted with 2 mL of ethyl acetate containing 2% ammonia solution. After addition of 20 µL MBTFA to prevent drug losses, eluates were evaporated to dryness at 40°C under a nitrogen stream. Residues were reconstituted and derivatized with 30 µL of MBTFA at 70°C for 45 min to obtain trifluoroacetyl derivatives of the analytes.

Immunoassay

Because no immunoassays were available for screening of MDMA in sweat at the time of the study, the STC Micro-plate EIA kit for qualitative/semiquantitative determination of methamphetamine in serum was used, in accordance with previous experiments on other drugs of abuse screening in sweat (15,16). Cross-reactivity of the EIA kit for methamphetamine to MDMA (at a concentration of 30 ng/mL) was 1513% and that of MDA (at a concentration of 5000 ng/mL) was 2.8%. Cross-reactivity for HMMA was not reported. Cross-reactivity for other sympathomimetic amines was as follows: 1.3% for *d*-amphetamine (at a concentration of 10,000 ng/mL); < 1% for *l*-ephedrine (at a concentration of 25,000 ng/mL); 2.5% for *l*-amphetamine (at a concentration of 10,000 ng/mL); 9.3% for *l*-methamphetamine (at a concentration of 2500 ng/mL); 65.2% for MDEA (at a concentration of 150 ng/mL); 1.3% for mephentermine (at a concentration of 25,000 ng/mL); 0.12% for phenylethylamine (at a concentration of 100,000 ng/mL); and 5.7.% for pseudoephedrine (at a concentration of 2500 ng/mL). Patch eluate isotonic solution (25 µL) was added to each well along with labelled enzyme and allowed to incubate for 30 min at room temperature, in the darkness. Following competition of binding to antibody sites immobilized on the surface of the well, each well was washed six times with distilled water. The color produced after a 30-min incubation was measured spectrophotometrically at 450 nm and at 630 nm. The absorbance was inversely proportional to the quantity of MDMA in the specimen. The calibrators and controls used consisted of isotonic solution from sweat pad elution with MDMA added at 0, 0.2, 1, 2, 5, 10, 15, 20, and 25 ng/patch.

The limit of detection (LOD) was obtained from the mean absorbance of the drug-free samples (A_0). Drug-free patches extracts ($n = 65$) from healthy subjects ($n = 11$) plus those obtained in the placebo condition of the clinical trial from the nine subjects at each time point ($n = 54$) were used for the mean and standard deviation (SD) calculations of this absorbance. Three times the standard deviation from this mean value was taken as a background noise of the analytical system. The LOD was estimated by interpolating $A_0 – 3SD$ in the calibration curve, and the LOQ was estimated by interpolating $A_0 – 10SD$ in the calibration curve (15). A cut-off concentration was also estimated from the mean zero absorbance (A_0) minus six times the SD as previously calculated. This estimated cut-off concentration was verified by pooling all patch results obtained from subjects in the placebo conditions ($n = 54$). A mean value and a standard deviation were calculated. The cut-off concentration in that case was calculated as 6 times this standard deviation.

GC–MS

The identification and quantitation of MDMA and eventual metabolites in sweat patches was performed using an HP6890 GC coupled to a model HP5973 quadrupole MS (Agilent, Palo Alto, CA). The separation of trifluoroacetyl derivatives of the analytes was carried out with a cross-linked 5% phenyl-methylsilicone capillary column, 12-m × 0.2-mm i.d. and 0.33-mm film thickness (Ultra-2, Hewlett-Packard). The samples were injected in splitless mode and helium was used as carrier gas at a flow rate of 1.2 mL/min (measured at 180°C). The MS was operated in the electron ionization and selected ion monitoring acquisition mode. Qualifying ions were *m/z* 154, 162, and 288 for MDMA *N*-TFA derivative; *m/z* 161, 134, and 274 for MDA *N*-TFA derivative; *m/z* 154, 110, and 260 for (HMMA)-O-TFA;

N-TFA derivative, *m/z* 158, 164, and 294 for MDMA-d_5 *N*-TFA derivative; and *m/z* 136, 167, and 280 for MDA-d_5*N*-TFA derivative. Ion ratios acceptance criterion was a deviation ≤ 20%.

Ions *m/z* 154 for MDMA *N*-TFA derivative, and HMMA-O-TFA, N-TFA derivative; *m/z* 162 for MDA *N*-TFA derivative; *m/z* 158 for MDMA-d_5 *N*-TFA derivative; and *m/z* 167 for MDA-d_5 *N*-TFA derivative were selected for quantitation.

This methodology has previously been validated for plasma and saliva samples, and it was reapplied for sweat patch samples in the present study (24,27).

Calibration curves were prepared in drug-free sweat patches by adding appropriate volumes of working isotonic solutions of the analytes under investigation. Drug-free patches were from study subjects administered with placebo (one of the treatment study conditions) and from healthy subjects wearing patches for 24 h. Peak-area ratios for each compound and its internal standard were used for calculation. Curves were linear over the 10–400 ng/patch concentration range for MDMA and HMMA and over the 2.5–40 ng/patch concentration range for MDA. Samples containing concentrations over the working ranges were repeated after appropriate dilution with phosphate buffer.

Analytical recoveries were calculated by comparing the peak areas obtained when calibration samples were analyzed by adding the reference substances and the internal standards in the extract from drug-free sweat patch prior to and after the previously mentioned extraction procedure. Mean analytical recovery, measured from five replicates, was 74% for MDMA and 82% for HMMA (20 ng/patch concentration) and 70% for MDA (20 ng/patch concentration).

Four replicate analyses were performed with drug-free sweat patches spiked with low concentrations of analytes (25 ng/patch MDMA and HMMA and 3 ng/patch for MDA). The standard deviation derived from quantitative values (at the aforementioned concentrations) was used as a measure of the noise for the calculation of the limit of detection (LOD = 3 SD) and quantitation (LOQ = 10 SD).

Table I. Immunoassay and GC–MS Analysis of Sweat Patches and Drugwipe Onsite Sweat Test of the Nine Subjects Administered 100 mg MDMA Orally

	Subject 1					Subject 2					Subject 3				
	EIA-STC		MDMA	Drugwipe		EIA-STC		MDMA	Drugwipe		EIA-STC		MDMA	Drugwipe	
Time (h)	Pcbo (ng/patch)	MDMA (ng/patch)	GC–MS (ng/patch)	Pcbo	MDMA	Pcbo (ng/patch)	MDMA (ng/patch)	GC–MS (ng/patch)	Pcbo	MDMA	Pcbo (ng/patch)	MDMA (ng/patch)	GC–MS (ng/patch)	Pcbo	MDMA
0	0.0	0.0	0.0	–	–	0.0	0.0	0.0	–	–	0.0	0.0	0.0	–	–
1.5	0.0	9.9	DNQ*	–	+	1.7	32.8	44.3	–	+	0.3	90.4	175.3	–	+
4	0.0	13.9	12.2	–	+	0.0	170.8	113.7	–	–	0.0	99.3	554.9	–	+
6	0.0	5.2	6.7	–	–	0.2	100.3	84.6	–	–	1.6	441.5	946.7	–	+
10	0.0	37.0	25.8	–	–	0.0	34.2	79.8	–	–	0.7	350.1	845.5	–	+
24	0.0	784.1	906.7	–	–	0.1	215.4	269.7	–	–	0.0	995.6	1326.1	–	–
	Subject 4					**Subject 5**					**Subject 6**				
	EIA-STC		MDMA	Drugwipe		EIA-STC		MDMA	Drugwipe		EIA-STC		MDMA	Drugwipe	
Time (h)	Pcbo (ng/patch)	MDMA (ng/patch)	GC–MS (ng/patch)	Pcbo	MDMA	Pcbo (ng/patch)	MDMA (ng/patch)	GC–MS (ng/patch)	Pcbo	MDMA	Pcbo (ng/patch)	MDMA (ng/patch)	GC–MS (ng/patch)	Pcbo	MDMA
0	0.0	0.0	0.0	–	–	0.2	0.0	0.0	–	–	0.0	0.0	0.0	–	–
1.5	0.2	96.3	19.2	–	+	0.7	8.3	4.5	–	+	2.0	9.1	14.5	–	+
4	0.1	436.8	433.4	–	+	0.3	44.0	183.3	–	+	1.6	82.4	86.9	–	+
6	0.6	387.4	625.6	–	+	0.6	58.0	55.4	–	+	0.3	105.7	123.9	–	+
10	0.8	645.0	487.8	–	+	0.0	5.3	6.7	–	+	1.6	17.6	50.2	–	+
24	2.3	319.7	398.6	–	+	0.0	101.5	241.3	–	+	0.0	498.7	377.2	–	–
	Subject 7					**Subject 8**					**Subject 9**				
	EIA-STC		MDMA	Drugwipe		EIA-STC		MDMA	Drugwipe		EIA-STC		MDMA	Drugwipe	
Time (h)	Pcbo (ng/patch)	MDMA (ng/patch)	GC–MS (ng/patch)	Pcbo	MDMA	Pcbo (ng/patch)	MDMA (ng/patch)	GC–MS (ng/patch)	Pcbo	MDMA	Pcbo (ng/patch)	MDMA (ng/patch)	GC–MS (ng/patch)	Pcbo	MDMA
0	0.8	0.0	0.0	–	–	2.0	0.0	0.0	–	–	0.0	0.0	0.0	–	–
1.5	0.0	33.9	DNQ	–	+	0.8	34.9	3.2	–	+	0.0	5.2	27.2	–	+
4	0.3	9.4	6.5	–	–	1.4	48.3	29.2	–	+	1.9	61.3	33.6	–	+
6	0.5	28.7	14.9	–	+	0.0	27.8	184.0	–	+	0.1	57.0	21.6	–	–
10	2.8	22.6	9.4	–	+	1.1	152.8	65.2	–	+	0.0	4.8	4.3	–	–
24	0.0	56.4	42.5	–	–	0.0	209.1	1023.8	–	+	0.0	201.5	293.2	–	–

* DNQ, detected, but not quantitated.

