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TITOLO TESI

DEVELOPMENT OF ORIGINAL ANALYTICAL METHODS FOR THE DETERMINATION OF DRUGS OF ABUSE IN BIOLOGICAL SAMPLES

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1. INTRODUCTION

1.1. Drug abuse and drug addiction

Drug abuse is a major global problem with a strong economic, personal and social impact. In the "World Drug Report" 2011 the United Nations Office on Drugs and Crime (UNODC) estimates that, in 2009, between 149 and 272 million people, that is 3.3% to 6.1% of the population aged 15-64, used illicit substances at least once in the previous year and that about half of that number used illicit drugs at least once during the month before the assessment [1]. The problem is not limited to illicit substances, but concerns also legal psychoactive drugs, such as alcohol, tobacco and medications. Recently, there has been an increase in the non-medical use of prescription drugs, such as a number of opioid analgesics (used in the treatment of pain), tranquillizers and sedatives (employed to treat anxiety and sleep disorders) and prescription stimulants (prescribed for the treatment of attention deficit hyperactivity disorder (ADHD)). While in Europe the extent of this phenomenon has not been fully evaluated (though some European countries have reported non-medical use of prescriptions drugs), in the USA the problem has dramatically increased over the last years [1,2].

Drug abuse can lead to the phenomenon of addiction, defined as a chronic, relapsing brain disease characterized by compulsive drug seeking and use, despite harmful consequences [3,4]. Addiction arises from the changes in the brain circuitry that the drugs produce over time. Drugs of abuse activate the brain's reward system, particularly the mesocorticolimbic dopaminergic pathway, producing positive reinforcing effects that powerfully compel people to keep using drugs, thus leading to addiction [4]. Moreover, long-term abuse causes neurobiological changes that impair cognitive function and may trigger mental disorders [3]. Besides effects on the brain, drug abuse has other serious health consequences, such as chronic diseases and organ failure associated with repeated use, blood-borne bacterial and viral infections (among injecting drug users, 17.9% is HIV positive and 50% is infected with HCV) and acute toxic effects, including overdose. Each year between 104,000 and 263,000 deaths are related to or associated with the use of illicit drugs and over half of these deaths are fatal

overdose cases occurring mainly in young people in the mid-thirties. In addition, drug abuse strongly affects society on multiple levels. For example, illicit drug use generates criminal activities, people under the influence of drugs can cause accidents and the risk of spreading infectious diseases is increased [1,3].

Different strategies can be employed to reduce drug abuse and limit its negative consequences. Prevention programs aimed to increase the awareness of drug-induced harms have proved to be effective in decreasing drug abuse [3]; controls carried out on people that perform particular tasks, such as those requiring high attention (e.g. driving), in order to ascertain drug intake can prevent accidents; proper medical treatment helps patients improve their health conditions and the quality of their lives and limits the social harm related to drug abuse.

Reliable analytical methods can play an important role in this setting. They can be employed to reveal drug intake, allowing the identification of drug users and to assess drug blood levels, assisting physicians in the management of the treatment. In fact, pharmacological therapy needs to be carefully monitored in order to optimize the dose scheduling according to the specific needs of the patient and to discourage improper use of the medication.

Therefore, the aim of this thesis work was to develop original analytical methods for the determination of drugs with a potential for abuse and of substances used in the pharmacological treatment of drug addiction in biological samples. In particular, methods have been developed for the detection of long-acting opioids, used in the management of opioid addiction; oxycodone, one of the most abused opioid analgesics in the United States; disulfiram and bupropion, prescribed for the treatment of alcohol and nicotine dependence, respectively; and ketamine, a dissociative anaesthetic increasingly used for recreational purposes. All the presented methods are based on high performance liquid chromatography (HPLC) coupled to various kinds of detectors (mass spectrometer, coulometric detector, diode array detector); biological sample pre-treatment was carried out using different extraction techniques, namely solid phase extraction and microextraction by packed sorbent.

The developed analytical methods will be individually described and discussed.

2. ANALYSIS OF NALOXONE AND LONG-ACTING OPIOIDS IN HUMAN PLASMA BY HPLC-ED

2.1. Introduction

Opioid dependence still represents a significant global problem affecting the lives of millions of people worldwide and is responsible for a higher demand of treatment than addiction to other drugs, thus reflecting the considerable harm associated with opioid abuse [1,5]. The combination of psychosocial support and opioid replacement therapy (ORT) with long-acting opioids (i.e. methadone and buprenorphine) has proved to be the most effective approach to opioid dependence [5].

Since its introduction in the 1960s, methadone ((RS)-6-(dimethylamino)-4,4-diphenylheptan-3-one, MTD, **Fig. 1**), a synthetic full μ-opioid receptor agonist, has been considered the treatment of choice for opioid addiction [6,7]. MTD blocks the euphoric effects of heroin, reduces craving and prevents withdrawal symptoms, thereby relieving the patient of the need to use heroin [8,9]. Thanks to its long duration of action (15-60 hours), a single daily dose is sufficient to stabilize the patient, avoiding the ups and downs experienced while on heroin [8]. MTD is administered by oral route at doses ranging from 60 to 120 mg day^{-1} or more [5]; it is metabolized in the liver by N-demethylation to inactive metabolites.

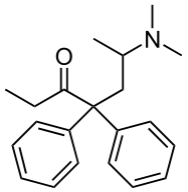
More recently, another long-acting opioid, buprenorphine ((2S)-2-[(-)-(5R,6R,7R,14S)-9α-cyclopropylmethyl-4,5-epoxy-6,14-ethano-3-hydroxy-6-methoxy-morphinan-7-yl]-3,3-dimethylbutan-2-ol, BPN, **Fig. 1**), has been introduced as an alternative treatment for opioid addiction [10]. BPN is a semisynthetic opioid derived from thebaine that acts as a partial μ-opioid receptor agonist and a k-opioid receptor antagonist [6]. It has also been shown to activate the opioid receptor-like (ORL-1) receptor, also known as nociceptine (NOP) receptor [11]. As a partial agonist, it produces minimal withdrawal symptoms and has a low potential for abuse and a low risk of overdose fatalities compared to full opioid agonist, thereby appearing to be a safer alternative to MTD [6]. BPN is extensively metabolized by glucuronidation and N-dealkylation. The latter is mainly mediated by cytochrome P450 (CYP 3A4 isoenzyme) and leads to the formation of the potent active metabolite norbuprenorphine

(N-BPN, **Fig. 1**) [6,12]. BPN is administered sublingually to avoid extensive first-pass hepatic metabolism [12]. The typical dosage that produces stable effects is in the range of 8-32 mg day^{-1} [5].

Although BPN has a low potential for abuse compared to other opioids, illicit diversion to the intravenous route has been reported [13]. Thus, in an effort to reduce parenteral abuse liability, a new formulation containing buprenorphine and naloxone in a 4:1 ratio (Suboxone®) has been developed [6,12]. Naloxone ((5α,17R)-4,5-epoxy-3,14-dihydroxy-17-(2-propenyl)-morphinan-6-one, NLX, **Fig. 1**) is an opioid antagonist with poor sublingual bioavailability [10]. Therefore, when taken sublingually as prescribed, the NLX moiety is not effective and does not interfere with the pharmacological actions of BPN. However, if an opioid-dependent individual abuses the medication intravenously, NLX becomes readily available, inhibits the opioid effects of BPN and precipitates opiate-withdrawal, thus reducing the potential for illicit diversion and intravenous misuse of BPN [6].

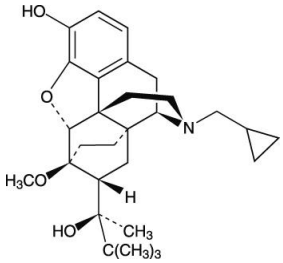
The outcome of ORT is highly variable depending on different factors, including individual metabolism. For this reason, it would be advisable to carefully monitor plasma levels of BPN and MTD in order to tailor the dose to each patient's needs. Moreover, since Suboxone® has been introduced in the market quite recently, further data should be collected to confirm the poor sublingual bioavailability of NLX. To these purposes, it is desirable to have at disposal high-sensitivity, low-cost analytical methods to simultaneously determine plasma levels of NLX and long-acting opioids. Some papers in the literature report the determination of MTD (with or without other substances) in human plasma/serum [14-17], urine [15,16,18,19], whole blood [20] or exhaled breath [21] samples by means of high performance liquid chromatography (HPLC) with UV/diode array detection (DAD) [14,16,19] or coupled with mass spectrometry (MS) [17,18,20,21]. Several papers deal with the analysis of BPN alone or with its metabolite or other drugs (e.g. NLX or MTD) in specimens such as urine [16,22-28], plasma [16,29,30], whole blood or dried blood spots [28,31,32], hair [28] and other tissues [33,34] using HPLC-MS [22,23,25,27-34], HPLC-DAD [16] or gas-chromatography (GC) with MS [24,26]. The simultaneous analysis of some illicit drugs,

including MTD, NLX and BPN, in human hair samples has been reported several years ago by means of HPLC with coularray detector [35]. More recently, a LC-MS/MS method for the determination of several opioid drugs (including BPN, N-BPN, MTD and NLX) in post-mortem blood and urine has been published [36]. To the best of knowledge, no analytical method based on the use of HPLC with coulometric detection is currently available for the simultaneous determination of NLX, BPN, N-BPN and MTD in plasma samples. As concerns the extraction of these drugs from biological specimens, it usually involves liquid–liquid extraction (LLE) [20,24,36], solid phase extraction (SPE) [15-17,25,28-30,34] or microwave assisted extraction [14,19] procedures. In the field of sample pre-treatment, microextraction by packed sorbent (MEPS) represents a recent technique for miniaturized solid phase extraction. The main difference between MEPS kit and SPE cartridge is that in the former the solid sorbent is inserted into a syringe as a plug and can be reused several times. MEPS greatly reduces the volume of sample and solvents used in the procedure from the millilitre to the microlitre range. Moreover, it can be connected on-line to liquid or gas chromatography without any modification of the chromatographic apparatus. Since MEPS takes only a few minutes for one analysis, it allows a rapid pre-treatment of biological samples. Given the advantages offered by this extraction technique, the aim of the present work was to develop a HPLC/coulometric detection method combined with sample preparation by MEPS for the simultaneous determination of NLX, BPN, N-BPN and MTD in human plasma in order to perform therapeutic drug monitoring in patients under treatment for heroin abuse and to ascertain NLX sublingual availability.



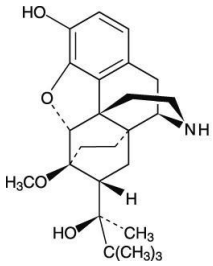
Methadone

(MTD)



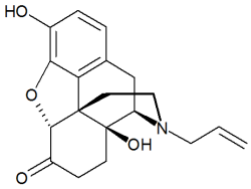
Buprenorphine

(BPN)



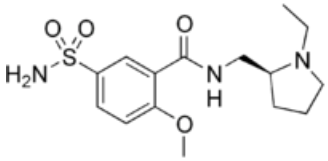
Norbuprenorphine

(N-BPN)



Naloxone

(NLX)



Levosulpiride

(IS)

Fig. 1

Chemical structures of methadone, buprenorphine, norbuprenorphine, naloxone and levosulpiride, used as the internal standard (IS).

2.2. Experimental

Chemicals

Methanolic stock solutions of BPN, N-BPN, MTD and NLX (0.1 mg mL^{-1}) were kindly provided by Dr. Matteo Conti from the Clinical Laboratory of Area Vasta Romagna (Ravenna, Italy). Levosulpiride, used as the internal standard (IS, **Fig. 1**), was purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile and methanol for HPLC, 85% (w/w) phosphoric acid and disodium hydrogen phosphate were also from Sigma Aldrich. Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA).

Preparation of stock and working solutions

Stock solutions of the IS (1 mg mL^{-1}) were prepared by dissolving 5 mg of pure substance in 5 mL of methanol and were stored at -20°C.

Working standard solutions of the analytes and the IS were prepared daily by diluting the primary stock solutions with the mobile phase.

Sample collection

Blood samples (3 mL) were drawn from patients subjected to ORT with Suboxone® at daily doses between 6 and 16 mg or with MTD at daily doses between 100 and 150 mg at Drug Addiction Treatment Centres (Ser.T.) in Cossato (Biella, Italy) and Bologna (Italy). Blood samples were usually collected in the morning from fasting patients, 12-18 hours after the last drug administration. The study was approved by local review board and informed consent was obtained for experimentation with human subjects. Blood was stored in glass tubes containing ethylenediaminetetraacetic acid (EDTA) as the anticoagulant and then centrifuged (within 2 hours from collection) at 4000 rpm for 15 minutes at 5°C. The supernatant (plasma) was then transferred to polypropylene tubes and stored at -80°C until analysis.

Blood samples from healthy volunteers, used as blank samples, were treated in the same way.

Equipment

The HPLC apparatus used for the analyses consisted of a Jasco (Tokyo, Japan) PU-1580 chromatographic pump and an ESA (Milford, MA, USA) Coulochem III coulometric detector equipped with a high sensitivity analytical cell having porous graphite working electrodes and α-hydrogen/palladium reference electrodes. MEPS was carried out by means of a BIN (Barrel Insert and Needle Assembly) containing 4 mg of solid phase silica-C8 sorbent inserted into a 250 µL gas-tight syringe from SGE Analytical Science (Melbourne, VIC, Australia). A Crison (Barcelona, Spain) MicropH 2000 pHmeter, a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge and an IKA (Staufen, Germany) RV 10 rotary evaporator were also used.

Data were handled by means of Chromatography Station (CSW 32 v. 1.4) software from DataApex (Prague, Czech Republic).

Chromatographic conditions

The chromatographic separation was achieved by isocratic elution on a Discovery® cyano column (3.0 x 250 mm, 5 µm) (Supelco, Bellefonte, PA, USA) kept at room temperature (25 ± 3°C). The mobile phase was a mixture (40:60, v/v) of acetonitrile and phosphate buffer (2.5 mM, pH 6.4). The flow rate was 0.6 mL min^{-1} and the samples were injected by means of a 50 µL loop. Prior to use, the mobile phase was filtered through Varian nylon filters (47 mm diameter, 0.2 µm pore size) and degassed by sonication.

The conditioning cell of the coulometric detector was set at +0.050 V; in the analytical cell, detector 1 (E1) was set at -0.200 V and detector 2 (E2) at +0.600 V, with a range of 10 nA and an output of +1.00 V. The analytes were monitored in oxidation at the analytical detector 2.

Sample pre-treatment: microextraction by packed sorbent

MEPS procedure was performed using a BIN containing 4 mg of C8 sorbent. The packed material was activated with 3 x 100 µL of acetonitrile and then conditioned with 3 x 100 µL of water. The volumes of acetonitrile and water were drawn up and then discarded every time at a flow rate of 20 µL sec^{-1}. A volume of 100 µL of plasma was added with 10 µL of the IS (on-column concentration 25 ng mL^{-1}) and was then diluted with 200 µL of water. A 100 µL aliquot of the resulting mixture was drawn up and down through the syringe 15 times (at a flow rate of 5 µL s^{-1}) without discarding it. The sorbent was washed twice with water (100 µL) and once with a mixture of acetonitrile and water (5:95, v/v; 100 µL) to remove biological interference, then the analytes were eluted with 2 x 250 µL of acetonitrile. The eluate was dried under vacuum using a rotary evaporator; the residues thus obtained were reconstituted with 100 µL of mobile phase and injected into the HPLC system. After each extraction, the sorbent was cleaned with 3×100 µL of acetonitrile followed by 3×100 µL of water. The cleaning step also acted as the conditioning step for the following extraction of the analytes. One packing bed was used for about 60 extractions, then it was discarded due to low analyte extraction yields and sorbent clogging.

Method validation

The method was validated according to USP XXXII [37] and "Crystal City" [38] guidelines.

- *Linearity, limit of quantitation, limit of detection*

Aliquots of 10 µL of analyte standard solutions at six different concentrations, containing the IS at a constant concentration, were added to 100 µL of blank plasma. The resulting calibration standards were subjected to the previously described MEPS procedure and then injected into the HPLC system. Calibrators were prepared in triplicate for each point and covered the following plasma ranges: 0.25-20.0 ng mL^{-1} for BPN and N-BPN, 3.0-1000.0 ng mL^{-1} for MTD and 0.13-10.0 ng mL^{-1} for NLX. The

concentration of the IS was 25 ng mL^{-1}. The analyte/IS peak-area ratios obtained were plotted against the corresponding concentrations of the analytes (expressed as ng mL^{-1}) and the calibration curves were constructed by means of the least-square method.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as the analyte concentrations which give rise to peaks whose heights are 3 and 10 times the baseline noise, respectively.

- *Extraction yield and precision*

For these assays plasma samples at three different concentrations of the analytes, corresponding to the lowest, the intermediate and the highest point of the calibration curve, were prepared. The samples, containing 0.25, 10.0 and 20.0 ng mL^{-1} of BPN and N-BPN, 3.0, 500.0 and 1000.0 ng mL^{-1} of MTD and 0.13, 5.0 and 10.0 ng mL^{-1} of NLX, were subjected to the previously described MEPS procedure and injected into the HPLC. The analyte peak areas of the spiked samples were compared to those obtained injecting standard solutions at the same theoretical concentrations and the extraction yield (expressed as percentage value) was calculated.

The assays described above were repeated six times within the same day to obtain repeatability (intraday precision) and six times over six different days to obtain intermediate precision (interday precision), both expressed as percentage relative standard deviation values (RSD%).

- *Selectivity*

The selectivity of the method was evaluated with respect to endogenous and exogenous compounds. Blank plasma samples from six different healthy volunteers were processed in the absence of the IS and the resulting chromatograms were checked for possible interference at the retention time of the analytes and the IS. Selectivity towards exogenous substances was investigated by injecting into the HPLC system standard solutions of several compounds that could be co-administered in clinical practice. A substance was classified as interfering if it gave rise to a peak that was not baseline separated from those of the analytes or the IS.

- *Stability*

Stock solution stability was tested by comparing the analyte/IS peak-area ratios of a standard solution (containing 10 ng mL^{-1} of BPN, N-BPN and NLX and 500.0 ng mL^{-1} of MTD) prepared from stock solutions stored for 1 month at -20°C with those of a standard solution at the same theoretical concentrations obtained from fresh stock solutions (n = 3). Stability assays were also carried out in blank plasma samples fortified with 10 ng mL^{-1} of BPN, N-BPN and NLX and 500.00 ng mL^{-1} of MTD. Sample stability was evaluated over five hours at room temperature, after three freeze-thaw cycles and after 1 month of storage at -80°C (n = 3 per storage condition).

- *Accuracy*

Analyte standard solutions at three different concentrations (in order to obtain analyte additions of 2.5, 5.0 and 7.5 ng $mL^{-1}$for BPN, N-BPN and NLX and 10.0, 250.0 and 500.00 ng mL^{-1} for MTD) were added to plasma samples from patients under ORT whose analyte concentrations had been previously determined (n = 3 for each level); then, the mixtures were subjected to the MEPS procedure. Accuracy was expressed as percentage recovery and was calculated according to the following formula: 100 × ([after spiking] – [before spiking]) / [added], where [after spiking] is the sum of the concentrations of the analyte in the real plasma and in the analyte standard solution added; [before spiking] is the concentration of the analyte in the real sample; and [added] is the concentration of the analyte standard solution added to the real sample.

2.3. Results and discussion

Development of the chromatographic conditions

The main problem faced during method development arose from the great lipophilicity difference between NLX (logP = 0.6) and all the other compounds (logP ~ 4) [39]. Different stationary phases were tested and several experiments were carried out on the composition of the mobile phase to obtain an acceptable simultaneous chromatographic separation of all analytes. Starting from an analytical method previously developed for the analysis of BPN, N-BPN and MTD by HPLC with DAD detector [16], a C8 column and a mobile phase consisting of a mixture of phosphate buffer, acetonitrile and methanol (40:10:50, v/v/v) were tried, but these conditions turned out to be unsuitable to provide a reasonable retention of NLX. The influence of the percentage of the aqueous phase (from 40% to 50%) and acetonitrile (from 10% to 0%) on the retention of NLX was then studied, keeping constant the percentage of methanol (less lipophilic than acetonitrile) at 50%. Unfortunately, none of these adjustments allowed the separation of NLX from the void peak, while the retention times (t_r) of BPN, N-BPN and MTD stretched over 25 minutes. It was therefore decided to change the stationary phase and hence the retention mechanism. More lipophilic phases than C8 (C18 and pentafluorophenylpropyl) were tested, but the results were unsatisfactory. Eventually, a cyanopropyl column was tried with a mobile phase consisting of a mixture of acetonitrile and phosphate buffer in the ratio 40/60 (v/v); the cyano column turned out to be sufficiently hydrophilic to provide greater retention of NLX and elution of the more lipophilic analytes (BPN, N-BPN and MTD) in a reasonable time. Afterwards, the ionic strength and the pH of the buffer used in the mobile phase were carefully optimized as they significantly affect the t_r of all analytes. The buffer concentration was studied in the range from 1 to 10 mM (pH = 6.4): a low ionic strength buffer led to a lengthening of the t_r of all analytes (**Fig. 2a**), providing a better chromatographic separation. With regard to the pH of the phosphate buffer, the retention times of BPN, N-BPN and MTD were more strongly influenced by small

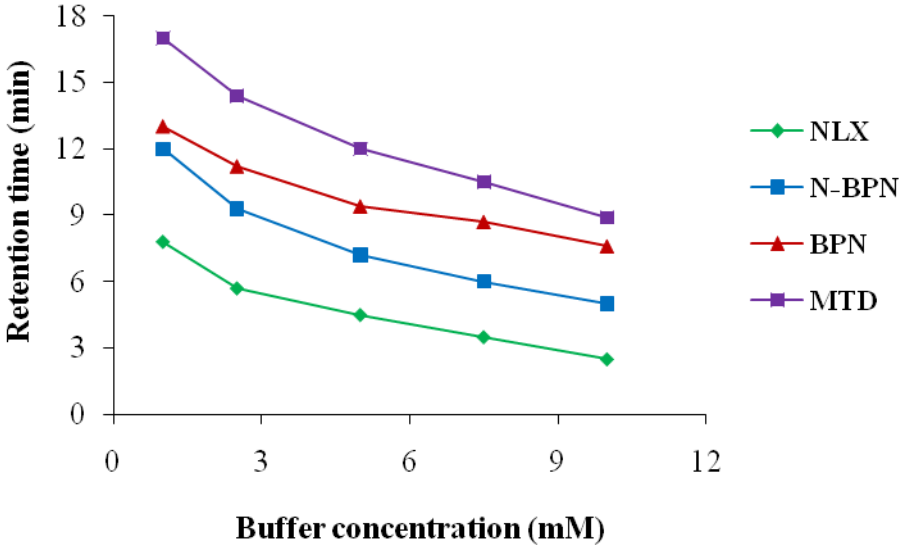
changes in the pH values (pH studied in the 5.0-7.5 range) than that of NLX **(Fig. 2b)**. Eventually, a 2.5 mM phosphate buffer at a pH value of 6.4 was chosen as the final component of the mobile phase.

Another challenging step was to find the best electrochemical conditions for the detection of the analytes, as BPN, N-BPN and MTD are hardly oxidizable compounds. Preliminary voltammetric studies revealed that a pH value of the mobile phase higher than 5.0 was necessary to oxidize the analytes. Moreover, several trials were carried out to improve the performance of the coulometric detector in terms of sensitivity and selectivity. An oxidation potential of +0.600 V was chosen for E2, resulting in good sensitivity; E1, used as the screening electrode, was set at a reduction potential of -0.200 V obtaining a satisfactory cut-off of biological interference.

As regards the selection of a suitable internal standard, some electroactive compounds such as substituted benzamides were tested. Among these, the most appropriate was found to be levosulpiride which showed a chromatographic behaviour similar to that of the analytes and did not lengthen the total run time.

The chromatogram of a standard solution containing 5.0 ng mL^{-1} of NLX, 10 ng mL^{-1} of BPN and N-BPN, 250.0 ng mL^{-1} of MTD and 25 ng mL^{-1} of IS is reported in **Fig. 3**. As can be seen, in the optimized chromatographic conditions the analytes are well resolved within an acceptable total run time and NLX is reasonably separated from the void peak. Retention times (t_r) are: NLX, t_r = 5.7 min; N-BPN, t_r = 9.3 min; BPN, t_r = 11.2 min; MTD, t_r = 14.4 min; IS, t_r = 7.5 min.

a



b

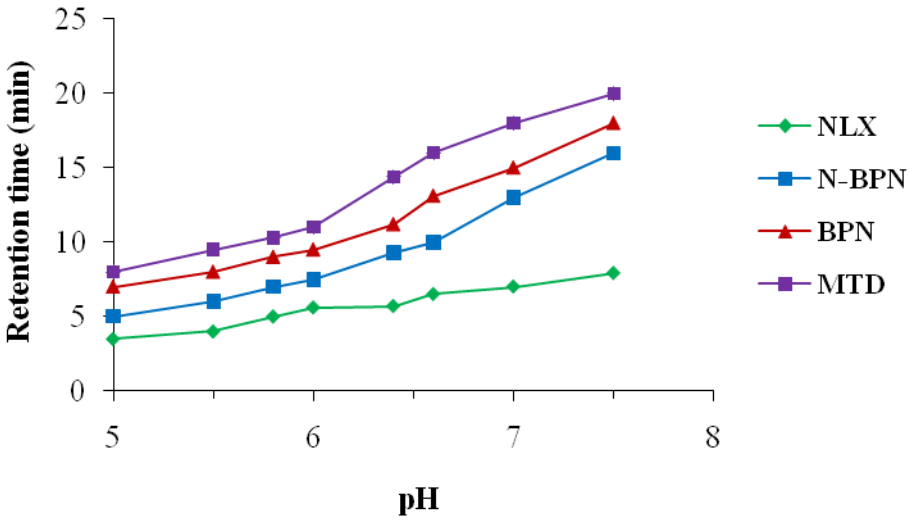


Fig. 2

Effect of the buffer concentration (a) and of the buffer pH (b) on the retention times of the analytes.

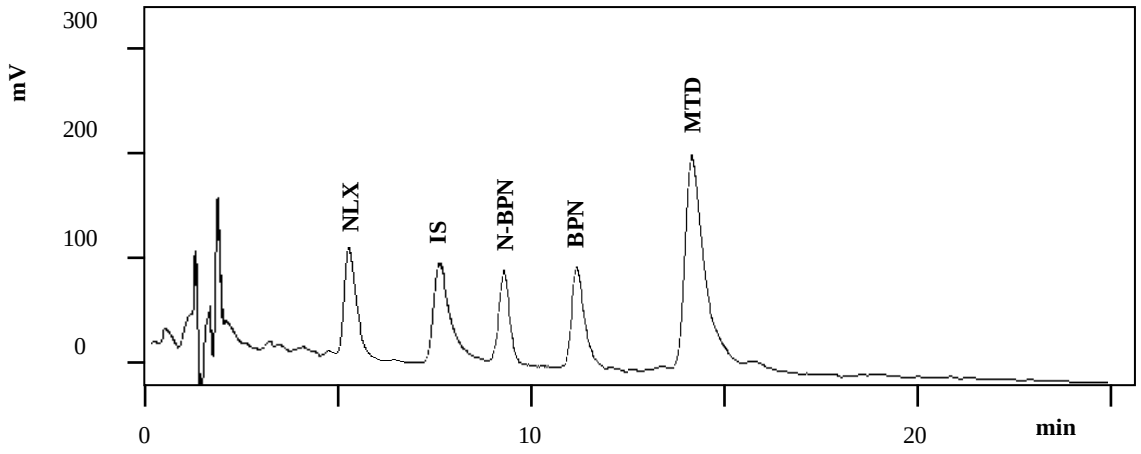


Fig. 3

Chromatogram of a standard solution containing 5.0 ng mL^{-1} of NLX, 10.0 ng mL^{-1} of BPN and N-BPN, 250.0 ng mL^{-1} of MTD and 25.0 ng mL^{-1} of IS.

Development of the MEPS procedure

MEPS is a novel approach in the field of sample preparation that provides notable advantages over the more widely used SPE and LLE procedures, namely miniaturization, short extraction times and consumption of small solvent volumes. Therefore, MEPS was chosen to perform sample clean-up.

The extraction of the analytes from plasma specimens was carried out using a MEPS BIN packed with a C8 sorbent and the factors affecting the performance of the procedure were thoroughly investigated. An aliquot of the sample volume can be drawn up and down through the sorbent at different speeds once or several times (cycles) without discarding it. Thus, in the loading step the number of cycles and the flow rate applied are two of the parameters that affect the retention of the analytes to the sorbent. Cycles and speeds were investigated in the range between 8-15 and 5-20 µL sec^{-1}, respectively, loading sample aliquots of 50 or 100 µL. A number of 15 extraction cycles carried out at 5 µL sec^{-1} gave mean extraction yield values of 90% for all analytes when 100 µL of sample were loaded (**Fig. 4**). The influence of different washing solutions on the purification of the matrix and on the absolute recovery of the analytes was investigated. Clean plasma extracts were obtained with 100 µL of water (drawn twice at a flow rate of about 20 µL sec^{-1}) followed by a further aliquot of a mixture containing acetonitrile and water (5:95, v/v) to better remove the interference from the biological matrix. The elution efficiency was tested using mobile phase, acetonitrile and methanol. Mobile phase proved to be almost ineffective (extraction yields < 40.0%) while better results were obtained using acetonitrile and methanol. Acetonitrile was chosen as the elution solvent and a linear relationship between the absolute recovery of the analytes and the volume of acetonitrile was established in the 100–500 µL range. Accordingly, 500 µL of acetonitrile was selected for the elution of the analytes.