LODs were 0.96 ng/patch for MDMA, 0.72 ng/patch for MDA, and 0.96 ng/patch for HMMA. LOQs were 3.2 ng/patch for MDMA, 2.4 ng/patch for MDA and 3.2 ng/patch for HMMA. Results that were under the LOQ but above the LOD were reported as detected-not quantified (DNQ).

Three replicates at three different concentrations of MDMA, HMMA, and MDA (12, 160, and 350 ng/patch for MDMA and HMMA and 3, 16, and 35 ng/patch for MDA) added to drug-free sweat patches were analyzed for the determination of intraassay precision and accuracy, and the interday precision and accuracy were determined for three independent experimental assays of the aforementioned replicates. These control samples (three concentration levels) were used daily to check the performance of the calibration curve.

Intraday precision (expressed as coefficient of variation for specific added target concentrations) and accuracy (expressed as percentage error of concentration found as compared with added target concentrations) were always lower than 4.1% for all the analytes under investigation. Similarly, interday precision and accuracy were lower than 5.5%.

Skin reabsorption of MDMA from the sweat patch

Preliminary results on MDMA in sweat showed an inflection in the kinetics at 10 h postadministration. This observation prompted a pilot study to evaluate the possibility that MDMA already incorporated in patches could be reabsorbed by the skin. A total amount of 200 ng of MDMA in isotonic solution was placed on patches and was applied on the back of two non-consumer subjects after their skin was cleaned with 70% isopropyl alcohol swabs. Four different patches were applied to each subject and then removed from the skin at 1.5, 4, 10, and 24 h after application and analyzed by GC–MS.

Results

Table I summarizes all the results obtained for immunoassay and GC–MS analysis of sweat patches and Drugwipe onsite sweat test of the nine subjects orally administered 100 mg MDMA.

Onsite sweat testing with Drugwipe

Figure 1 shows results obtained for onsite sweat testing with Drugwipe. Drugwipe could detect MDMA consumption in sweat already at 1.5 h and as long as 24 h after drug administration. Although all the subjects gave a positive result at 1.5 h after drug administration, at 4 h, two out of the nine subjects gave a negative result, and at 6 and 10 h after treatment, three subjects gave a negative result. At 24 h from MDMA treatment, it was still possible to detect consumption in three of the nine subjects. Theoretically, it would not be possible to compare results from Drugwipe with those of GC–MS. Indeed, GC–MS measured MDMA collected in the sweat patch during a certain time interval. On the contrary, Drugwipe test collected a punctual sample of sweat during the 10 s of Drugwipe application.

However, true and false positives and negatives for Drugwipe test were calculated, as reported by international literature (16), versus GC–MS results. Sensitivity of the device resulted to be 100% up to 24 h, and specificity was 81.5% in the first 6 h and dropped to 66.7% when considering all the results up to 24 h.

Immunoassay

The LOD was found to be 1.4 ng MDMA/patch. Using the LOD as positivity criterion to differentiate treatments (placebo vs. MDMA) in samples, 10 false-positive results (according to GC–MS) were observed in case of placebo samples (10 out of 54 samples). Two approaches to calculate cut-off concentrations were used as previously described. Using the calibration curve a cut-off concentration of 4.3 ng MDMA/patch, was estimated. From the pooled concentration data of subjects wearing patches in the placebo condition (mean ± SD, 0.5 ± 0.7 ng/patch) the cut-off calculated was 4.4 ng MDMA/patch. Both approaches for the calculation of cut-off concentrations gave identical results. By applying this threshold concentration (4.4 ng/patch), no false-positive or false-negative results were observed.

Positive results were observed at 1.5 h and as long as 24 h after single drug administration in sweat patches from all participants. All samples, screened by immunoassay irrespective of the positive or negative result, were subsequently analyzed by GC–MS. All samples showing results outside the dynamic range of the calibration curve were diluted accordingly with extracts from worn drug-free patches.

GC–MS

Figure 2A shows the time course of MDMA concentrations in sweat patches in a 24 h period for each of the nine subjects (mean values can be found in Figure 3). MDMA appeared in sweat prior to 1.5 h after drug administration, (mean = 41.2 ng/patch, range: 3.2–175.3 ng/patch) in seven subjects, but in two volunteers MDMA was detected for the first time in the 4-h postadministration sweat patch. Four of nine individuals presented a first peak concentration at 4 h following administration, (range: 12.2–183.3 ng MDMA/patch), and five subjects presented a peak at 6 h (range: 6.7–946.7 ng MDMA/patch). Following the peak concentration, at 10 h postadministration MDMA levels in sweat patch declined to a mean concentration of 175.0 ng MDMA/patch (range: 4.3–845.5 ng MDMA/patch). At 24 h after drug intake, the highest concentrations of MDMA in sweat patches were observed, with a mean value of 542 ng MDMA/patch and a range from 42.5 to 1326.1 ng MDMA/patch. Concentrations of MDMA encountered in the 24 h postadministration patches were used to estimate total amount of MDMA excreted in the 24 h as the ratio between area of the patch and total body surface area of each volunteer (Figure 4). Mean concentration of MDMA excreted in sweat was 0.6 mg (confidence interval 95%, 0.28–0.92 mg), which is equivalent to about 0.6% of the administered dose, approximating sweating at different body sites to the excretion obtained from the backs of individuals.

Intersubject variability of MDMA concentration in the sweat patches was large. MDMA concentration variability ranged from a factor of 30 at 24 h to a factor of 140 at 6 h. It should be noted that two of the nine subjects (subjects 3 and 4 at Figure 2A) had exceedingly high MDMA concentrations. These two individuals also showed high MDMA concentrations in other biological

fluids (plasma and saliva) after being administered 100 mg MDMA (5).

Sweat patch specimens were also analyzed for MDMA metabolites by GC–MS. HMMA, the major plasma metabolite, was not detected in sweat at an LOD of 0.96 ng/patch. On the other hand, MDA was found only in trace amounts in seven out of nine participants, and quantitation was not possible. However, in the two subjects with the highest MDMA concentrations, MDA could be quantitated from 6 to 24 h postadministration. Concentrations ranged from 24.8 and 18.1 ng MDA/patch at 6 h to 23.7 and 8.9 ng MDA/patch at 24 h postadministration (Figure 2B).

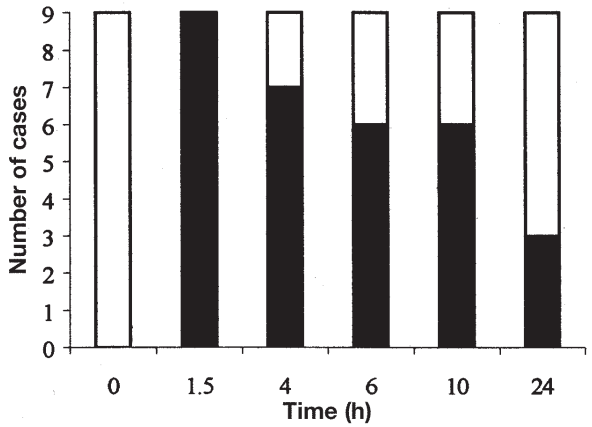

Figure 1. Performance of the Drugwipe onsite test directly applied for 10 s on the armpit of the nine subjects administered 100 mg MDMA.

Skin reabsorption of MDMA from the sweat patch

Figure 5 shows the results obtained in the experiment concerning skin reabsorption of MDMA from the sweat patch.

At 1.5 and 4 h after patch application on the back of two subjects, amounts of MDMA in the sweat patches remained almost unchanged. Conversely, at 10 and 24 h postapplication, in both subjects, a decrease in MDMA concentration in the patch was observed. In fact, the 10-h patch, from volunteer A, showed a 12% decrease in the amount of MDMA initially present, and this result was confirmed in the 24 h patch. Volunteer B, exercising