The chromatograms of a blank plasma sample and of the same sample fortified with known amount of the analytes and the IS are reported in **Fig. 5**. As can be seen, no endogenous interference was present at the retention times of the analytes, showing that the developed MEPS procedure provided good cleaning of the biological matrix.

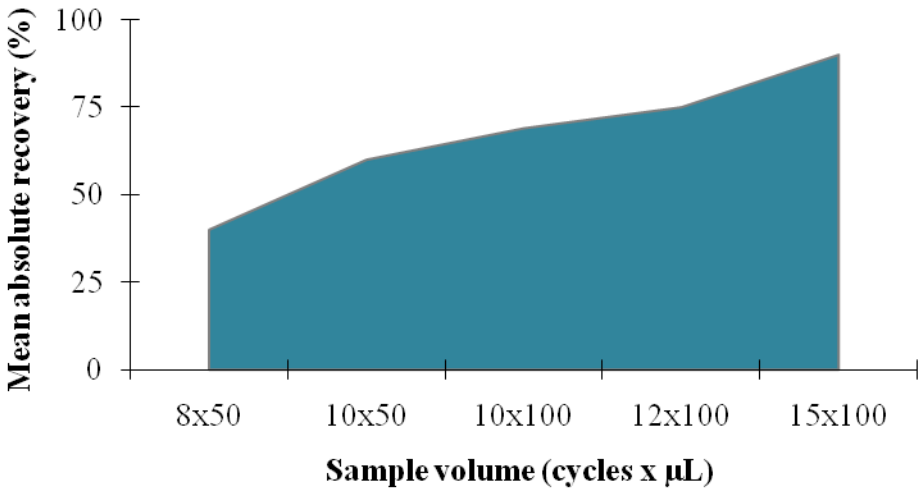


Fig. 4

Mean absolute recovery of NLX, BPN, N-BPN and MTD as a function of applied sample volumes.

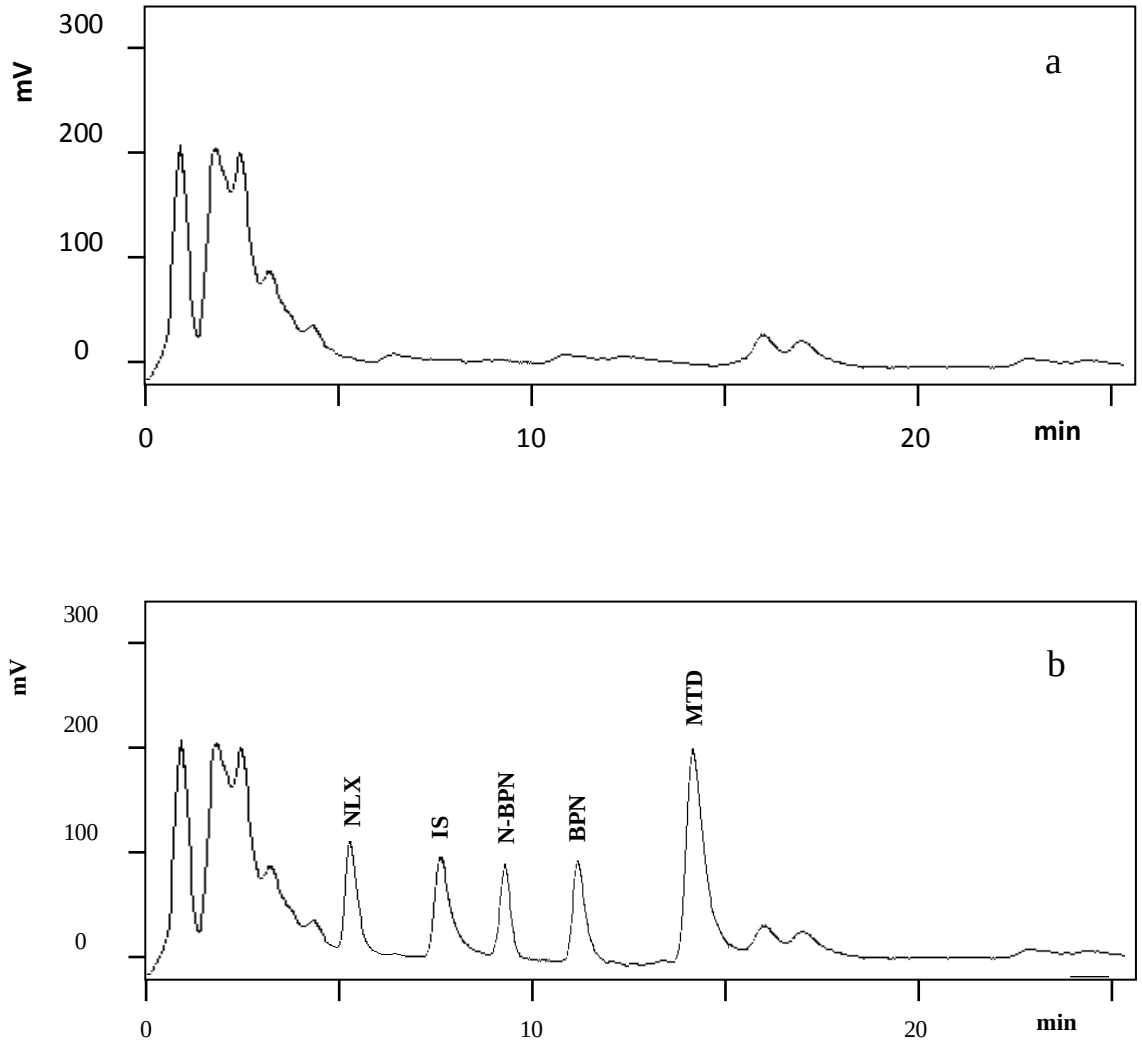


Fig. 5

Chromatograms of a blank plasma sample (a) and of the same sample spiked with 5.0 ng mL^{-1} of NLX, 10.0 ng mL^{-1} of BPN and N-BPN, 250.0 ng mL^{-1} of MTD and 25.0 ng mL^{-1} of IS (b).

Method Validation

Calibration curves were set up for all analytes and good linearity ($r^2 > 0.9994$) was found over the studied concentration ranges. LOD and LOQ values, reported in **Table 1**, show the good sensitivity of the method.

Extraction yield and precision assays were carried out at three different concentrations, corresponding to the lowest, the intermediate and the highest point of each calibration curve. The results of these assays are reported in **Table 2**. As can be seen, good extraction yield was obtained with values higher than 85%; the mean extraction yield of the IS was 95%. Precision was also satisfactory, with RSD values always lower than 4.3% for all analytes.

- *Selectivity*

The analysis of plasma samples from six different healthy volunteers showed no evidence of unacceptable interference from endogenous compounds at the retention time of the analytes and the IS. Several compounds that could be co-administered during clinical practice, such as antipsychotics, antidepressants and sedative-hypnotics, were tested for possible interference; none of them gave rise to peaks that could interfere with the determination of the analytes.

- *Stability*

The mean difference in the analyte/IS peak-area ratios between a standard solution of the analytes prepared from stored stock solutions and a standard solution at the same nominal concentration obtained from fresh stock solutions was -0.1%, indicating that all the analytes were stable in methanol when stored at -20°C for 1 month.

Stability was also assessed in plasma samples fortified with known amount of the analytes and stored under various conditions. The concentrations of the analytes found after storage were compared to the respective theoretical concentrations. The mean differences observed were -0.6% for plasma sample kept at room temperature for 5 hours, -1.0% for samples subjected to three freeze-thaw cycles and -1.2% for samples

stored at -80°C for 1 month. The loss of the analytes was not significant, indicating that the compounds are stable under the tested storage conditions.

Analyte	Linearity range (ng mL^{-1})	Equation coefficients (y = ax + b) [a]		r^2 [b]	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
		a	b			
BPN	0.25-20.0	0.1032	0.0035	0.9996	0.08	0.25
N-BPN	0.25-20.0	0.1217	-0.0057	0.9994	0.08	0.25
MTD	3.0-1000.0	0.0131	0.0047	0.9997	0.90	3.0
NLX	0.13-10.0	0.1819	0.0023	0.9994	0.04	0.13

[a] y = analyte/IS peak-area ratio; x = analyte concentration (ng mL^{-1})
[b] r^2 = correlation coefficient

Table 1

Linearity parameters.

Analyte	Concentration (ng mL^{-1})	Extraction yield (%)[a]	Repeatability (RSD%)[a]	Interday precision (RSD%)[a]
BPN	0.25	96	3.45	3.83
	10.0	94	3.15	3.65
	20.0	89	2.95	3.11
N-BPN	0.25	95	3.09	3.85
	10.0	93	2.87	3.54
	20.0	88	2.64	3.26
MTD	3.0	94	3.80	4.29
	500.0	92	3.72	3.95
	1000.0	90	3.45	3.77
NLX	0.13	93	3.56	3.96
	5.00	91	3.33	3.72
	10.0	86	3.16	3.53

[a] n = 6

Table 2

Validation parameters: extraction yield and precision data.

Analysis of plasma samples from patients

The validated method was applied to the analysis of plasma specimens from some patients under ORT with Suboxone® or MTD. As an example, the chromatogram of a plasma sample from a patient taking 16 mg day^{-1} of BPN and 4 mg day^{-1} of NLX is reported in **Fig. 6a**; the plasma levels found were: 4.30 ng mL^{-1} for BPN; 3.43 ng mL^{-1} for N-BPN; plasma levels of NLX were below the LOQ value (0.13 ng mL^{-1}). The chromatogram of a plasma sample from a patient treated with 150 mg day^{-1} of MTD is shown in **Fig. 6b**; plasma level found was 325 ng mL^{-1}. Plasmatic concentrations were in the therapeutic range for either drugs (i.e. 0.5-10 ng mL^{-1} for BPN [13,40] and 40-1000 ng mL^{-1} for MTD [41]), so both patients were considered "responder" to the therapy. NLX plasma levels were lower than the method LOQ in all subjects analysed, thus confirming the low sublingual bioavailability of this opioid antagonist. As concerns the plasmatic concentrations of MTD and BPN, a notable interindividual variability was found among the analysed patients (**Fig. 7**). These results support the importance of an appropriate monitoring to optimize ORT, especially during the beginning of the therapy or in case of switching from MTD to BPN alone or in association with NLX.

- *Accuracy*

Method accuracy was evaluated by means of recovery assays. Standard solutions of the analytes at three different concentrations were added to plasma samples from patients under ORT (n = 3 for each level) previously analysed and the percentage recovery was calculated. Results were satisfactory, as recovery values were always higher than 88.0% for all analytes.

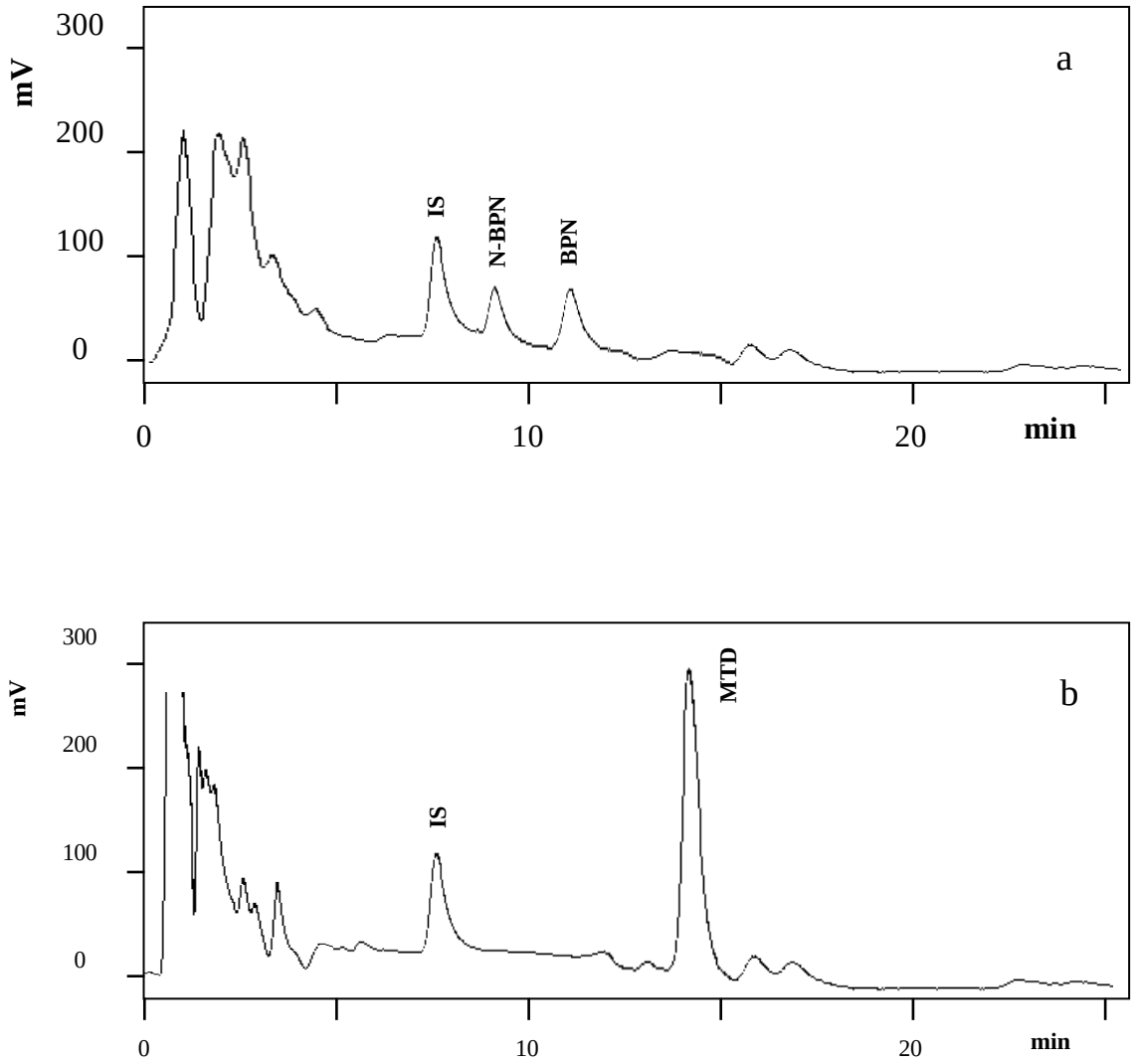
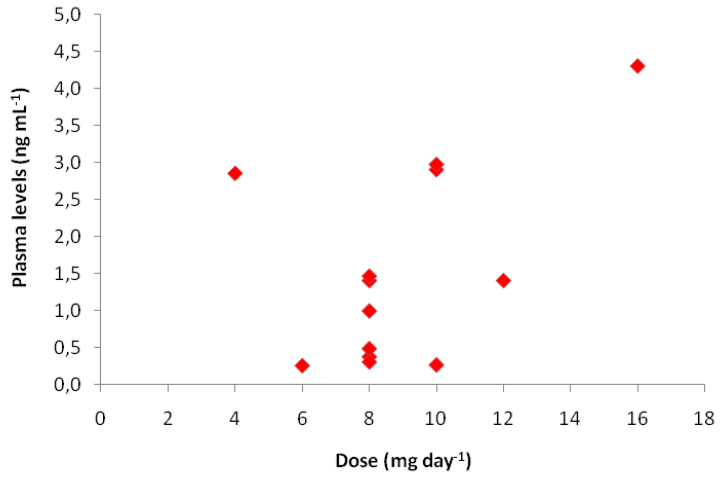