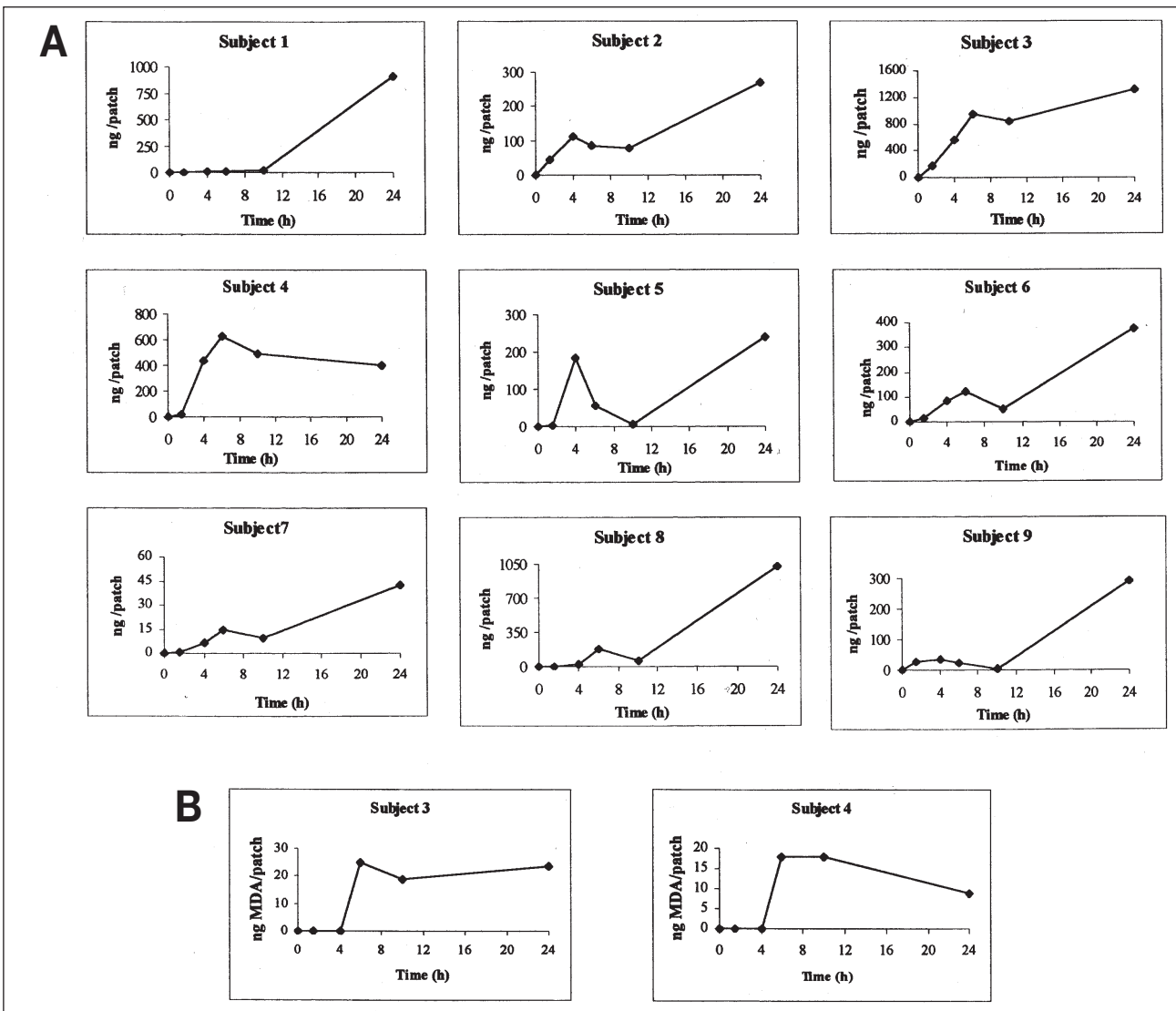

Figure 2. Concentration time profiles of MDMA contained in sweat patches applied to the back of the nine subjects and removed at different time intervals (A). Concentration time profiles of MDA contained in sweat patches from subjects 3 and 4 at different time intervals (B).

in the gymnasium, showed a 49% reduction of MDMA concentration in the 10-h postapplication patch, whereas in the subsequent 24-h patch, some recovery of MDMA concentration (22% over the previous value) but a net decrease (40%) over the initial amount spiked was observed.

Discussion

Drug testing in sweat through patches was originally designed as an approach of detecting drug consumption in subjects wearing them for a 7-day time period (7). In the present study, sweat patches have been used for a shorter period of time (up to 24 h) as a tool to monitor MDMA excretion in sweat and to evaluate the reliability of available onsite testing devices by immunoassay (i.e., Drugwipe).

The present results show for the first time that MDMA is excreted in sweat after a controlled administration of a single 100-mg dose. Excretion of an amphetamine analogue, *l*-dimethylamphetamine, in human sweat was first demonstrated in 1972 (20) and more recently other amphetamine derivatives have been detected in the sweat of drug users (15,19,22).

Drugs are generally incorporated into sweat by passive diffusion because of a concentration gradient in which only the free fraction of drug (unbound to proteins) diffuses through lipid membranes from plasma to sweat (8,20). Furthermore, MDMA is a basic drug with a pK_a around 9.9 [data derived from methamphetamine (28)] and because, in normal conditions, sweat is more acidic than blood (mean value of 6.3) and its normal pH is maintained under non-occlusive dressings (29), MDMA is converted to its ionized form and should accumulate in sweat (8). The excretion of a basic drug in sweat can be theoretically calculated by a modification of the Henderson-Hasselbalch equation (8). In the case of MDMA, the theoretical sweat/plasma ratio should be around 12, indicating a significant accumulation of the drug in the sweat matrix. Unfortunately, this hypothesis could not be verified in our study because the sweat patch, which loses water content during wear, did not allow the calculation of volume of sweat collected or accurate sweat pH. In any case, sweat patch technology permitted the monitoring of MDMA accumulation in sweat during the 24 h after administration. This information was used to calculate total amount of MDMA excreted in sweat. This calculation was most probably an overestimation of the real figures as perspiration is not homogeneous in the whole body surface but is a first approach for estimating the amount of drug excreted through this biological fluid. Estimations suggest that a non-negligible fraction of the dose is excreted through sweat, providing a basis for sweat testing of MDMA exposure.

Analytical methodology applied in this study, immunological screening and GC–MS confirmation, appeared to be adequate for monitoring single drug use within 24 h.

No immunoassays were available at the time of the study for detection of drugs in sweat; however, a commercial test kit for plasma samples was adapted for this purpose. A threshold concentration of 4.4 ng MDMA/patch clearly differentiated drug consumption. By applying this cut-off concentration, no false-positive or false-negative results were observed. Nevertheless, these excellent results are limited to the population of samples examined in the study. A better validation of the immunoassay for use with alternative specimens would have required the analysis of samples from populations encountered in screening situations.

On the other hand, the advantage of a controlled administration study is that the capability of a sweat patch technology for diagnosing the use of even a single dose of MDMA could be clearly defined.

GC–MS analysis was performed in all samples irrespective of EIA results, with a good correlation (r = 0.85) between values obtained by immunoassay screening and GC–MS confirmation (Figure 3). Nonetheless, it has to be mentioned that lengthy extraction procedures had to be applied for a supposedly "simple" biological matrix such as sweat because of the complexity of

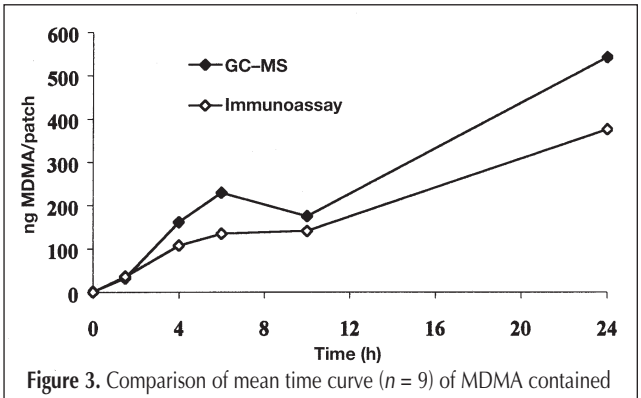

Figure 3. Comparison of mean time curve (n = 9) of MDMA contained in sweat patches applied to the backs of the nine subjects by immunoassay and GC–MS.

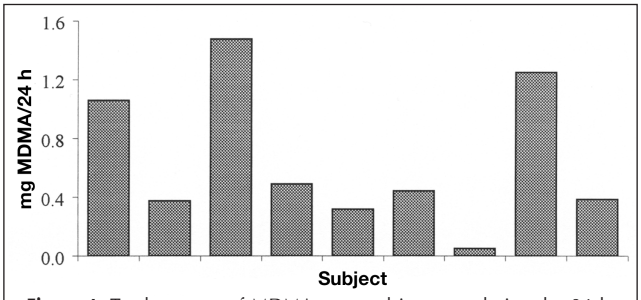

Figure 4. Total amount of MDMA excreted in sweat during the 24 h postadministration for the nine subjects.

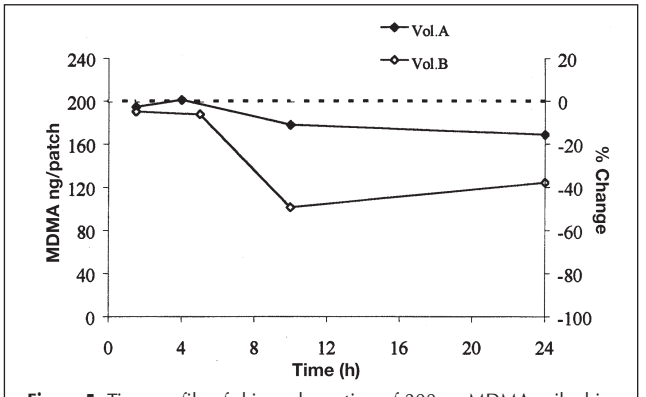

Figure 5. Time profile of skin reabsorption of 200 ng MDMA spiked in sweat patches applied to the backs of two control subjects.

many different components contained in the collector, in order to avoid analytical interferences both in screening and confirmation assays and to set up a robust and reproducible chromatographic system. In particular, the extraction used for the immunoassay resulted in several chromatographic interferences in the GC–MS determination of analytes. Another procedure was applied in order to increase method selectivity, although lower analytical recovery was achieved.

Using this sweat collector, a full investigation of MDMA pharmacokinetics in sweat was not possible. However, cumulative excretion, as reported, and the time-course of the excretion of this drug in sweat could be defined. MDMA was detected in sweat by 1.5 h after administration, corresponding to its concentration peak in plasma (24), and at least until 24 h postadministration. MDMA concentrations tended to progressively increase during the 24 h, with an influx at 10 h from consumption. It can be postulated that the decrease in sweat patch content of MDMA was probably due to a reabsorption by the skin. This phenomenon has also been observed for other drugs, such as cocaine, diazepam, or MBDB (22,30,31) and was confirmed in our pilot experiment on skin reabsorption of MDMA from the sweat patch in non-consumer subjects.

It is known that temperature and physical exercise can change sweat flow and pH. A rise in body temperature or physical exercise could increase sweat flow, producing an increase in sweat pH and the volume of sweat in contact with the patch, facilitating drug transfer to the skin (30). Indeed, in our study, the subject (volunteer B) that had a higher percentage of skin reabsorption participated in moderate gymnasium exercise during the experiment. This observation may be of relevance considering MDMA consumption patterns (rave parties, overcrowded, with high ambient temperature and physical exercise associated to dancing). Nevertheless, the impact of this observation in sweat testing performed in 'normal' conditions (i.e., 7 days wearing patch) is unclear. What can be nevertheless concluded is that the final amount of MDMA collected in the patch is the result of a more dynamic process (excretion vs. reabsorption) than expected by authors and by other research groups.

Different perspiration rates and volumes of sweat between individuals could also explain the large intersubject variability in sweat excretion of the same administered dose of MDMA. The evidence of inter-subject variability in sweat excretion of the same dosage of drug was also reported after controlled administration of 90 mg oral codeine (13) and 50 and 126 mg intranasal cocaine (7). However, a relevant difference between MDMA and the other drugs of abuse administered in controlled settings resides in the total amount of drug excreted in the sweat patch. Whereas in the case of codeine (90 mg/dose), cocaine (126 mg/dose), and MBDB (100 mg/dose), maximum concentrations hardly exceeded 100 ng/patch (7,13,22), 100 mg of administered MDMA gave rise to sweat patch maximum concentrations in the order of micrograms per patch, confirming the theoretical calculation of a high sweat/plasma ratio for this drug. The present study only considers a 24-h collection period, but it can be easily foreseen that as drug users have more than a single dose per rave, the application of the patch technology for monitoring MDMA consumption for longer periods of patch wearing has to be hypothesized as very successful.

Sweat excretion, although highly variable between individuals and induced by MDMA pharmacological activity on body temperature regulation (32), most probably explains the acceptable results obtained with the application of an onsite test for rapid screening of MDMA in sweat.

The Drugwipe information sheet reported sensitivity for methamphetamine of 10 ng, which was understood as the minimum quantity of drug on the collection pad giving rise to a positive immune reaction. From what is known of saliva (33), the device cannot detect concentrations down to 100 ng/mL of MDMA. Several studies were performed administering single doses of several over-the-counter phenylalkylamines (i.e., phenylpropanolamine and ephedrine) and no positive reactions with the device were observed (intramural data). Drugwipe could detect MDMA consumption in sweat as early at 1.5 h and as long as 24 h after drug administration. These findings are in agreement with results obtained in a pilot study including two subjects (24). However, although all the subjects gave a positive result at 1.5 h after drug administration, in the following 4–10 h, two or three subjects produced negative results. An overall false-negative rate of 18% may be estimated from results of the first 6 h post MDMA administration, where pharmacological and subjective effects are measurable. No false-positive results were observed during the study in either recreational users of MDMA or in drug-free healthy subjects (intramural data). These findings are in accordance with those obtained when wiping the Drugwipe over the tongue of individuals administered MDMA in gelatin capsules for a rapid test in saliva. Also in that case, positivity to the test decreased noticeably at 6 and 10 h after MDMA administration. The most reasonable explanation for the negative results was the insufficient amount of biological matrix (sweat and saliva) which could be collected wiping the armpit or tongue with the test pad of the device (32). Indeed, this hypothesis was confirmed by the fact that when a pre-selected volume of saliva was applied to the device, the test performed better, giving negative results only in individuals which showed the lowest salivary MDMA concentrations. Unfortunately, in the case of sweat, it was not possible to verify if negative results were also attributable to a low MDMA recovery on the collection pad.

In any case, if the objective of onsite sweat testing is not only to detect the consumption of a given drug but also to determine if an individual is under the effects of the drug, onsite sweat testing with Drugwipe appeared to fit this purpose. Indeed, 1.5 h corresponds to maximal effects for some physiological and psychomotor variables. Those effects may last at least 6 h, unless there is a repeated administration (33). However, to draw any definite conclusion, a study in a large population sample is needed, given that present results were obtained in a controlled setting with a small number of participants.

Conclusions

MDMA appears in sweat and can be quantitated in the first few hours after a single-dose administration of 100 mg drug. This dosage was chosen as the one commonly encountered in clandestine preparations of the illicit market (34,35). MDMA tends

to accumulate in sweat 24 h postadministration with a large intersubject variability in the amount of MDMA excreted, preventing a correlation with the consumed dose. Although some traces of MDA were observed, the parent drug was the principal analyte found in sweat samples. An equilibrium between skin reabsorbance and sweat excretion of MDMA seems to exist, although preliminary results should be confirmed by more extensive investigations. The accumulation of MDMA in sweat allowed the application of a non-invasive onsite test, the Drugwipe, which appeared to be feasible to detect MDMA in the first hours after administration, when an individual is known to be under the effects of the drug.

Sweat patch technology proved to be useful for monitoring single-dose MDMA (100 mg) administration in the 24 h after consumption. Further field studies should be performed to investigate the routine application of patch technology. Nevertheless, taking into account these preliminary results it can be seen that sweat patches can be applied in some analytical toxicology applications for monitoring MDMA consumption.

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References

1. H. Zhao, R. Brenneisen, A. Scholer, A. McNally, M.A. Elsohly, T. Murphy, and S.J. Salamone. Profile of urine samples taken from ecstasy users at rave parties: analysis by immunoassays, HPLC, and GC–MS. *J. Anal. Toxicol.* **25:** 258–269 (2001).
2. J.A. Henry, K.J. Jeffreys, and S. Dawling. Toxicity and deaths from 3,4-methylenedioxymethamphetamine ("ecstasy"). *Lancet* **340:** 384–387 (1992).
3. N. Samyn and C. Van Haeren. On-site testing of saliva and sweat with drugwipe and determination of concentration of drugs of abuse in saliva, plasma and urine of suspected users. *Int. J. Legal Med.* **113:** 150–154 (2000).
4. J. Ortuño, N. Pizarro, M. Farré, M. Mas, J. Segura, J. Camí, R. Breinnessen, and R. de la Torre. Quantification of 3,4-methylenedioxymethamphetamine and its metabolites in plasma and urine by gas chromatography with nitrogen-phosphorus detection. *J. Chromatogr. B* **723:** 221–232 (1999).
5. M. Navarro, S. Pichini, M. Farré, J. Ortuño, P.N. Roset, J. Segura, and R. de la Torre. Usefulness of saliva for measurement of MDMA and its metabolites: correlation with plasma drug concentrations and effect of salivary pH. *Clin. Chem.* **47:** 1788–1795 (2001).
6. C. Jurado, M.P. Gimenez, T. Soriano, M. Menedez, and M. Repetto. Rapid analysis of amphetamine, methamphetamine, MDA, and MDMA in urine using solid-phase microextraction, direct on-fiber derivatization, and analysis by GC–MS. *J. Anal. Toxicol.* **24:** 11–16 (2000).
7. M. Burns and R.C. Baselt. Monitoring drug use with a sweat patch: an experiment with cocaine. *J. Anal. Toxicol.* **19:** 41–48 (1995).
8. D. Kidwell, J. Holland, and S. Athanaselis. Testing for drugs of abuse in saliva and sweat. *J. Chromatogr. B* **713:** 111–135 (1998).
9. W. Schramm, R.H. Smith, P.A. Craig, and D.A. Kidwell. Drugs of abuse in saliva: a review. *J. Anal. Toxicol.* **16:** 1–9 (1992).
10. E.J. Cone. Saliva testing for drugs of abuse. *Ann. N.Y. Acad. Sci.* **694:** 91–127 (1993).
11. K.L. Preston, M.A. Huestis, C.J. Wong, A. Umbricht, B. Goldberger, and E.J. Cone. Monitoring cocaine use in substance-abuse treatment patients with sweat and urine testing. *J. Anal. Toxicol.* **23:** 313–321 (1999).
12. R. Haeckel and P. Hanecke. The application of saliva, sweat and tear fluids for diagnostic purposes. *Ann. Biol. Clin.* **50:** 903–910 (1993).
13. P. Kintz, A. Tracqui, C. Jamey, and P. Mangin. Detection of codeine and phenobarbital in sweat collected with a sweat patch. *J. Anal. Toxicol.* **20:** 197–201 (1996).
14. P. Kintz, A. Tracqui, and P. Mangin. Sweat testing in opioid users with a sweat patch. *J. Anal. Toxicol.* **20:** 393–397 (1996).
15. J. Fay, R. Fogerson, D. Schoendofer, R.S. Niedbala, and V. Spiehler. Detection of metamphetamine in sweat by EIA and GC–MS. *J. Anal. Toxicol.* **20:** 398–403 (1996).
16. R. Fogerson, D. Schoendorfer, J. Fay, and V. Spiehler. Qualitative detection of opiates in sweat by EIA and CG–MS. *J. Anal. Toxicol.* **21:** 451–457 (1997).
17. E.J. Cone, M.J. Hillsgrove, A.J. Jenkins, and R.M. Keenan. Sweat testing for heroin, cocaine and metabolites. *J. Anal. Toxicol.* **18:** 298–305 (1994).
18. P. Kintz, V. Cirimele, and B. Ludes. Codeine testing in sweat and saliva with the drugwipe. *Int. J. Legal Med.* **111:** 81–86 (1997).
19. N. Samyn and C. Van Haeren. On-site testing of saliva and sweat with drugwipe and determination of concentration of drugs of abuse in saliva, plasma and urine of suspected users. *Int. J. Legal Med.* **113:** 150–154 (2000).
20. T.B. Vree, A.T. Muskens, and J.M. Van Rossum. Excretion of amphetamines in human sweat. *Arch. Int. Pharmacodyn. Ther.* **199:** 311–317 (1972).
21. P. Kintz and N. Samyn. Determination of "Ecstasy" components in alternative biologic specimens. *J. Chromatogr. B* **733:** 137–143 (1999).
22. P. Kintz. Excretion of MBDB and BDB in urine, saliva, and sweat following single oral administration. *J. Anal. Toxicol.* **21:** 570–575 (1997).
23. M.A. Huestis, E.J. Cone, C.J. Wong, A. Umbricht, and K.L. Preston. Monitoring opiate use in substance abuse treatment patients with sweat and urine drug testing. *J. Anal. Toxicol.* **24:** 509–521 (2000).
24. R. Pacifici, M. Farré, S. Pichini, J. Ortuño, P.N. Roset, P. Zuccaro, J. Segura, and R. de la Torre. Sweat testing of MDMA with the Drugwipe analytical device: a controlled study with two volunteers. *J. Anal. Toxicol.* **25:** 144–146 (2001).
25. Y. Wang, J. Moss, and R. Thisted. Predictors of body surface area. *J. Clin. Anesth.* **4:** 4–10 (1992).
26. B. Schmid, J. Bircher, R. Preisig, and A. Küpfer. Polymorphic dextromethorphan metabolism: co-segregation of oxidative o-demethylation with debrisoquin hydroxylation. *Clin. Pharmacol. Ther.* **38:** 618–624 (1985).
27. R. de la Torre, M. Farré, P.N. Roset, C. Hernández López, M. Mas, J. Ortuño, E. Menoyo, N. Pizarro, J. Segura, and J. Camí. Pharmacology of MDMA in humans. *Ann. N.Y. Acad. Sci.* **914:** 225–237 (2000).
28. R.C. Baselt, and R.H. Cravey. *Disposition of Toxic Drugs and Chemicals in Man*, 4th ed. Chemical Toxicology Institute, Foster City, CA, 1995, pp 44 and 475.
29. C. Aly, S.B. Cunico, and H. Mainbach. Effect of prolonged occlusion on the microbial flora, pH, carbon dioxyde and transepidermal water loss on human skin. *J. Invest. Dermatol.* **71:** 378–386 (1978).
30. D.A. Kidwell and F.P. Smith. Susceptibility of PharmCheck™ drugs of abuse patch to environmental contamination. *Forensic Sci. Int.* **116:** 89–106 (2001).
31. P. Kintz, A. Tracqui, and P. Mangin. Sweat testing for benzodiazepines. *J. Forensic Sci.* **41:** 851–854 (1996).
32. J. Camí, M. Farré, M. Mas, P.N. Roset, S. Poudevida, A. Mas, L. San,