Fig. 6

Chromatograms of a plasma sample from a patient treated with Suboxone® (16 mg day^{-1} BPN/4 mg day^{-1} NLX) (a) and of a plasma sample from a patient treated with MTD (150 mg day^{-1}) (b).

a



b

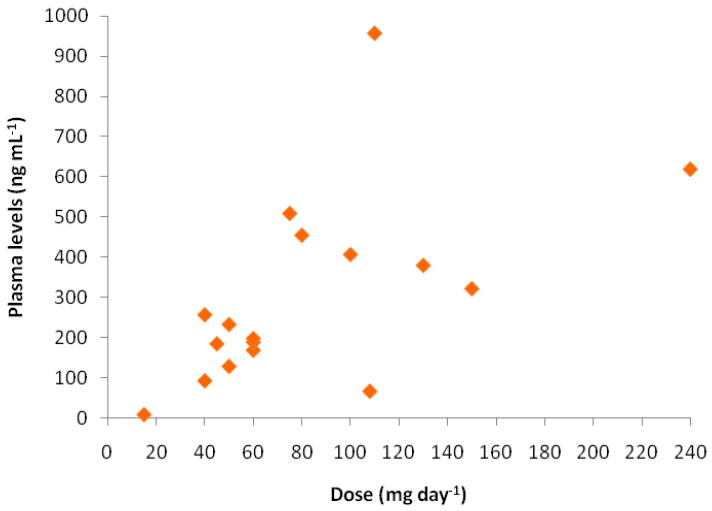


Fig. 7

Plasma levels of BPN (a) and MTD (b) found in patients under ORT plotted against the daily administered dose.

2.4. Conclusions

An analytical method based on the use of an HPLC system with coulometric detector and of microextraction by packed sorbent has been developed for the simultaneous determination of NLX, BPN, N-BPN and MTD in human plasma samples.

One of the novelties of this work is represented by the simultaneous analysis of these compounds using a coulometric detector, taking advantage of their electroactive properties. When compared to other methods reported in the literature which make use of mass spectrometry [17,18,20-23,25,27-34,36], coulometric detection represents a good alternative, as it offers high sensitivity and selectivity, requires less maintenance and is less expensive. Moreover, it is less prone to suffer from matrix effect, which could be a drawback in mass spectrometry detection. In addition, most of the LC-MS/MS based assays reported so far deal with the determination of only some of the analytes. In comparison with a method previously developed in the laboratory based on the use of HPLC-DAD for the determination of BPN, N-BPN and MTD [16], the present one is more sensitive, precise and needs a smaller aliquot of plasma sample to carry out a single analysis (100 µL instead of 300 µL). The HPLC-coulometric detector used herein is more advantageous than the method based on the use of coularray detector (different from the coulometric one in structure and function of the electrochemical apparatus) reported by Achilli et al. [35], because it is cheaper and more feasible. Furthermore, the method by Achilli et al. is not validated for plasma samples and does not include N-BPN that being an active metabolite can give important information for the management of the therapy.

Another important novelty of the present work is the development of an original MEPS procedure for the clean-up of the biological matrix. This new approach is faster and cheaper than the reported SPE or LLE procedures [15-17,20,24,25,28,30,34,36] and requires small volumes of sample (100 µL).

The method was successfully applied to the analysis of plasma specimens from patients treated for heroin addiction, supporting physicians in the pharmacological management of opioid replacing therapy.

3. DETERMINATION OF METHADONE IN DRIED BLOOD SPOTS BY HPLC-ED

3.1. Introduction

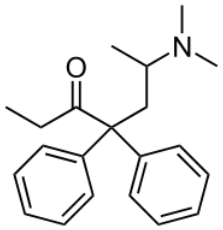
Methadone ((RS)-6-(dimethylamino)-4,4-diphenylheptan-3-one, MTD, **Fig. 1**), a synthetic full μ-opioid receptor agonist, is currently one of the drugs most frequently used in the management of opioid addiction [7]. Devoid of the euphoric effects of heroin, MTD is able to reduce craving and withdrawal symptoms thereby relieving the patient of the need to use heroin [8]. When administered orally at typical maintenance doses between 80 and 120 mg day^{-1}, MTD is readily absorbed via the gastrointestinal tract resulting in a high but variable bioavailability of 40–100% depending on the individual patient [42]. The main biotransformation of the drug mostly takes place in the liver and combines N-demethylation and cyclization to give the inactive metabolite 2-ethyl-1,5-dimethyl-3,3diphenyilpyrrolidine (EDDP) [43].

One of the main difficulties physicians have to face in the management of methadone maintenance treatment (MMT) is represented by a heterogeneous response pattern which is partly due to MTD large interindividual pharmacokinetic variability [42]. In fact, different studies have evidenced that MTD plasma levels vary for a given dose, so contributing to variability in clinical response [44]. Wide-ranging variations in the relationship between dose and plasma concentration are characteristic of drugs that are mainly metabolised and/or transported by polymorphic proteins. Genetic polymorphism in genes encoding for MTD metabolising enzymes and transporter proteins as well as for μ-opioid receptors may partly explain the observed interindividual variation in the pharmacokinetics and pharmacodynamics of MTD. Different isoforms of cytochrome P450 (CYP), in particular CYP 3A4, 2B6, 2C19 and 2D6, have been identified as the main CYP isoforms involved in MTD metabolism and some of these are subjected to genetic polymorphism [45-47]. Moreover, MTD is a P-glycoprotein substrate (ABCB1 isoform) and genetic polymorphisms of ABCB1 can also contribute to the interindividual variability of MTD kinetics and influence dose requirements [48]. In addition, during MMT, many patients take concomitant medications that might induce or inhibit some of the CYP isoforms involved in MTD metabolism, thus affecting its

pharmacokinetics. This accentuates interindividual response variability and consequentially influences the clinical effects and the safety profile of the drug. On the whole, MMT is well tolerated from a long-term perspective, however, deaths due to MTD have been reported and they seem related to the risk of overdose during induction of the therapy and to the practice of giving the patient take-home doses, with implications such as self-administration errors, combination with other substances, self-harm and diversion to the "grey market" [49]. The measurement of haematic concentrations of MTD can help assess patient compliance, tailor the dose to the patient's needs, minimize the risk of dose-related toxicity and can be useful for clinical decision making. Thus, an accurate therapeutic drug monitoring (TDM) of MTD should be carried out in patients under MMT.

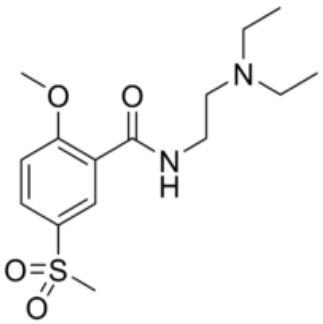
As blood (plasma, serum or whole blood) is the optimal matrix for TDM, the use of dried blood spot (DBS) sampling technique can be an attractive approach. In fact, DBSs are easily obtained via a finger prick collecting the resulting drops of blood onto filter paper and letting dry. The use of DBSs offers numerous advantages: it avoids venous blood withdrawal, thus reducing patient discomfort, it simplifies storage and transport as no refrigeration is required and it decreases the risk of infection with blood-borne pathogens [50].

To the best of knowledge, no analytical method is currently available for the determination of MTD in DBSs from patients under MMT. In fact, papers from the literature deal with the determination of MTD in human plasma/serum [14-17], urine [15,16,18,19,51], whole blood [20], exhaled breath [21] or hair [35] samples by means of HPLC methods with UV/diode array detection (DAD) [14,16,19], electrochemical detection [35] or coupled with mass spectrometry (MS) [17,18,20,21,51]. Therefore, given the importance of TDM for MMT and the advantages offered by DBS sampling technique, the aim of the present study was to optimize the method previously developed for the determination of long-acting opioids in human plasma (see page 4) for the analysis of MTD in DBS.



Methadone

(MTD)



Tiapride

(IS)

Fig. 1

Chemical structures of methadone and tiapride, used as the internal standard (IS).

3.2. Experimental

Chemicals

Methanolic stock solution of MTD (1 mg mL^{-1}) was purchased from LGC Standards (Teddington, UK). Tiapride, used as the internal standard (IS, **Fig. 1**), was from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile and methanol, 85 % (w/w) phosphoric acid and disodium hydrogen phosphate were also obtained from Sigma Aldrich. Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA).

Preparation of stock and working solutions

IS stock solutions were prepared at a concentration of 1 mg mL^{-1} by dissolving the appropriate amount of pure substance in methanol. Stock solutions were stable for at least 3 months when stored at -20°C (as assessed by HPLC assays).

Working standard solutions were prepared fresh every day by diluting primary stock solutions with the mobile phase.

Sample collection

DBS samples were obtained by puncturing the subjects (healthy volunteers and patients under MMT) on a finger with single use lancing device. The first drop of blood was wiped away using dry sterile gauze, then the blood drops were collected on a FTA® card, paying attention not to go outside the pre-marked circles. The blood spots thus obtained were left to dry for 2 h in the dark at room temperature and then stored in a sealed plastic bag containing a suitable desiccant (i.e. silica gel).

For plasma analysis, blood from the same subjects was collected by venipuncture into tubes containing ethylenediaminetetraacetic acid (EDTA) as the anticoagulant. The blood samples were centrifuged (4000 rpm, 10 min, 5°C) and the supernatant plasma was separated, transferred into polypropylene vials and stored at -20°C.

All the blood samples were collected between 8.00 a.m. and 10.00 a.m. from fasting patients under MMT at local Drug Addiction Treatment Centres (Ser.T.); this use for TDM was already authorised at the time of sampling.

Equipment

The HPLC apparatus used for the analyses consisted of a Jasco (Tokyo, Japan) PU-1580 chromatographic pump and an ESA (Milford, MA, USA) Coulochem III coulometric detector equipped with a high sensitivity analytical cell having porous graphite working electrodes and α-hydrogen/palladium reference electrodes. Microextraction by packed sorbent (MEPS) was carried out by means of a BIN (Barrel Insert and Needle Assembly) containing 4 mg of silica-C18 sorbent, inserted into a 250 µL gas-tight syringe from SGE Analytical Science (Melbourne, VIC, Australia). A Crison (Barcelona, Spain) MicropH 2000 pHmeter, a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge, an IKA (Staufen, Germany) RV 10 rotary evaporator and a microwave oven were also used. Whatman (Maidstone, UK) FTA® classic cards (WB120205) were used for DBS sample collection.

Data were handled by means of Chromatography Station (CSW 32 v. 1.4) software from DataApex (Prague, Czech Republic).

Chromatographic conditions

The chromatographic separation was achieved by isocratic elution on a cyano column (4.6 x 250 mm, 5 µm) from Waters (Milford, MA, USA) maintained at room temperature (25 ± 3°C). The mobile phase consisted of 25 mM phosphate buffer (pH 6.4) and acetonitrile (45:55, v/v). The flow rate was kept constant at 1.4 mL min^{-1} and the samples were injected by means of a 50 µL loop. Prior to use, the mobile phase was filtered through Varian nylon filters (47 mm diameter, 0.2 µm pore size) and degassed by sonication.

The conditioning cell of the coulometric detector was set at +0.050 V; in the analytical cell, detector 1 (E1) was set at +0.500 V and detector 2 (E2) at +0.700 V. The analytes were monitored in oxidation at the analytical detector 2.

Extraction from DBS

One or more DBS disks were cut out of the card after the addition of 10 µL of IS solution (on column concentration 50 ng mL^{-1}) and were placed into a vial with 250 µL of a mixture containing phosphate buffer/acetonitrile (20:80, v/v). The vial was put in a microwave oven for 80 s, then the extract was brought to dryness under vacuum using a rotary evaporator, redissolved with 250 µL of phosphate buffer (50 mM, pH 7.4) and subjected to the MEPS procedure (see section below).

The volume of blood contained in the blood spot disks was determined by pipetting known volumes of blood (from 5 to 50 µL) onto the FTA® paper cards with a micropipette: the diameter of the regular-shaped blood spots was then measured and a calibration curve constructed. A power equation was fitted and the equation was used to determine the volume of blood contained in the DBS disks from patients:

$$y = 0.931\ x^{0.352}$$

where y and x were the diameter and the volume of blood spot, respectively. The volume of blood determined in this way was used for calculating the concentration of MTD in all samples analysed.

Sample pre-treatment: microextraction by packed sorbent

The microextraction by packed sorbent (MEPS) procedure was carried out on a C18 sorbent activated with 3 x 100 µL of acetonitrile and then conditioned with 3 x 100 µL of water. The volumes of acetonitrile and water were drawn up and then discarded every time. A volume of 10 µL of IS and 100 µL of water were added to 50 µL of plasma sample. A 100 µL aliquot of this mixture or of the extract from the processed DBS was drawn up and down through the syringe 15 times without discarding it. The sorbent was washed once with water (100 µL) and once with a mixture acetonitrile/water (5:95, v/v;

50 µL) to remove biological interference; then, the analytes were eluted with 2 x 250 µL of acetonitrile. The eluate was dried under vacuum using a rotary evaporator, reconstituted in 100 µL of mobile phase and injected into the HPLC system. After each extraction, 3×100 µL of acetonitrile followed by 3×100 µL of water were passed through the sorbent in order to clean it and to avoid carry-over. These steps also acted as the conditioning step for the next extraction of the analytes. One packing bed was used for about 40 extractions; then it was discarded due to low analyte extraction yields and sorbent clogging.

Method validation

The method was validated following USP XXXII [37] and "Crystal City" [38] guidelines.

- *Linearity, limit of quantitation, limit of detection*

Aliquots of 10 µL of MTD standard solutions at six different concentrations (in order to obtain on-column concentrations over the 4-500 ng mL^{-1} range), containing the IS at a constant concentration (in order to obtain on-column concentration of 50 ng mL^{-1}), were added to DBS disks or to 50 µL of blank plasma (after the addition DBS samples were left to dry for at least 1 h). The resulting fortified DBS or plasma specimen were subjected to the previously described sample preparation and injected into the HPLC system. This procedure was done in triplicate for each point. The analyte/IS peak-area ratios were plotted against the corresponding concentrations of the analyte (expressed as ng mL^{-1}) and the calibration curve was constructed by means of the least-square method.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as the analyte concentrations which give rise to peaks whose heights are 3 and 10 times the baseline noise, respectively.