and R. de la Torre. Human pharmacology of 3,4-methylenedioxymethamphetamine ("ecstasy"): psychomotor performance and subjective effects. *J. Clin. Psychopharmacol.* **4:** 455–466 (2000).
33. S. Pichini, M. Navarro, M. Farré, J. Ortuño, P.N. Roset, R. Pacifici, P. Zuccaro, J. Segura, and R. de la Torre. On-site testing of 3,4 methylenedioxymethamphetamine (Ecstasy) in saliva with Drugwipe and Drugread: a controlled study in controlled users. *Clin. Chem.* **48:** 174–176 (2002).
34. K. Wolff, A.W.M. Hay, K. Sherlock, and M. Conner. Contents of "ecstasy". *Lancet* **346:** 1100–1101 (1995).
35. M. Baggott, B. Heifets, R.T. Jones, J. Mendelson, E. Sferios, and J. Zehnder. Chemical analysis of ecstasy pills. *J. Am. Med. Assoc.* **284:** 2190 (2000).

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Development and validation of a gas chromatography-mass spectrometry assay for hair analysis of amphetamine, methamphetamine and methylenedioxy derivatives

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Abstract

A procedure based on gas chromatography-mass spectrometry (GC-MS) is described for the determination of amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA, ecstasy), 3,4-methylenedioxyethylamphetamine (MDE or MDEA) and *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB) in hair. Hair samples were digested with 1 M sodium sulfide at 37 °C (by shaking for 3 h and was kept at room temperature overnight), and extracted with two sequential extraction procedures: liquid-liquid extraction with *tert*-butyl methyl ether and solid-phase extraction with Bond-Elut Certify columns. Extracted analytes were derivatised with *N*-methyl-bis(trifluoroacetamide), separated by a 5% phenylmethylsilicone column and determined by a mass spectrometer detector in selected ion monitoring mode. A good reproducibility (intra-assay R.S.D. = 1.5–15.7%), accuracy (intra-assay error = 2.0–11.7%) and sensitivity (LOD = 0.03–0.08 ng/mg hair) were attained. The method was successfully applied to the analysis of the proximal (1 cm) hair segment to assess recent self-reported use in "ecstasy" consumers. Otherwise, further studies are needed to validate methodology developed in case of amphetamine consumption.

Keywords: Derivatization, GC; Amphetamine; Methamphetamine

1. Introduction

Recreational use of methylenedioxyphenylderivatives of amphetamine (referred to as designer drugs: 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) 3,4-methylenedioxyethylamphetamine (MDE or MDEA), *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB) either alone or in combination with other drugs, such as alcohol and cannabis, has become increasingly popular among young people [1,2]. In particular, MDMA is the most commonly consumed designer drug [2]. Acute medical complications of drug use include malignant hyperthermia, seizures, cerebral haemorrhage, hepatitis, rhabdomyolisis, disseminated intravascular coagulation, and acute renal failure [3]. There are a number of reports concerning severe intoxication and death after MDMA consumption [4].

Acute intoxication by designer drugs is diagnosed by urine and blood analysis and well documented literature is available [5]. Hair drug testing is an alternative approach to monitor past drug use in settings other than acute intoxication cases (i.e. forensic science) [6].

An individual's past history of drug abuse is usually collected through structured questionnaires. As this information is entirely based on the credibility of subjects which is questioned in many cases, drug testing in hair has been proposed as an objective alternative to self-reported drug

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abuse histories [6]. Furthermore, when performing analyses of different hair sections corresponding to different periods of time—segmental hair analysis—eventual changes in patterns of consumption can be theoretically defined and associated to eventual alterations observed in cognitive processes or incidence of psychopathology [7]. Hair accumulation of both classical amphetamines and methylenedioxy derivatives have been extensively reported in consumers and in fatal cases, with gas chromatography-mass spectrometry (GC-MS) methods being applied for quantification purposes and to investigate disposition of parent drugs and metabolites in hair [8–12]. The majority of developed methods detected classical amphetamines (amphetamine and methamphetamine) or methylenedioxy derivatives, with few assays simultaneously testing the two classes of drugs [13–16].

The objective of the present report was to develop a sensitive and selective analytical method for hair analysis of amphetamine derivatives that meets the accepted criteria for bioanalytical method validation [17]. Since it has been shown that amphetamines consumed in the dance scene are mainly "ecstasy" pills (containing not only MDMA but also MDA, MDEA, and eventually MBDB), amphetamine and methamphetamine [14], a methodology for hair analysis of both classical amphetamines and methylenedioxy derivatives has been set up, validated and applied in a follow-up study of "ecstasy" abusers. In this paper, preliminary results concerning segmental hair analysis in selected cases will be presented.

2. Materials and methods

2.1. Chemicals and reagents

Amphetamine (AP), methamphetamine (MA), MDMA, MDA, MDEA, [$^2H_5$]MDMA, [$^2H_5$]MDA, [$^2H_5$]AP and [$^2H_8$]MA were supplied by Cerilliant (Austin, TX, USA). MBDB was provided by Lipomed (Cambridge, MA, USA). Bond Elut Certify® solid-phase extraction (SPE) columns were obtained from Varian Corp. (Harbor City, CA, USA). Gas chromatography grade *N*-methyl-bis(trifluoroacetamide) (MBTFA) was purchased from Macherey-Nagel (Düren, Germany). Analytical grade sodium sulfide nonahydrate was supplied by Aldrich (Steinheim, Germany). All other reagent grade chemicals were supplied by Merck (Darmstadt, Germany).

2.2. Standards and solutions

Separate stock solutions of AP, MA, MDMA, MDA, MDEA and MBDB (1 mg/ml) were prepared in HPLC-grade methanol and stored at −20 °C. From stock solutions, working solutions of 10, 1, and 0.1 μg/ml were made and used for the preparation of calibration curves and quality control (QC) samples. Internal standards (ISTDs) ([$^2H_5$]AP, [$^2H_8$]MA, [$^2H_5$]MDA and [$^2H_5$]MDMA) were diluted in methanol to give a working solution at a concentration of 1 μg/ml and stored at −20 °C.

Daily standard curves were obtained by analyzing hair samples (10 mg blank control samples) fortified with the drugs at 0, 0.5, 5, 10, 15 and 20 ng/mg hair. QC samples (10 mg blank control samples fortified with 0.6, 7.5, 17.5 ng drug/mg hair) were also prepared daily from working solution of stock sources with different lot numbers from the reference materials used to prepare the calibration standards. QC samples were included in each analytical batch to check calibration, accuracy and precision.

2.3. Hair samples collection

Hair samples were collected within the framework of a two-year follow-up study of 40 ecstasy abusers and matched controls. Subjects, with age ranging from 18 to 34 years, completed a structured questionnaire which asked about: initial and final age of consumption, last consumption, and total consumption in the previous month and in the last 6 and 12 months, respectively in their history with drugs. Subjects provided information for several drugs of abuse, among others: MDMA or ecstasy, methamphetamine, other psycho stimulants like amphetamine and cocaine, cannabis, and LSD. Hair analysis was performed for several drugs, but this study describes only findings related to the methodology described: methamphetamine and methylenedioxyderivatives.

Hair samples (as an entire strand) were cut close to the scalp in the vertex region using a stainless steel scissors. From all the samples collected for the study, 17 hair samples were used within the method development to assess association between hair analysis and self-reported use of designer drugs in the last month. The selection was made according to several patterns of consumption representative of the population studied. Consumers recruited for the study were divided in two different categories of drug consumption: occasional users (eventual use, less than once a week) and regular users (weekly to daily use). Hair strands from the 17 subjects (all with natural brown hair colour) were divided in segments. The first was cut at 1 cm from the proximal region, representing hair growth in the last month. Hair segment was finely cut and a duplicate of 10 mg weight was obtained. Results reported in the present study, refer to the analytical findings in this segment supposed to be related with information collected in the questionnaire for the last month consumption.

Hair from 10 ecstasy users was pooled, homogenised and used as positive control to check different digestion and extraction procedures and it was included in each analytical batch as internal QC of the methodology developed. Drug-free human hair samples obtained from 10 non-consumers were reduced in short cuts, analysed during method validation to exclude any source of chromatographic interferences and mixed to obtain a homogeneous pool of blank hair.

2.4. Hair sample preparation and extraction

The pooled drug free hair and hair samples (10 mg) were washed three times (2 min) with 3 ml dichloromethane in an ultrasonic water-bath and allowed to dry at room temperature.

Then, samples, calibrators, QC samples (drug free hair spiked with drugs at concentrations and working standard solutions other than those used for calibrators) and the internal QC positive sample (pooled hair from MDMA users) were fortified with 50 μl of 1 μg/ml [$^2H_5$]AP, [$^2H_8$]MA, [$^2H_5$]MDA and [$^2H_5$]MDMA, as internal standards.

Four different digestion and extraction procedures were tested with the pool of blank hair fortified with analytes under investigation and in a pool of hair samples from consumers. Two digestions were performed in alkaline conditions and consisted of 1 ml of 1 M sodium sulfide (Na_2S) or 1 M NaOH placed in silanized glass tubes with 10 mg hair samples with periodic shaking for 3 h and then kept at room temperature overnight [18]. After digestion, hair samples were ultracentrifuged at 12000 rpm for 10 min and the aqueous layer was transferred in a new silanized glass tube. Analytes were extracted from the aqueous layer with two subsequent portions of 3 ml of *tert*-butyl methyl ether by rocking mixing for 30 min and centrifuged at 3500 rpm for 5 min. The organic phase was separated and evaporated to dryness under nitrogen stream at 23 °C with a c.a.10 psi pressure. Sample extracts were reconstituted with 1 ml of 0.1 M sodium phosphate buffer (pH 6). Reconstituted extracts underwent a solid–liquid extraction (SPE) with Bond Elut Certify columns according to a previously reported method [19]. Two acid digestions, were assayed as follows: hair samples were placed in silanized glass tubes with 1 ml methanol/5 M HCl (v/v 20:1) or methanol/trifluoroacetic acid (v/v 9:1) [20,21] and allowed to stand 1 h under ultrasonication. Then the solution was left to stand at room temperature overnight. Afterwards, the organic phase was separated from hair samples and evaporated to dryness under nitrogen stream at 23 °C (c.a.10 psi pressure). Sample extracts were reconstituted with 1 ml of 0.1 M sodium phosphate buffer (pH 6). Reconstituted extracts underwent a solid–liquid extraction (SPE) with Bond Elut Certify columns according to a previously reported method [20].

Eluates from SPE after both basic and acid digestion, were added with 20 μl of MBTFA to prevent amphetamines losses, were evaporated to dryness under nitrogen stream at 40 °C (c.a.10 psi pressure). Trifluoroacyl derivatives were formed by reaction with 50 μl of MBTFA as derivatization agent in a dry bath at 70 °C during 45 min.

2.5. GC-MS analysis

GC-MS analysis was performed in a Hewlett Packard 6890 gas chromatograph coupled to an HP 5973 quadrupole mass spectrometer detector (Palo Alto, CA). The gas chromatograph was fitted with an HP 7683 auto sampler injector.

Samples were injected in splitless mode into a 12 m × 0.2 mm i.d., 0.33 μm film thickness 5% phenylmethylsilicone column (Ultra 2-Hewlett Packard).

The oven temperature was initially maintained at 70 °C during 2 min and programmed to 160 °C at 30 °C per min, then to 170 °C at 5 °C per min, to 200 °C at 15 °C, and finally to 290 °C at 30 °C per min.

The injector and the interface were operated at 280 °C. Helium was used as carrier gas at a flow rate of 1.2 ml/min.

The mass spectrometer was operated in electron impact ionization mode at 70 eV. Qualifying ions selected for analytes under investigation were: *m/z* 91, 118, 140 for AP-*N*-TFA, *m/z* 91, 118, 154 for MA-*N*-TFA, *m/z* 154, 162, 289 for MDMA-*N*-TFA, *m/z* 135, 162, 275 for MDA-*N*-TFA, *m/z* 162, 168, 303 for MDEA-*N*-TFA, *m/z* 168, 176, 303 for MBDB-*N*-TFA, *m/z* 96, 123, 140 for [$^2H_5$]AP-*N*-TFA, *m/z* 92, 113, 161 for [$^2H_8$]MA-*N*-TFA, *m/z* 136, 167, 280 for [$^2H_5$]MDA-*N*-TFA, *m/z* 158, 164, 294 *m/z* for [$^2H_5$] MDMA-*N*-TFA. Ion ratio acceptance criterion was a deviation ≤20% of the average of ion ratios of all the calibrators. The ions: *m/z* 118 for AP-*N*-TFA, *m/z* 154 for MA-*N*-TFA and MDMA-*N*-TFA, *m/z* 162 for MDA-*N*-TFA and MDEA-*N*-TFA, *m/z* 168 for MBDB-*N*-TFA, *m/z* 123 for [$^2H_5$]AP-*N*-TFA, *m/z* 161 for [$^2H_8$]MA-*N*-TFA, *m/z* 136 for [$^2H_5$]MDA-*N*-TFA, *m/z* 158 for [$^2H_5$] MDMA-*N*-TFA were used for quantification.

2.6. Method validation

Analytical recoveries were calculated by comparison between peak areas of the calibration samples analyzed with the normal procedure and those obtained after adding the same amounts of reference substances and ISTDs to blank hair after extraction. Recoveries were analyzed at three different concentrations, 0.5, 10 and 20 ng/mg hair, using four replicates for each evaluated concentration. Linearity was determined by checking different calibration curves (n = 10 in four consecutive days) at five different concentrations (limit of quantification, 5, 10, 15 and 20 ng/mg for all compounds). Peak area ratios between compounds and internal standards were used for calculations.

[$^2H_5$]AP was used as internal standard for AP, [$^2H_8$]MA for MA, [$^2H_5$]MDA for MDA and [$^2H_5$]MDMA for MDMA, MDEA and MBDB. A weighted least-square regression analysis was used (SPSS for Windows 9.0.1).

Five replicates of blank samples added with 5 ng/mg of ISTDs were used for calculating the limits of detection and quantification. Standard deviation (S.D.) of the analytical background response was used to determine the detection limit (LOD = 3.3 S.D.) and the quantification limit (LOQ = 10 S.D.).

Five replicates at three different concentrations of the analytes (0.6, 7.5 and 17.5 ng/mg hair) spiked in blank hair were used for the determination of intra-assay precision (expressed as coefficient of variation for specific added target concentrations) and accuracy (expressed as percentage error

of concentration found as compared with target added concentrations). Inter-day precision and accuracy were determined in three different experimental days.

Mid-term stability test was performed for hair samples stored at ambient temperature. Hair pool from 10 ecstasy consumers, used as internal QC, was included in each analytical batch during a 3 months period. The stability was expressed as a percentage of the initial concentration (first analyzed batch) of the analytes in pooled hair.

Selectivity tests were performed with 10 hair samples from non consumers extracted and analysed for assessment of potential interferences from endogenous substances. The apparent response at the retention times of the analytes under investigation was compared to the response of analytes at the limit of quantification. Furthermore, potential interferences from principal drugs of abuse (opiates, cocaine and main metabolites, cannabinoids-Δ-9-tetrahydrocannabinol and 11-nor-9-carboxy-tetrahydrocannabinol-), were also evaluated spiking 10 mg of blank hair spiked with 10 ng of the aforementioned substances and carried through the entire procedure.

3. Results and discussion

3.1. Analytical method

Recently, it was suggested [18] that hair digestion with 1 M sodium sulfide increased drug recovery from keratin matrix in case of nicotine and flunitrazepam. Hence, this digestion agent was tested during method development and compared with other digestion approaches commonly used to extract drugs of abuse and in particular amphetamines from hair samples, such as sodium hydroxide, methanol/HCl and methanol/trifluoroacetic acid [18–21].