- *Extraction yield and precision*

Aliquots of 10 µL of MTD standard solutions at three different concentrations (in order to obtain on-column concentrations of 4, 250 and 500 ng mL^{-1}), containing the IS at a constant concentration (in order to obtain on-column concentration of 50 ng mL^{-1}) were added to DBS disks or to 50 µL of blank plasma. The resulting spiked DBS or plasma samples were subjected to the previously described pre-treatment procedures and finally injected into the HPLC system. The analyte peak areas were compared to those obtained injecting standard solutions at the same theoretical concentrations and the absolute recovery was calculated.

The assays described above were repeated six times within the same day to obtain repeatability (intraday precision) and six times over six different days to obtain intermediate precision (interday precision), both expressed as percentage relative standard deviation values (RSD%).

- *Selectivity*

Blank DBS or plasma samples from six different healthy volunteers were subjected to the sample pre-treatment procedure and injected into the HPLC system. The resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was no interfering peak higher than an analyte peak corresponding to its LOD. Furthermore, standard solutions of several different compounds that could be co-administered in clinical practice were injected into the HPLC system. A substance was classified as interfering if it gave rise to a peak that was not baseline separated from those of the analytes or the IS.

- *Stability*

Stability assays were carried out in DBS samples from three patients stored at room temperature (25 ± 3°C) over a period of 1 month (n = 3). DBSs were kept in the dark and in plastic bags with a suitable desiccant (i.e. silica gel). The concentrations of MTD found in the stored DBSs were compared to those obtained from the corresponding samples extracted and analysed immediately after initial spotting and drying.

- *Accuracy*

Accuracy was evaluated by means of recovery assays. Aliquots of 10 µL of MTD standard solutions at three different concentrations (i.e. 10, 50 and 150 ng mL^{-1} of MTD on-column concentrations) containing the IS at a constant concentration (in order to obtain on-column concentration of 50 ng mL^{-1}) were added to real DBS or plasma samples from subjects under MMT whose content of MTD was previously determined. Recovery values were calculated according to the following formula: 100 × ([after spiking] – [before spiking]) / [added].

3.3. Results and discussion

Development of the chromatographic conditions

Initially, the same chromatographic conditions used for the analysis of naloxone and long-acting opioids in human plasma (i.e. a cyanopropyl column with small inner diameter combined with a mixture of acetonitrile/ phosphate buffer (40:60, v/v) as the mobile phase) were applied (see page 10). However, for cheapness and simplicity the previous column was replaced by another one with the same functional group but with a conventional inner diameter (4.6 mm instead of 3.0 mm). Consequently, the mobile phase was adapted to suit the chromatographic behaviour of the analyte and the IS on the new column: a mixture of phosphate buffer (25 mM, pH 6.4) and acetonitrile (45:55, v/v) gave satisfactory results.

Some trials were carried out to improve the performance of the coulometric detector in terms of selectivity and sensitivity. To minimize the typically large solvent front due to DBS matrix effect, the screening action of the first electrode was exploited, so that the analyte could be easily detected at the second one without interference. This was achieved applying oxidation potentials of +0.500 V and +0.700 V at E1 and E2, respectively. The application of a high voltage at the second electrode avoided loss of sensitivity.

Tiapride was selected as the IS, since it showed physico-chemical properties similar to those of MTD and did not lengthen the run time.

The chromatogram of a standard solution containing 250 ng mL^{-1} of MTD and 50 ng mL^{-1} of IS is shown in **Fig. 2**. Retention times (t_r) are: MTD, t_r = 13.0 min; IS, t_r = 8.6 min.

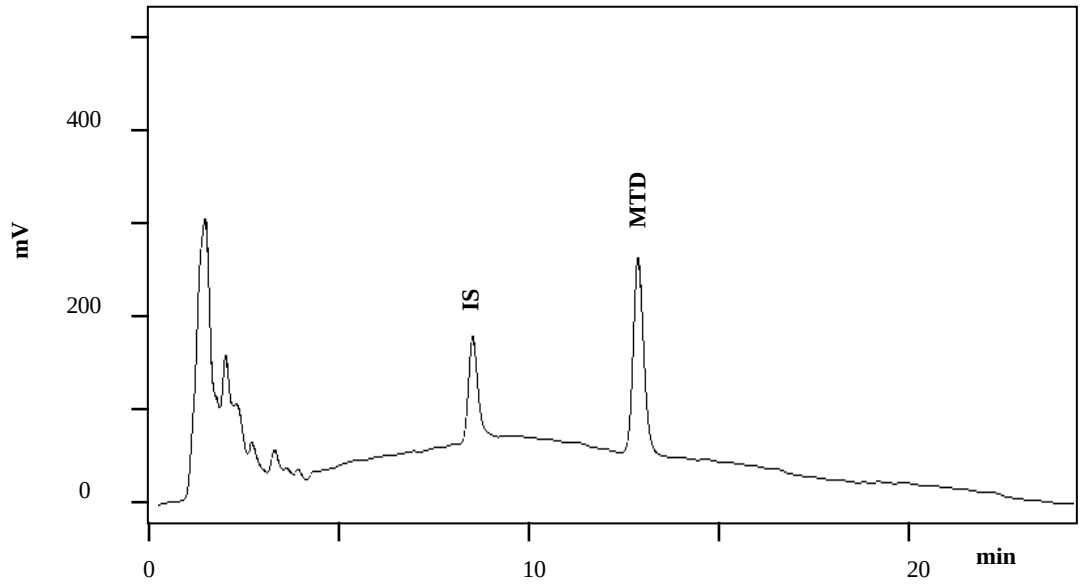


Fig. 2

Chromatogram of a standard solution containing 250 ng mL^{-1} of MTD and 50 ng mL^{-1} of IS.

Development of the extraction from DBS

Extraction efficiency of molecules from DBSs may vary depending on the optimal combination of some operating parameters, such as nature of the solvent employed, duration of the extraction procedure and technique used to aid the removal of the compounds from the filter paper. Therefore, several assays were carried out to optimize extraction efficiency and to obtain reliable results.

Spiked DBSs were treated with different solvents or mixtures (acidic or basic buffers, methanol, acetonitrile, buffer/organic solvent mixtures): pure organic solvents proved to be unsuitable to remove MTD from the paper (extraction yields < 50%), while aqueous buffers eluted red blood cells which interfered with the assay. Only phosphate buffer/acetonitrile mixture (20:80, v/v) gave promising results in terms of sample cleaning and extraction yields (> 90 %). Extraction time and mixing technique were also tested. In particular, microwave-assisted extraction, ultrasound and vortex agitation were investigated. The extraction efficiency for vortex and ultrasound agitation increased while increasing the duration of the extraction time from 5 to 10 minutes giving satisfactory extraction yield, but the employment of microwave-assisted extraction provided good results in terms of removal efficiency within 80 seconds. Hence, the latter technique was adopted.

Development of the MEPS procedure

The complexity of biological matrices such as DBS and plasma require the development of selective and reliable sample pre-treatments to minimize endogenous interference. The MEPS procedure previously developed for the analysis of NLX, BPN, N-BPN and MTD in human plasma (see page 11) proved to be suitable also for purification of the extracts from DBSs. Few minor changes were introduced to optimize the procedure for the new, more complex matrix. C18 sorbent was used instead of the C8 one as it gave cleaner extracts maintaining at the same time good extraction yields. The analyte was sufficiently retained by the sorbent after 15 drawing/discarding cycles of the loading mixture, irrespective of the matrix; good purification was obtained with

100 µL of water followed by 50 µL of acetonitrile/water 5/95 (v/v); as previously tested, two successive elution steps with 250 µL of acetonitrile gave the best elution efficiency.

Good extraction yields of MTD and the IS (> 90.0%) were obtained, while eliminating all endogenous interference from both matrices, as can be seen in **Fig 3**. and **Fig**. **4**.

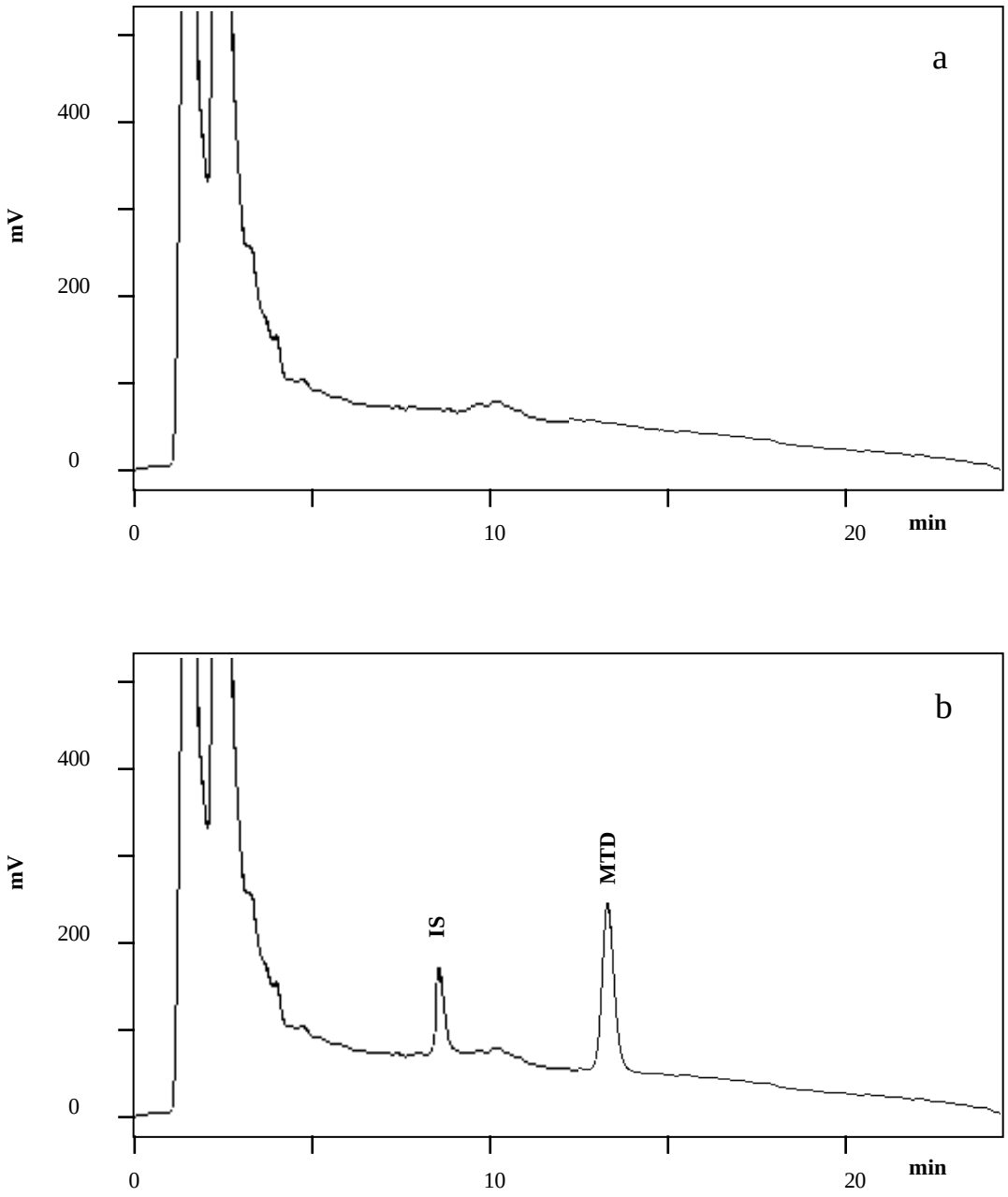


Fig. 3

Chromatograms of a blank DBS sample (a) and of the same sample spiked with a known amount of MTD and IS (b) (on-column concentrations of 250 ng mL^{-1} and 50 ng mL^{-1}, respectively).

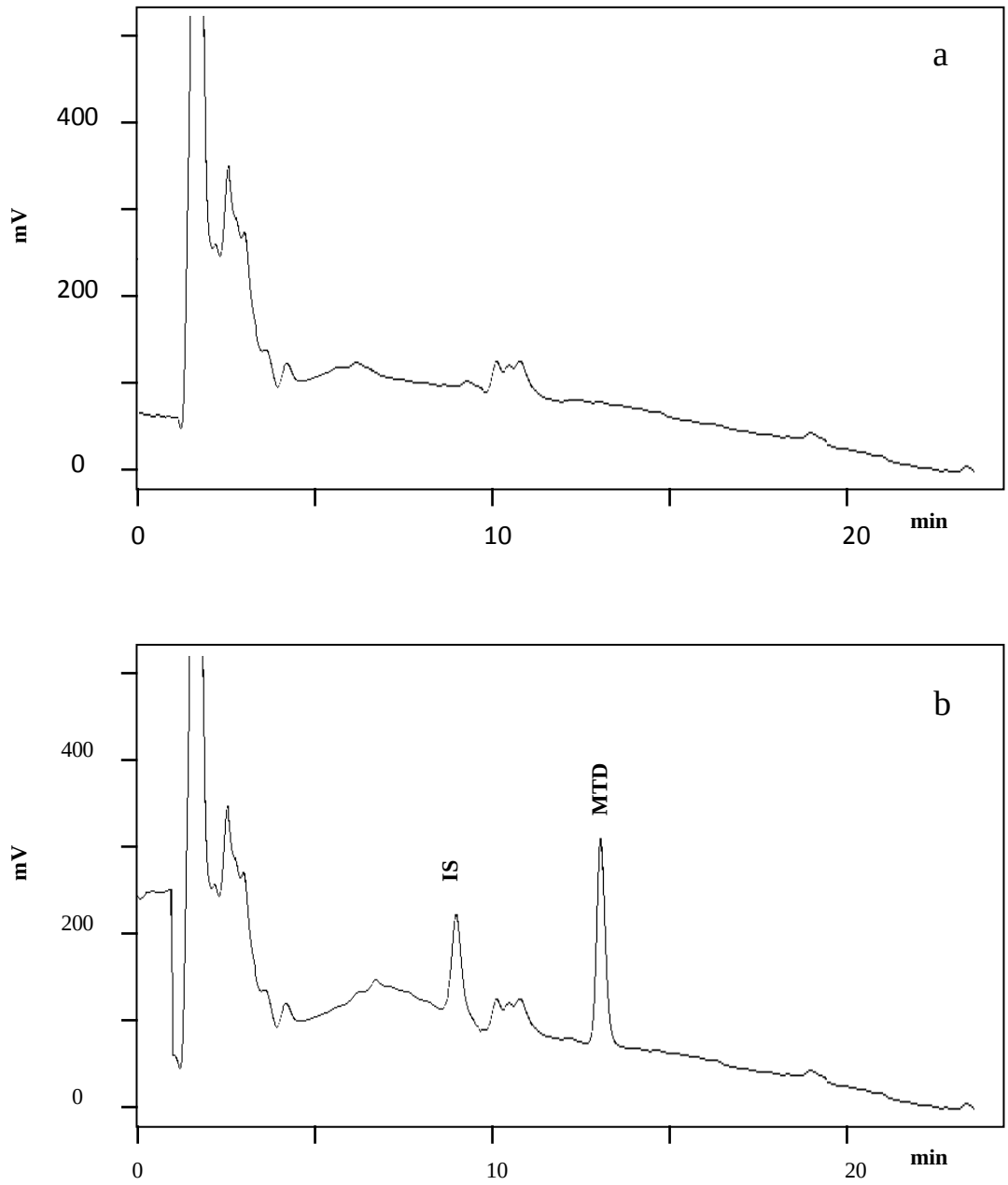


Fig. 4

Chromatograms of a blank plasma sample (a) and of the same sample spiked with a known amount of MTD and IS (b) (on-column concentrations of 250 ng mL^{-1} and 50 ng mL^{-1}, respectively).