Table 1 shows the recovered amounts of AP, MA and methylendioxy derivatives in blank hair fortified with 2.5 ng/mg of each analyte and in a pool of hair samples from consumers following different digestions and extraction procedures.

Complete dissolution of hair samples, as obtained with sodium sulfide or sodium hydroxide, required a subsequent two-step extraction procedure in order to isolate analytes under investigation from substances released from keratin matrix. In contrast, acid digestions are usually described associated with a single SPE extraction step. However, extracts obtained following acid digestion and SPE resulted dirty and hence presented problems during GC injection giving rise to many chromatographic interferences in sample analysis by GC-MS. On the other hand, if a liquid–liquid extraction was applied before SPE to acid digestion in order to improve sample clean-up, analyte recoveries resulted sensibly lowered (intramural data not shown).

Digestion with 1 M sodium sulfide, followed by a liquid–liquid extraction and a SPE with Bond-Elut Certify resulted to be the best compromise between recovery of analytes from hair matrix, clean-up of extracts and absence of chromatographic interferences.

Consequently, this procedure was used to examine hair from ecstasy consumers. A comparison of some digestion and extraction procedures for amphetamines in hair has been reported by Kintz and Cirimele [22]. The authors concluded that best recoveries were observed after alkaline hydrolysis with NaOH 1 N. This result is not in contrast with our findings. Indeed, apparent best recoveries here obtained after acid extractions were due to the fact that in this case only a SPE extraction was used after digestion and when a liquid–liquid step was introduced before SPE, recoveries were lower than those obtained by alkaline hydrolysis with NaOH. Nonetheless, sodium sulfide, which was not evaluated by aforementioned authors gave better results.

Fig. 1 shows the fragmentograms from an extract of a blank hair (A), an extract of a blank hair fortified with 1 ng of each analyte per mg hair (B), and an extract of hair from a “ ecstasy” consumer containing 0.63 ng/mg of MDA, 4.53 ng/mg of MDMA and 0.70 ng/mg of MDEA. Hair from non consumers, analyzed as separate and pooled samples, did not interfere with the assay.

Data on method validation are reported in Tables 2–4. Standard curve plots for the analytes were linear in the range of tested concentrations with a coefficient of correlation (r^2) higher than 0.99. Intra- and inter-assay accuracy and precision results satisfactorily met current acceptance criteria in the validation of bioanalytical methods [18]. Analytical recoveries and calculated limits of detection and quantification were considered adequate for the purpose of the study.

Table 1
Recovered amount of amphetamine, methamphetamine and methylendioxyderivatives in blank hair fortified with 2.5 ng/mg analytes and pool of consumers following different digestions and extraction procedures

Analyte	Fortified hair (ng/mg)				Pool of consumers (ng/mg)			
	Na_2S 1 M	MeOH–HCl	MeOH–TFA	NaOH 1 M	Na_2S 1 M	MeOH–HCl	MeOH–TFA	NaOH 1 M
AP	0.91 ± 0.10	1.15 ± 0.87	1.28 ± 0.09	0.99 ± 0.29	N.D.	N.D.	N.D.	N.D.
MA	1.80 ± 1.11	0.57 ± 0.26	1.94 ± 0.49	0.46 ± 0.11	N.D.	N.D.	N.D.	N.D.
MDA	2.38 ± 0.11	2.77 ± 0.25	0.69 ± 0.23	0.51 ± 0.06	0.33 ± 0.08	0.18 ± 0.05	0.11 ± 0.02	N.D.
MDMA	2.22 ± 0.08	2.50 ± 0.44	1.25 ± 0.82	0.70 ± 0.20	3.71 ± 0.27	2.96 ± 0.25	3.55 ± 0.12	1.67 ± 0.40
MDEA	1.85 ± 0.19	2.56 ± 0.26	1.22 ± 0.24	0.48 ± 0.10	N.D.	N.D.	N.D.	N.D.
MBDB	1.98 ± 0.12	2.54 ± 0.30	1.68 ± 0.32	0.54 ± 0.16	N.D.	N.D.	N.D.	N.D.

Results are expressed as mean ± S.D. ($n = 4$). N.D.: not detected.

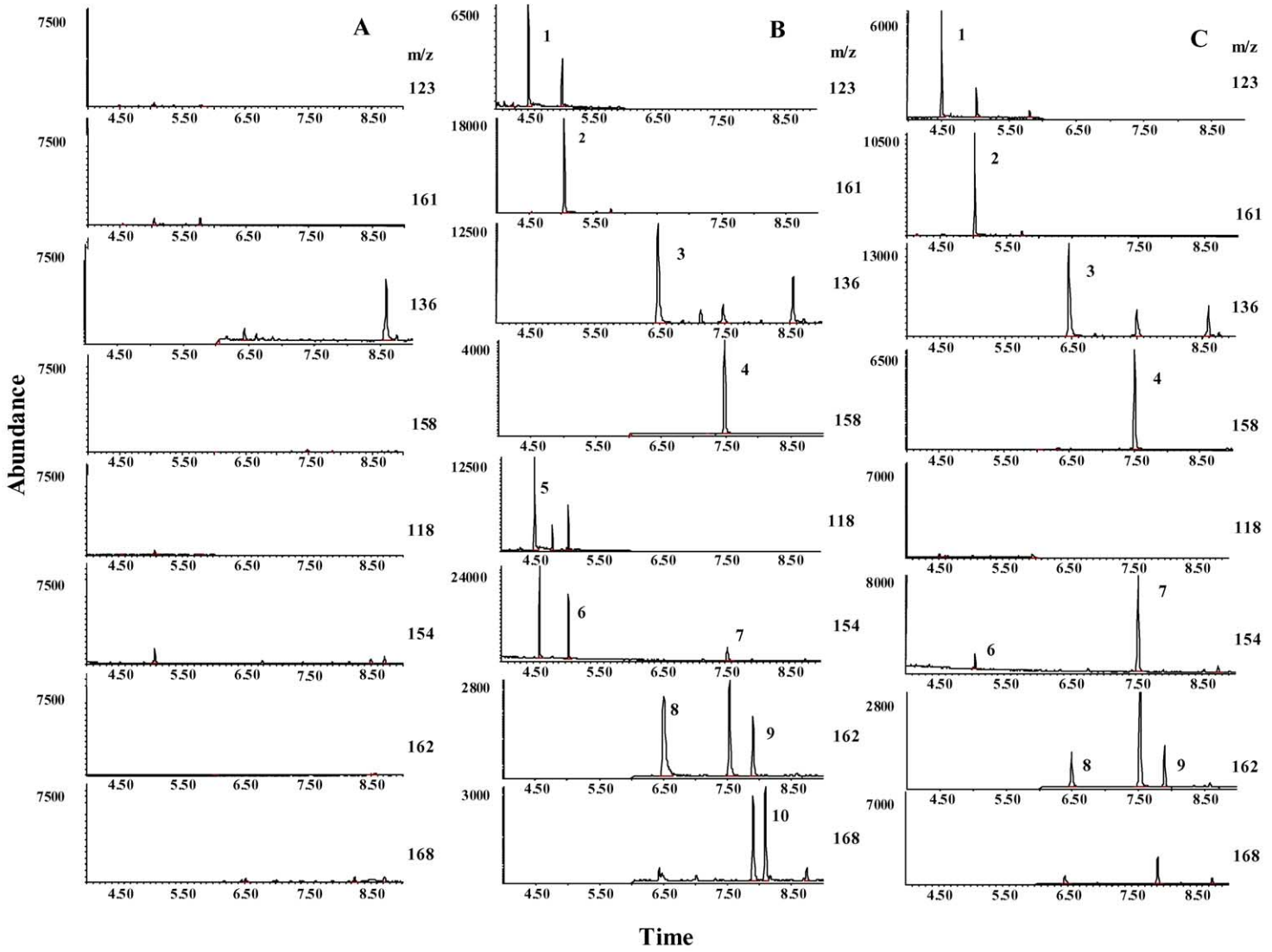

Fig. 1. GC-MS-SIM Fragmentograms of TFA-derivatised extracts from (A) human blank hair, (B) human blank hair spiked with 1 ng/mg analytes under investigation and (C) subject scalp hair. (1) [$^2H_5$]amphetamine, 123 *m/z*; (2) [$^2H_8$]methamphetamine, 161 *m/z*; (3) [$^2H_5$]MDA, 136 *m/z*; (4) [$^2H_5$]MDMA, 158 *m/z*; (5) amphetamine, 118 *m/z*; (6) methamphetamine, 154 *m/z*; (7) MDMA, 154 *m/z*; (8) MDA, 162 *m/z*; (9) MDEA, 162 *m/z*; (10) MBDB, 168 *m/z*.