Method Validation

Calibration curves were set up in blank matrices fortified with different concentrations of MTD and a constant concentration of the IS. Good linearity was found in the 4-500 ng mL^{-1} on-column concentration range (**Table 1**). The LOD and LOQ values were 1.2 ng mL^{-1} and 4 ng mL^{-1}, respectively.

Extraction yield and precision assays were carried out at three different concentration levels of MTD, corresponding to the lowest, the intermediate and the highest point of the calibration curve (**Table 2**). The results were satisfactory, being the extraction yield values higher than 90%; the mean extraction yield of the IS was 92%. Precision was also good, with RSD values always lower than 5.8%.

- *Selectivity*

The analysis of blank DBS and plasma samples from six healthy volunteers showed no evidence of unacceptable interference from endogenous compounds at the retention times of MTD and the IS. Standard solutions of some opioids and some antipsychotic, antidepressant and sedative-hypnotic drugs were injected into the HPLC system at concentrations higher than the upper limit of the calibration curve. None of them gave rise to peaks that could interfere with the determination of MTD.

- *Stability*

Stability was evaluated in DBS samples from 3 patients stored for 1 month at room temperature. The concentrations of MTD found in the specimens after storage were compared with those determined in the fresh samples, revealing a mean difference of -8.3%. This percentage of loss is not significant, especially if the relative long time of storage (1 month) is considered. Thus, MTD can be considered stable in DBS under the tested condition of storage.

Analyte	Matrix	Linearity range (ng mL^{-1}) [a]	Equation coefficients (y = ax + b) [b]		r^2 [c]
			a	b	
MTD	DBS	4-500	0.0048	0.0333	0.9988
	Plasma	4-500	0.0049	0.0290	0.9992

[a] on-column concentration
[b] y = analyte/IS peak-area ratio; x = analyte concentration (ng mL^{-1})
[c] r^2 = correlation coefficient

Table 1

Linearity parameters.

Analyte	Matrix	Concentration (ng mL^{-1})[a]	Extraction yield (%)[b]	Repeatability (RSD%)[b]	Interday precision (RSD%)[b]
MTD	DBS	4.0	95	4.8	5.3
		250	92	4.5	5.0
		500	90	4.4	4.8
	Plasma	4.0	96	5.2	5.8
		250	94	5.0	5.3
		500	91	4.9	5.0

[a] on-column concentrations
[b] n = 6

Table 2

Validation parameters: extraction yield and precision data.

Analysis of samples from patients

The method was applied to the analysis of DBS and plasma samples collected from 16 former heroin addicted subjects under MMT. As an example, the chromatogram of a DBS sample from one of these patients treated with 80 mg day^{-1} of MTD is reported in **Fig. 5a**, while the chromatogram of the plasma sample from the same subject is shown in **Fig. 5b**. As can be seen in **Fig. 6**, a good correlation (r^2 = 0.998) was obtained between the concentrations of MTD found in DBS and those found in the corresponding plasma samples, taking into account the presence of hematocrit in DBS and its absence in plasma. In fact, since the hematocrit is normally about 40–54% for men and 36-46% for women (mean 45%), the concentrations found in DBS samples were multiplied by a correction factor of 1.79 to obtain the corresponding plasma concentrations. For example, the MTD level found in the DBS sample reported in Fig. 5a was 253 ng mL^{-1}, which transformed for hematocrit correction factor becomes 456 ng mL^{-1}. The latter value is in good agreement with the one found in the corresponding plasma sample (Fig. 5b), which was 456 ng mL^{-1}.

The analysis of MTD in DBSs from 16 patients under MMT (dosage range from 40 to 240 mg day^{-1}) showed a notable interindividual variability in the blood disposition of MTD even for the same given dose (**Fig. 7**). For example, it can be noted that patients 1 and 2 who received the same dose (i.e. 40 mg day^{-1}) had blood levels of MTD of 53 and 143 ng mL^{-1}, respectively. This is partly due to the reported large variation in the pharmacokinetics of MTD and results in variable clinical responses so requiring dose adjustments tailored to each patient.

- *Accuracy*

Method accuracy was evaluated by means of recovery assays. Standard solutions of the analytes at three different concentrations were added to DBS or plasma samples from patients previously analysed and the percentage recovery was calculated. Accuracy was satisfactory, being recovery values always higher than 87.0%.

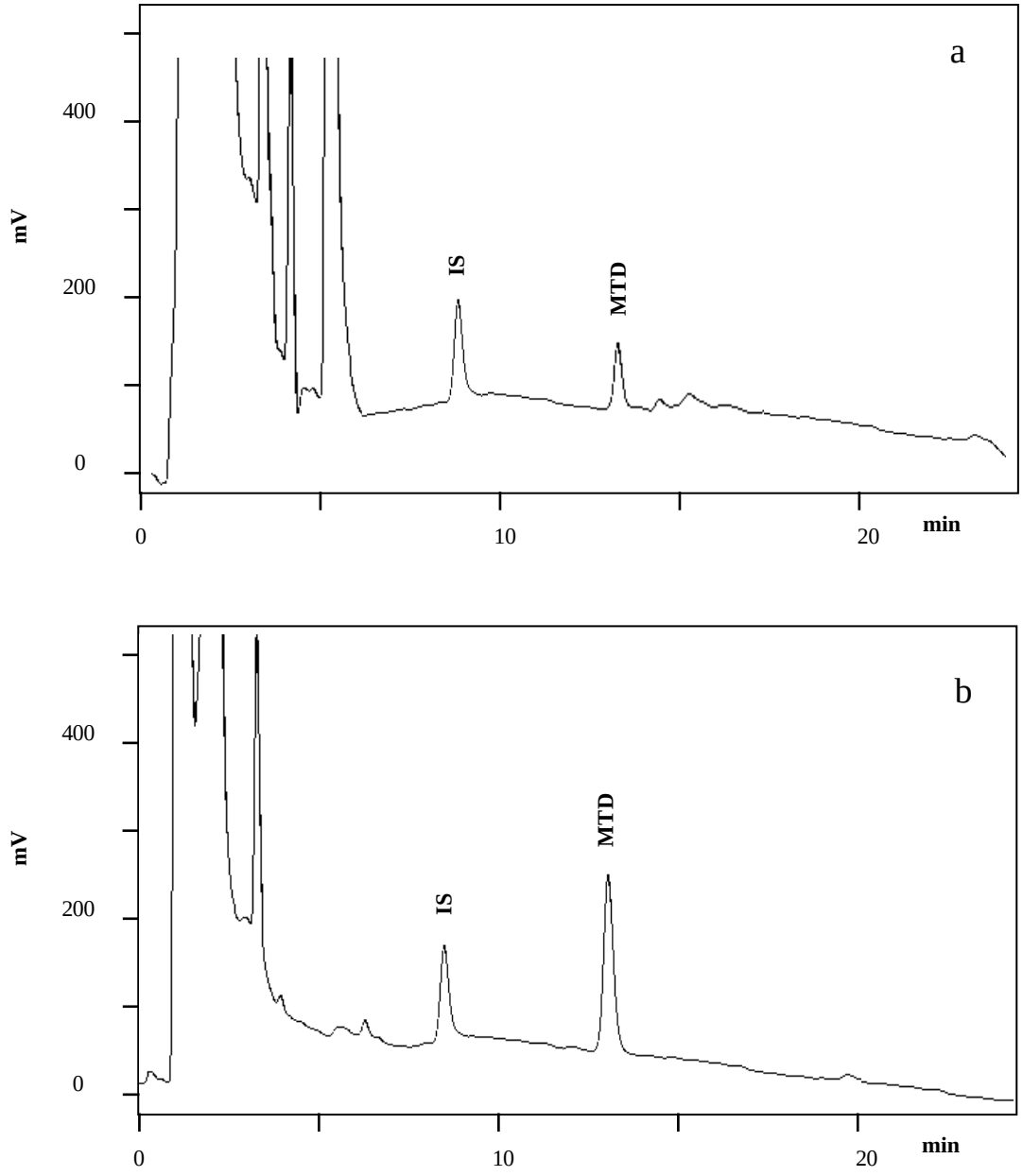


Fig. 5

Chromatograms of a DBS sample from a patient taking 80 mg day^{-1} of MTD (a) and of the corresponding plasma sample from the same patient (b).

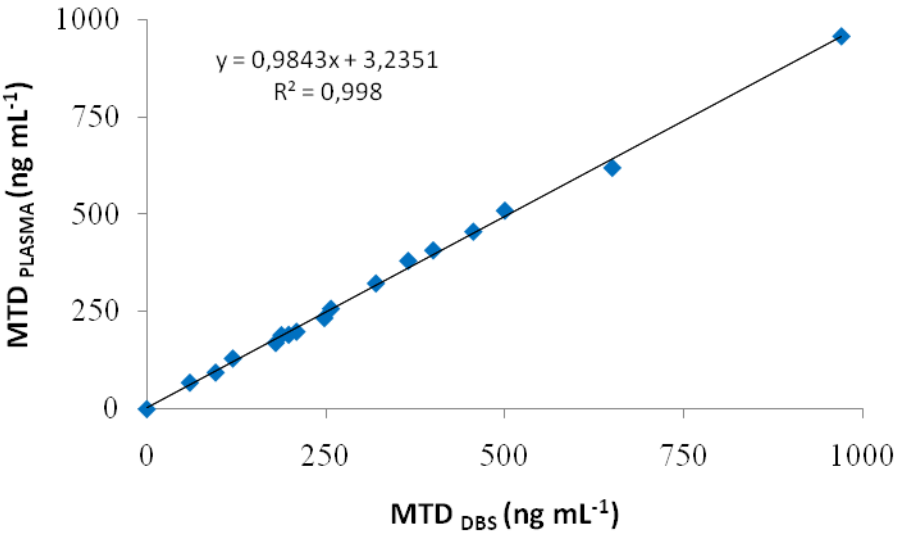
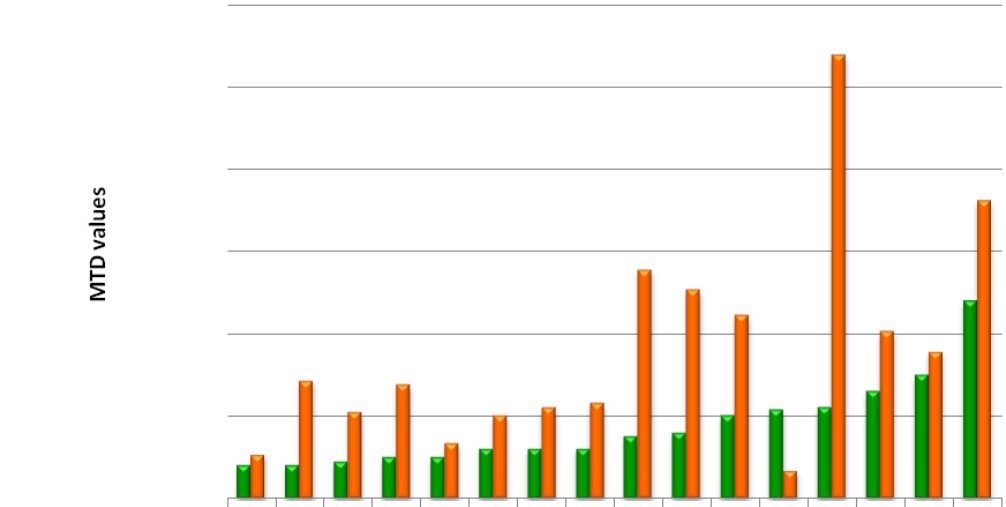


Fig. 6

Linear regression plot of MTD concentrations in DBSs (multiplied by hematocrit correction factor (i.e. 1.79)) vs MTD concentrations in plasma samples.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Dose (mg/day)	40	40	45	50	50	60	60	60	75	80	100	108	110	130	150	240
Blood level (ng/mL)	53	143	104	138	67	100	110	116	278	253	222	33	539	203	178	361

Fig. 7

MTD blood disposition for a given dose in 16 patients under MMT.

3.4. Conclusions

A reliable HPLC method with coulometric detection has been optimized for the determination of MTD in DBS and human plasma samples. MTD removal from DBSs was performed by means of microwave-assisted extraction with a suitable solvent and was followed by a MEPS procedure for the clean-up of the extracts. The reliability of DBS specimens in the determination of MTD blood levels was evaluated: the analyte proved to be stable in the DBS matrix for at least one month and the concentrations of MTD obtained from the analysis of DBS samples were perfectly comparable with those found in the corresponding plasma specimens. Since the outcome of MMT is highly variable depending on different factors, such as individual metabolism, it is of paramount importance to accurately determine MTD blood levels in order to optimize the dosage in each subject. The developed method allows to perform TDM of patients under MMT with all the advantages offered by DBS technique, including low invasiveness of the sampling procedure and low cost of sample collection, transport and storage.

4. ANALYSIS OF DISULFIRAM AND BUPROPION IN HUMAN PLASMA BY HPLC-DAD

4.1. Introduction

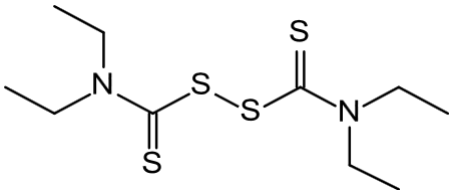
Alcohol and nicotine are the most commonly abused addictive drugs and they have a high propensity to cause illnesses and death as a result of chronic use [1,52]. According to a recent study, alcohol and nicotine appear to be more harmful than cannabis, lysergic acid diethylamide and ecstasy [53]. A substantial number of individuals are co-dependent on these two drugs and this lead to an increased risk of negative health consequences and medical complications. Many factors may contribute to determine alcohol and nicotine co-dependence; in particular, the genetic predisposition for both substance addiction, a specific isoform of cytochrome P450 (CYP) induced by alcohol (CYP 2E1 metabolizes nicotine) and the combination of the neurochemical effects of the two drugs that can lead to positive reinforcement [54]. Currently, there is no approved single treatment for alcohol and nicotine co-dependence, so each addiction is addressed with a different drug in association with psychosocial support.

Disulfiram (tetraethylthiuram disulphide, DSF, **Fig. 1**) was the first substance proposed for the management of alcoholism [55]. It blocks the enzyme aldehyde dehydrogenase, leading to an accumulation of acetaldehyde following alcohol intake. This in turn causes flushing, shortness of breath, tachycardia, headache and nausea, thus discouraging alcohol ingestion. DSF is given orally in the range dose of 200-400 mg day^{-1} generally for a period no longer than 6 months [56]. It is rapidly reduced to its main metabolite diethyldithiocarbamate by the glutathione reductase system mainly in the erythrocytes. Plasma concentrations of DSF are subjected to notable interindividual variability that might result from its marked lipid solubility, differences in plasma protein binding, or enterohepatic cycling [56]. While the primary pharmacological action of this drug involves the inhibition of the enzyme aldehyde dehydrogenase, recent studies have evaluated uncovered potential anti-craving effects as well as direct effects of DSF also on cocaine abuse, highlighting some possible benefits DSF may have through the inhibition of dopamine beta-hydroxylase [57].

Bupropion (1-(3-chlorophenyl)-2-[(1,1-dimethylethyl)amino]-1-propanone, BPP, **Fig. 1**) is an antidepressant of the aminoketone class, chemically unrelated to other known antidepressant agents. BPP is used in the range dose of 150-300 mg day^{-1} in the pharmacological treatment of smoking cessation in combination with motivational support [58]. It is a relatively weak inhibitor of the reuptake of norepinephrine and dopamine as well as a α3β4-nicotinic receptor antagonist [59]. The exact mechanism responsible of the efficacy of BPP as smoking-cessation aid is not completely understood yet, but it might involve its ability to support positive mood by inhibiting dopamine reuptake. Thanks to its action on the dopaminergic system, BPP is currently under investigation for the treatment of cocaine dependence [60]. BPP is extensively metabolized by the cytochrome P450 system and three of its metabolites have been shown to be active, namely hydroxybupropion and the amino-alcohol isomers threohydrobupropion and erythrohydrobupropion [58].

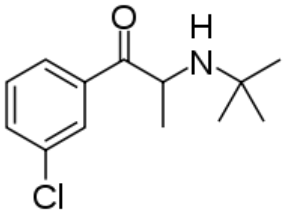
Only few papers (published more than 20 years ago) can be found in the literature for the determination of DSF in biological fluids. The analyses were based on the use of high performance liquid chromatography (HPLC) with UV detection [61-64] and sample pre-treatment was carried out by means of time-consuming derivatization and liquid-liquid extraction (LLE) procedures [63,64] or by direct injection of the biological sample [61,62]. As regards the analysis of BPP, HPLC methods with UV detection [65-67] or mass spectrometry (MS) [68,69] were reported for its determination in plasma/serum and urine. The extraction of the analyte and its metabolites from the biological matrices was carried out by LLE [65,66,68], protein precipitation [67] or solid phase extraction (SPE) [69] procedures. To the best of knowledge, no analytical method is currently available for the simultaneous analysis of DSF and BPP in human plasma. Considering the frequent co-administration of these two drugs in alcohol and nicotine abusers and the need for therapeutic drug monitoring, it is advisable to have at disposal analytical methods to simultaneously determine both drugs, thus saving time and money. Therefore, the aim of this work was to develop a feasible HPLC method for the simultaneous quantification of DSF and BPP in human plasma. The analytes were

monitored by diode array detection (DAD) and sample clean-up was accomplished through an original SPE procedure.



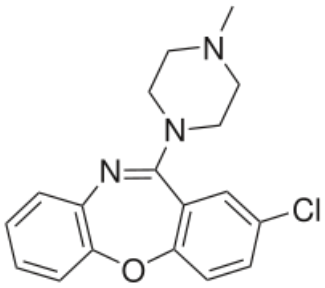
Disulfiram

(DSF)



Bupropion

(BPP)



Loxapine

(IS)

Fig. 1

Chemical structures of disulfiram, bupropion and loxapine, used as the internal standard (IS).

4.2. Experimental

Chemicals

DSF and BPP were purchased from Sigma-Aldrich (St. Luis, MO, USA). Loxapine, used as the internal standard (IS, **Fig. 1**), was kindly provided by Lederle Laboratories (Gosport, Hampshire, UK). Acetonitrile and methanol for HPLC, 85% (w/w) phosphoric acid, potassium dihydrogen phosphate and 2N sodium hydroxide, all pure for analysis, were from Sigma-Aldrich. Triethylamine, pure for analysis (> 99.5%), was purchased from Fluka Chemie (Buchs, Switzerland). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA).

Preparation of stock and working solutions

Standard stock solutions of DSF and BPP (1.0 mg mL^{-1}) were prepared by dissolving 10.0 mg of pure substance in 10.0 mL of methanol. IS stock solutions (1.0 mg mL^{-1} of pure loxapine) were prepared by dissolving 14.6 mg of loxapine succinate in 10.0 mL of methanol (14.6 mg of loxapine succinate corresponds to 10 mg of pure loxapine).

Working standard solutions at different concentrations were obtained by diluting standard stock solutions with the mobile phase. Stock solutions were stable for at least 2 months when stored at -20°C (as assessed by HPLC assays); working standard solutions were prepared fresh every day.

Sample collection

Blood samples were obtained from patients under treatment for alcohol and/or nicotine dependence at a local Drug Addiction Treatment Centre (Ser.T.). Patients were administered Antabuse® (DSF) or Zyban® (BPP) tablets at daily doses between 200-400 mg for DSF and 150-300 mg for BPP. Blood samples were collected in glass tubes containing ethylenediaminetetraacetic acid (EDTA) as the anticoagulant and centrifuged at 4000 rpm for 15 min at 5°C within 2 h from collection. The supernatant (plasma) thus

obtained was transferred into polypropylene tubes and stored at -80°C until HPLC analysis.

Blood samples from healthy volunteers, used as blank plasma, were treated in the same way as samples from patients.

Equipment

HPLC analyses were carried out on an Agilent (Santa Clara, CA, USA) 1100 series chromatographic system equipped with a diode-array detector. The sample clean-up procedure was carried out on IST (Hengoed, UK) Isolute ethyl (C2) cartridges (50 mg, 1 mL) using a Vac Elut apparatus. A Crison (Barcelona, Spain) MicropH 2000 pHmeter, an Universal 32 R centrifuge from Hettich (Tuttlingen, Germany) and a Branson (Danbury, CT, USA) 1510 ultrasonic bath were also used.

Data were handled by means of ChemStation (Rev. A.09.01) software (Agilent, Santa Clara, CA, USA).

Chromatographic conditions

Separation of the analytes was achieved on a Varian (Harbor City, CA, USA) Microsorb reversed-phase C8 column (4.6 x 250 mm, 5 μm) kept at room temperature (25 ± 3°C). The mobile phase was composed of phosphate buffer (67 mM, pH 7.0) and acetonitrile (50:50, v/v) and contained 0.25% (v/v) triethylamine. The flow rate was 1.5 mL min^{-1}. The samples were injected into the HPLC system by means of a 50 μL loop. Prior to use, the mobile phase was filtered through Phenomenex (Torrance, CA, USA) nylon filters (47 mm diameter, 0.2 μm pore size) and degassed by sonication.
The diode-array detector wavelength was set at 250 nm.

Sample pre-treatment: solid phase extraction

Plasma sample clean-up was carried out on C2 cartridges activated with 3×1 mL of methanol and then conditioned with 3×1 mL of water. A 50 μL aliquot of IS solution was added to 500 μL of plasma. The resulting mixture was diluted with 500 μL of

phosphate buffer (50 mM, pH 3.0) and loaded onto the previously conditioned cartridge. After loading, the sorbent was washed with 1 mL of phosphate buffer to remove biological interference. The analytes were then eluted with 250 μL of a mixture of acetonitrile and phosphate buffer (65:35, v/v) and the eluate was directly injected into the HPLC system.

Method validation

The method was validated according to official guidelines (USP XXXII [37] and "Crystal City" guidelines [38]).

- *Linearity, limit of quantitation, limit of detection*

Aliquots of 50 μL of DSF and BPP standard solutions at six different concentrations (to obtain plasma concentrations over the 5–500 ng mL^{-1} range), containing the IS at a constant concentration (to obtain plasma concentration of 250 ng mL^{-1}), were added to 500 μL of blank plasma. The resulting fortified samples were subjected to the previously described SPE pre-treatment and injected into the HPLC system. This procedure was done in triplicate for each point. The analyte-to-IS peak-area ratios were plotted against the corresponding concentrations of the analytes (expressed as ng mL^{-1}) and the calibration curves were constructed by means of the least-squares method.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as the analyte concentrations which give rise to peaks whose heights are 3 and 10 times the baseline noise, respectively.

- *Extraction yield and precision*

Aliquots of 50 μL of DSF and BPP standard solutions at three different concentrations (to obtain analyte plasma concentrations of 5, 250 and 500 ng mL^{-1} for both analytes), containing the IS at a constant concentration (to obtain plasma concentration of 250 ng mL^{-1}), were added to 500 μL of blank plasma. The resulting spiked plasma samples were subjected to the previously described SPE procedure and

injected into the HPLC system. The analyte peak areas were compared to those obtained injecting standard solutions at the same theoretical concentrations and the extraction yield was calculated.

The assays described above were repeated six times on the same day to obtain repeatability (intraday precision) and six times over six different days to obtain intermediate precision (interday precision), both expressed as percentage relative standard deviation (RSD%).

- *Selectivity*

The selectivity of the method was evaluated with respect to endogenous and exogenous compounds. Blank plasma samples from six healthy volunteers not receiving DSF or BPP treatment were processed in the absence of the IS and the resulting chromatograms were checked for possible interference at the retention time of the analytes and the IS. Selectivity towards exogenous substances was ascertained by injecting into the HPLC system standard solutions of several drugs which are often co-administered in clinical practice during treatment for alcohol and nicotine addiction. If the tested compounds co-eluted with the analytes or the IS, blank plasma samples were spiked with the interfering drug and subjected to the SPE procedure. The resulting chromatograms were then checked again for interference.

- *Accuracy*

Accuracy was evaluated by means of recovery assays. Aliquots of 50 μL containing analyte standard solutions at two different concentrations (i.e. analyte plasma additions of 20 and 100 ng mL^{-1} for both analytes) and the IS at a constant concentration were added to 500 μL of samples from patients whose analyte concentrations had been previously determined. The mixtures were then subjected to the SPE procedure described above. Accuracy was expressed as recovery values and was calculated according to the following formula: 100 × (concentration after spiking – concentration before spiking)/concentration added.

4.3. Results and discussion

Development of the chromatographic conditions

Preliminary chromatographic assays were carried out on a C8 column with a mixture of phosphate buffer (pH 3.0) and acetonitrile (50:50, v/v) as the mobile phase. Under these conditions, BPP was not sufficiently separated from the void peak. The decrease of the organic solvent to 45% increased the retention time of DSF without improving BPP retention on the column. Since BPP, DSF and the IS have very different pKa values (7.16, 0.86 and 7.32, respectively), the influence of the buffer pH on the chromatographic behavior of the analytes was investigated in a range between 3.0 and 7.0. A value of 7.0 provided satisfactory retention for BPP and complete resolution of the analytes in an acceptable total run time.

A detection wavelength of 250 nm was chosen as a good compromise between sensitivity and selectivity. In fact, DSF, BPP and the IS show two absorbance maxima at 220 and 250 nm. The 250 nm wavelength affords the method higher selectivity than 220 nm, reducing the potential interference from the biological matrix while maintaining satisfactory sensitivity.

A representative chromatogram of a standard solution containing 500 ng mL^{-1} of DSF, BPP and IS is shown in **Fig. 2.** Retention times were 5.1, 8.6 and 12.3 min for BPP, DSF and the IS, respectively.

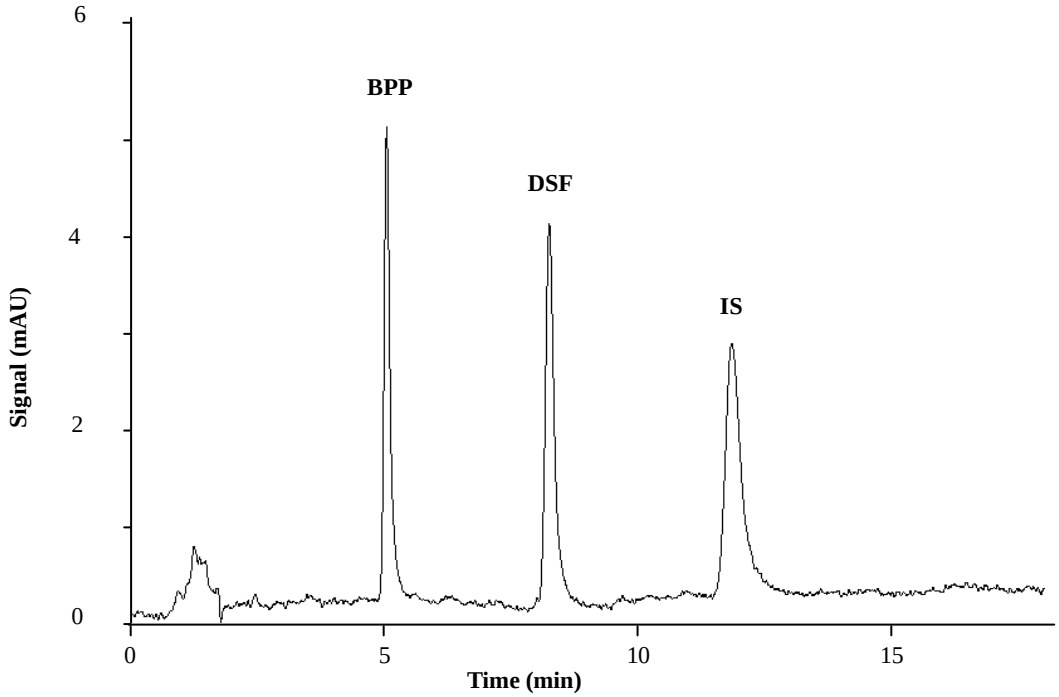


Fig.2

Chromatogram of a standard solution containing 500 ng mL^{-1} of DSF, BPP and IS.