Table 2
Method calibration

Analyte	Calibration slope	Calibration intercept	Correlation coefficient (r^2)	Limit of detection (LOD) (ng/mg)	Limit of quantification (LOQ) (ng/mg)
AP	0.3704 ± 0.0282	0.0362 ± 0.0091	0.9958 ± 0.0056	0.08	0.25
MA	0.1361 ± 0.0075	0.0151 ± 0.0112	0.9932 ± 0.0029	0.05	0.15
MDA	0.0712 ± 0.0083	0.0086 ± 0.0042	0.9912 ± 0.0009	0.03	0.10
MDMA	0.2131 ± 0.0034	0.0021 ± 0.0012	0.9974 ± 0.0009	0.05	0.15
MDEA	0.0582 ± 0.0110	0.0048 ± 0.0020	0.9940 ± 0.0031	0.05	0.15
MBDB	0.2792 ± 0.0125	−0.0168 ± 0.0082	0.9984 ± 0.0016	0.08	0.25

Table 3
Intra-assay ($n = 5$) and inter-assay ($n = 15$) precision and accuracy calculated for the determination of MDMA, AP, MA, MDA, MDEA and MBDB in hair

Analyte	Concentration (ng/mg)	Intra-assay		Inter-assay	
		Precision (R.S.D.%)	Accuracy (error%)	Precision (R.S.D.%)	Accuracy (error%)
AP	0.6	4.5	9.1	7.5	13.5
	7.5	13.9	10.4	11.9	10.6
	17.5	9.3	9.0	12.7	10.1
MA	0.6	7.2	6.6	9.0	7.9
	7.5	13.9	9.8	12.7	14.1
	17.5	2.0	2.0	5.3	4.3
MDA	0.6	14.3	10.5	11.7	10.9
	7.5	5.4	7.0	11.8	8.1
	17.5	5.8	4.0	10.2	7.9
MDMA	0.6	15.7	11.7	15.0	9.9
	7.5	3.1	2.2	5.3	3.9
	17.5	4.5	3.0	4.8	4.2
MDEA	0.6	2.8	2.6	19.5	19.2
	7.5	5.5	9.6	13.3	10.3
	17.5	5.1	3.6	5.9	5.4
MBDB	0.6	8.4	6.6	16.3	12.4
	7.5	14.6	11.0	11.6	8.1
	17.5	1.5	3.9	7.8	5.9

None of the drugs of abuse other than analytes under investigation carried through the entire procedure interfered with the assay. In reference to mid-term stability test, no relevant degradation was observed in the pooled positive control analysed in a 3 months period, with differences when compared to the initial concentration lower than 5%.

Table 5 presents the results obtained after applying the developed analytical methodology to proximal 1 cm hair segments of individuals claiming use of “ecstasy” and methamphetamine.

Concordance between the self-reported data on last month “ecstasy” consumption and MDMA concentration was good ($r^2 = 0.76$) even in the limited number of individuals examined. Recent findings show that drug incorporation in hair depends from melanin concentration in hair [23]. Indeed, the hair color of the subjects recruited for this study was quite homogeneous, being all the subjects brown-haired, as is the majority of Spanish population. Furthermore, in those hair samples in which MDA could be also determined and other amphetamines were absent, the metabolite/parent drug (MDA/MDMA) ratio ranged between 0.04 and 0.06. When comparing the self-report data for the use of methamphetamine with AP and MA hair concentration, results were disappointing in case of AP. AP could never be detected in hair samples of subjects claiming methamphetamine consumption while it was found, together with MA, in a hair

Table 4
Analytical recoveries at three different concentrations ($n = 4$)

Concentration (ng/mg)	AP (mean ± S.D.)	MA (mean ± S.D.)	MDA (mean ± S.D.)	MDMA (mean ± S.D.)	MDEA (mean ± S.D.)	MBDB (mean ± S.D.)
0.5	92.8 ± 12.1	81.0 ± 2.4	75.1 ± 6.3	91.3 ± 16.4	84.6 ± 15.9	85.0 ± 9.5
10	97.8 ± 23.9	77.3 ± 2.8	98.0 ± 3.8	97.9 ± 2.9	98.7 ± 17.4	96.2 ± 5.3
20	92.5 ± 7.7	85.0 ± 7.8	96.2 ± 13.9	93.9 ± 4.7	80.5 ± 12.9	90.4 ± 3.3

Table 5
Amphetamine-type stimulant consumed and results of hair for study volunteers

Vol.	Consumed substance	Declared consumption last 30 days	Analyte found in the proximal 1 cm hair (ng/mg)				
			MDMA	MDA	MA	AP	MDEA
002	Ecstasy, methamphetamine	2 tablets	3.68	N.D.	N.D.	N.D.	N.D.
003	Ecstasy, methamphetamine	2 tablets, 500 mg	3.08	0.15	N.D.	N.D.	N.D.
007	Ecstasy, methamphetamine	2 tablets, 250 mg	3.14	0.16	N.D.	N.D.	N.D.
011	Ecstasy, methamphetamine	4 tablets	6.13	N.D.	N.D.	N.D.	N.D.
015	Ecstasy, methamphetamine	12 tablets, 1000 mg	12.60	9.00	2.18	N.D.	N.D.
016	Ecstasy, methamphetamine	1 tablet	2.35	0.10	N.D.	N.D.	N.D.
018	Ecstasy, methamphetamine	3 tablets	4.34	0.25	N.D.	N.D.	N.D.
020	Ecstasy, methamphetamine	2 tablets	1.20	N.D.	N.D.	N.D.	N.D.
023	Ecstasy, methamphetamine	1.5 tablets	1.70	N.D.	N.D.	N.D.	N.D.
037	Ecstasy, methamphetamine	2.5 tablets	6.98	0.59	N.D.	N.D.	N.D.
038	Ecstasy, methamphetamine	3 tablets, 150 mg	2.98	2.02	0.54	N.D.	N.D.
040	Ecstasy, methamphetamine	–	N.D.	N.D.	1.04	0.74	N.D.
051	Ecstasy, methamphetamine	4 tablets, 750 mg	4.15	2.10	0.57	N.D.	N.D.
075	Ecstasy, methamphetamine	0.75 tablets, 250 mg	4.53	0.63	0.22	N.D.	0.70
103	Ecstasy, methamphetamine	3 tablets, 150 mg	4.68	0.30	N.D.	N.D.	N.D.

*N.D.: not detected.

sample of an individual, which did not declare consumption. This finding is in agreement with what reported by other authors [11] which affirmed that LOD and LOQ of AP higher than those for the other compounds could decrease the number of potentially positive results. MBDB was never detected in the examined subjects, as this amphetamine derivative seemed not to be present in Spain, nor as at the moment in any other European country. Conversely, in a single case out of 17, MDEA could be detected.

4. Conclusions

The GC-MS method reported in this paper to simultaneously analyze amphetamine, methamphetamine and methylendioxy derivatives in hair was validated according to internationally accepted criteria [14]. The method consists of sample digestion in sodium sulfide followed by a liquid–liquid and solid phase extraction, chromatographic separation on a 5% phenylmethylsilicone column and detection in SIM mode by GC/MS. The method showed adequate range of linearity, intra and inter-assay accuracy and precision for its application in hair analysis of MDMA and MDA for assessment of recent self-reported “ecstasy” use. Assessment of methamphetamine use requires further investigation to improve sensitivity to detect hair AP and analysis of a larger number of samples.

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References

[1] J.D. Wright, L. Pearl, BMJ 310 (1995) 20.
[2] H. Zhao, R. Brenneisen, A. Scholer, A. McNally, M.A. Elsohly, T. Murphy, S.J. Salamone, J. Anal. Toxicol. 25 (2001) 258.
[3] U. McCann, S.O. Slate, G.A. Ricaurte, Drug Saf. 15 (1996) 107.
[4] J.A. Henry, K.J. Jeffreys, S. Dawling, Lancet 340 (1992) 384.
[5] T. Kraemer, H.H. Maurer, J. Chromatogr. Biomed. Appl. 713 (1998) 163.
[6] S. Pichini, I. Altieri, P. Zuccaro, R. Pacifici, Clin. Pharmacokinet. 30 (1996) 211.
[7] D.J. McHenna, S.J. Peroutka, J. Neurochem. 54 (1990) 14.
[8] P. Kintz, V. Cirimele, A. Tracqui, P. Mangin, J. Chromatogr. Biomed. Appl. 670 (1995) 162.
[9] Y. Nakahara, Forensic Sci. Int. 70 (1995) 135.
[10] M. Rothe, F. Pragst, K. Spiegel, T. Harrach, K. Fischer, J. Kunkel, Forensic Sci. Int. 89 (1997) 111.
[11] R. Kronstrand, R. Grundin, J. Jonsson, Forensic Sci. Int. 92 (1998) 29.
[12] G.A.A. Cooper, D.L. Allen, K.S. Scott, J.S. Oliver, J. Ditton, I.D. Smith, J. Forensic Sci. 45 (2000) 400.
[13] H. Sachs, P. Kintz, J. Chromatogr. B. 713 (1998) 147.
[14] J. Röhrich, G. Kauert, Forensic Sci. Int. 84 (1997) 179.
[15] P. Kintz, N. Samyn, J. Chromatogr. B 733 (1999) 137.
[16] J. Liu, K. Hara, S. Kashimura, M. Kashiwagi, M. Kageura, J. Chromatogr. B 758 (2001) 95.
[17] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, May 2001 (http://www.fda.gov/cder/guidance/4252fnl.htm).
[18] D.J. Claffey, P.R. Stout, J.A. Ruth, J. Anal. Toxicol. 24 (2000) 54.
[19] J. Ortuño, N. Pizarro, M. Farré, M. Mas, J. Segura, J. Camí, R. Brenneisen, R. de la Torre, J. Chromatogr. B 723 (1999) 221.
[20] R. Kikura, Y. Nakahara, Biol. Pharm. Bull. 18 (1995) 1694.
[21] J. Segura, C. Stramesi, A. Redón, M. Ventura, C.J. Sánchez, G. González, L. San, M. Montagna, J. Chromatogr. B 724 (1999) 9.
[22] P. Kintz, V. Cirimele, Forensic Sci. Int. 84 (1997) 151.
[23] R. Kronstrand, S. Förstberg-Peterson, B. Kågedal, J. Ahlner, G. Larson, Clin. Chem. 45 (1999) 1485.