Development of the SPE procedure

Protein precipitation is the easiest and less time consuming way to remove endogenous interference from biological matrices. Therefore, a protein precipitation procedure with acetonitrile was initially tested, but it highly diluted the sample and gave a very low extraction yield for DSF (less than 30%). Consequently, it was decided to exploit SPE technique, since it is more selective and allows the pre-concentration of the sample. Different cartridges were tested: C2, C8, C18, phenyl, cyanopropyl, diol and hydrophilic–lipophilic balance polymeric sorbents. The more lipophilic cartridges (C8, C18 and phenyl) gave unsatisfactory absolute recovery for BPP (60%) and DSF (10%), whereas more polar sorbents (cyanopropyl and diol) provided worse extraction yields for both analytes (less than 15%). As the chemical properties of DSF and BPP are quite different, hydrophilic–lipophilic balance (Oasis HLB) polymeric sorbents were also tried, but these gave neither an adequate clean-up of the matrix nor satisfactory extraction yields. Only the weakly lipophilic cartridges C2 provided good results for both analytes, hence they were selected for the pre-treatment procedure. The pH value of the loading, washing and eluting solutions was investigated. Among the different solvents tested (ultrapure water, phosphate buffer in the 3.0–7.0 pH range and pH 10.0 carbonate buffer), acidic phosphate buffer gave the best results. Among those tried, a mixture of acetonitrile and pH 3.0 phosphate buffer (65:35, v/v) was found to be the most appropriate to elute the analytes. A volume of 250 μL was sufficient to provide good extraction yields, thus it was possible to obtain a two-fold pre-concentration of the sample, increasing method sensitivity, without any further evaporation step.

The developed SPE procedure effectively cleaned the biological matrix. In fact, as can be seen in **Fig**. **3**, no endogenous interference is present at the retention times of the analytes and the IS in a blank plasma sample subjected to the SPE pre-treatment.

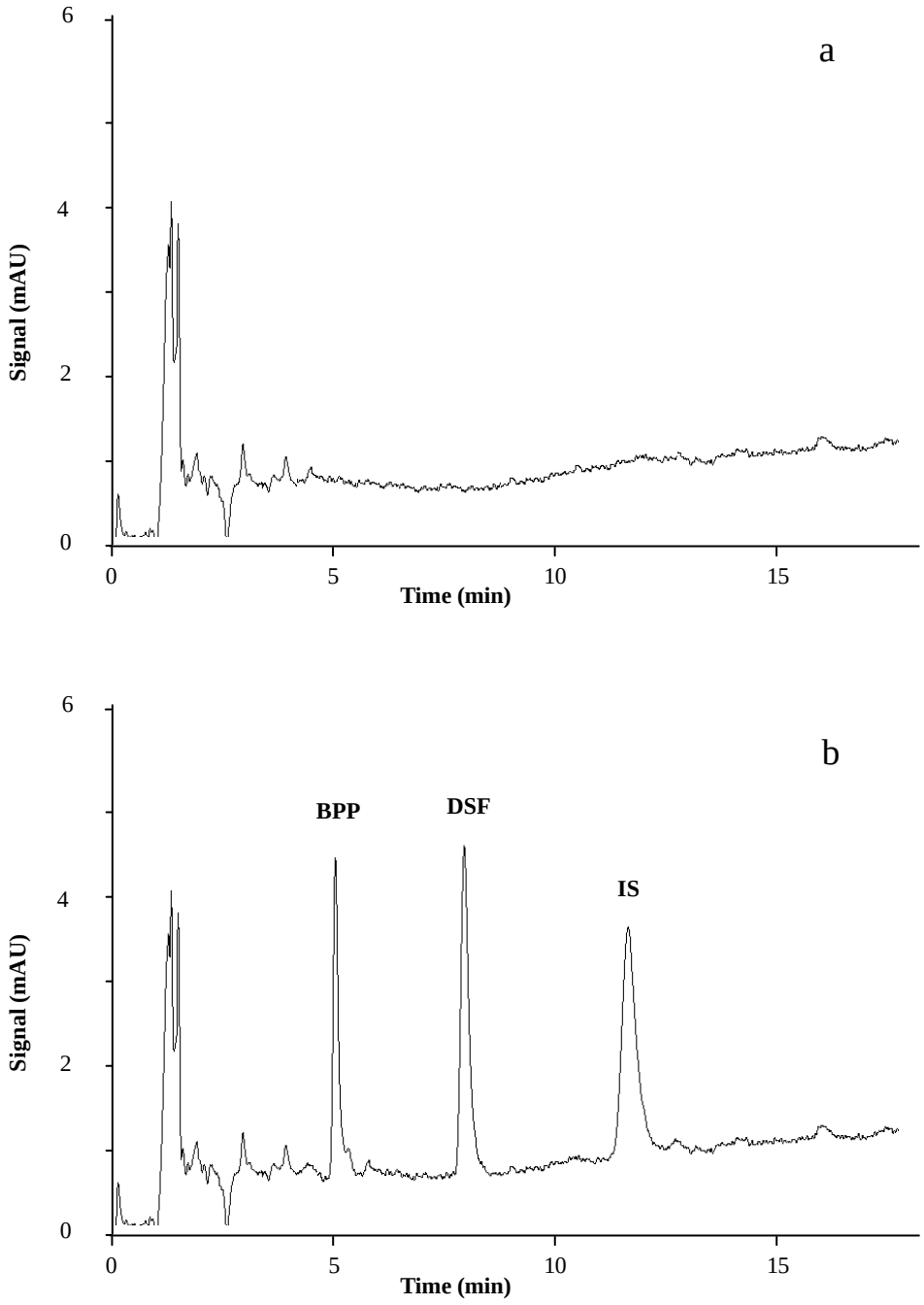


Fig. 3

Chromatograms of a blank plasma sample (a) and of the same sample spiked with 250 ng mL^{-1} of DSF, BPP and IS (b).

Method Validation

Calibration curves were set up on blank plasma fortified with different concentrations of the analytes and a constant concentration of the IS. Satisfactory linearity was found in the 5–500 ng mL^{-1} plasma concentration range (**Table 1**). The LOD and LOQ values were 1.5 and 5 ng mL^{-1}, respectively, for both analytes.

Extraction yield and precision assays were carried out on blank plasma spiked with three different concentrations of DSF and BPP, corresponding to the lowest the intermediate and the highest point of each calibration curve (**Table 2**). Extraction yield was satisfactory, as it ranged between 84% and 90%. Mean extraction yield of the IS was 90%. Precision was also good, with RSD values always lower than 5.9%.

- *Selectivity*

The analysis of blank plasma samples from six healthy volunteers showed no evidence of unacceptable interference from endogenous compounds at the retention times of DSF, BPP and the IS. In addition, several compounds belonging to the antipsychotic, antidepressant, antiepileptic and sedative-hypnotic classes were tested for exogenous interference. Among these, risperidone, duloxetine and imipramine interfered with the determination of DSF and BPP. Consequently, blank plasma samples were spiked with standard solutions of the three interfering drugs and were subjected to the SPE procedure, resulting in no extraction of the three compounds. Hence, none of the tested drugs gave rise to peaks that could interfere with the determination of the analytes.

Analyte	Linearity range (ng mL^{-1})	Equation coefficients, $y = ax + b$ [a]		r^2 [b]
		a	b	
DSF	5.0-500.0	1.0194	3.8055	0.9927
BPP	5.0-500.0	0.7962	3.9788	0.9989

[a] y = analyte/IS peak-area ratio; x = analyte concentration (ng mL^{-1})
[b] r^2 = correlation coefficient

Table 1

Linearity parameters.

Analyte	Concentration (ng mL^{-1})	Extraction yield (%)[a]	Repeatability (RSD%)[a]	Interday precision (RSD%)[a]
DSF	5.0	85	5.5	5.9
	250.0	84	3.7	4.1
	500.0	84	3.2	4.0
BPP	5.0	89	4.8	4.8
	250.0	88	3.5	3.6
	500.0	88	3.4	3.5

[a] n = 6

Table 2

Validation parameters: extraction yield and precision data.

Analysis of samples from patients

The validated method was applied to the analysis of plasma samples from patients treated with Antabuse® (DSF) or Zyban® (BPP). The chromatograms of a plasma sample from a patient taking 200 mg day^{-1} of DSF and from a patient taking 150 mg day^{-1} of BPP are reported in **Fig. 4a** and **Fig. 4b**, respectively. The concentrations found in these samples were 13.4 ng mL^{-1} for DSF and 37.2 ng mL^{-1} for BPP. DSF levels were lower than the therapeutic range (i.e. 50–400 ng mL^{-1}), probably because the patient was at the beginning of the therapy, whereas BPP levels were within the therapeutic range reported for the drug (i.e. 10–100 ng mL^{-1}) [70].

- *Accuracy*

Method accuracy was evaluated by means of recovery assays. Standard solutions of the analytes at two different concentrations were added to plasma samples from patients under treatment with DSF or BPP previously analysed and the percentage recovery was calculated. Results were satisfactory, with recovery always higher than 85.0% for both analytes.

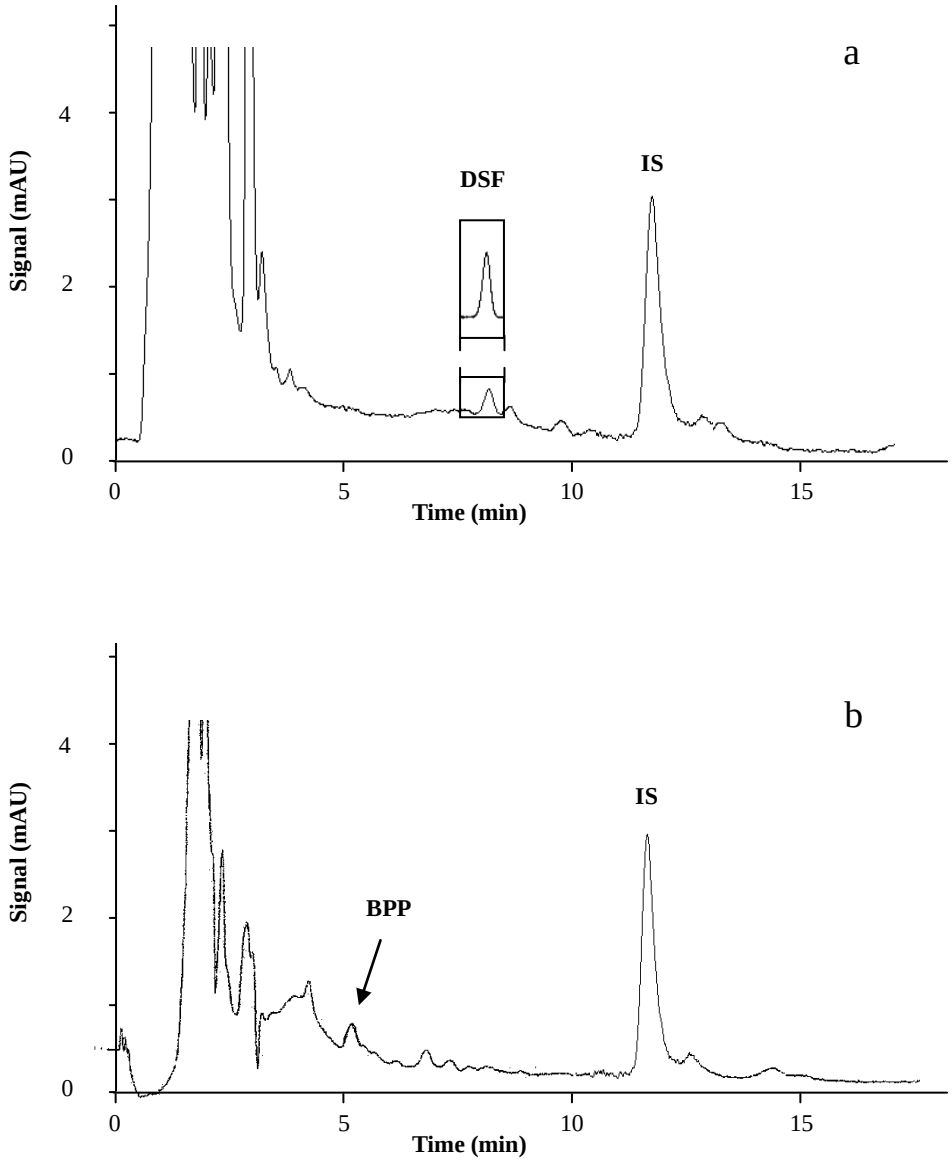


Fig. 4

Chromatograms of a plasma sample from an alcohol abuser taking 200 mg day^{-1} of DSF (a) and of a plasma sample from a nicotine abuser taking 150 mg day^{-1} of BPP (b).

4.4. Conclusions

A HPLC–DAD method for the simultaneous determination of DSF and BPP in human plasma has been developed. Sample pre-treatment was carried out by means of an original SPE procedure on C2 cartridges. When compared to HPLC–UV assays with LLE formerly reported for the analysis of DSF [63,64], the method presented herein shows better precision and extraction yield. Moreover, it has the advantage of using a smaller volume of plasma (500 μL vs 1 mL) [63-65] and of reducing the time necessary for sample pre-treatment. As regards BPP analysis, compared to previous assays based on the use of liquid chromatography–mass spectrometry [68,69], the presented HPLC–DAD method is less expensive and needs less maintenance; furthermore, if compared to previous HPLC–UV methods [65-67], it gives better precision (RSD < 5.9% instead of values up to 15%) [65,66] and higher extraction yields [65].

The developed method has been successfully applied to the analysis of plasma samples from some alcohol and nicotine abusers under treatment with DSF and/or BPP, proving to be suitable for the therapeutic monitoring of the two drugs.

5. DETERMINATION OF KETAMINE AND ITS MAIN METABOLITE IN DRIED BLOOD SPOTS AND HUMAN PLASMA BY HPLC-DAD

5.1. Introduction

Ketamine ((RS)-2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one, KET, **Fig. 1**) is a phencyclidine structural analogue first introduced as an anaesthetic agent in the early 1960's [71]. It was initially well received as it does not produce respiratory and cardiovascular depression unlike other drugs [72]. However, early reports of post-anaesthetic complications, such as delusions, hallucinations, delirium and confusion, soon limited its clinical use [71,73]. Today, KET is primarily employed in paediatric, geriatric and veterinary anaesthesia (children and elderly patients appear to be less sensitive to KET-induced emergence reactions) [73,74]. Moreover, thanks to its good safety profile, KET is still the anaesthetic of choice in parts of the world that have limited availability of resuscitation equipment [71].

While its role in the anaesthetic field is well established, the potential usefulness of the drug in the management of pain and treatment-resistant depression is currently under investigation. A growing body of evidence supports KET short-term use for neuropathic and nociceptive pain, whereas further studies are needed to better ascertain its long-term efficacy and to address safety/toxicity issues [75,76]. The potential utility of KET in preemptive analgesia is examined with great interest as well. In fact, KET seems to prevent spinal neuron sensitization to painful stimuli, hence sub-anaesthetic doses given before, during and after surgery could improve post-operative pain relief [71,75].

As regards KET utility in the treatment of depression, recent clinical studies have demonstrated that a single sub-anaesthetic dose of KET produces fast-acting, long-lasting antidepressant responses in patients suffering from major depressive disorder, although the underlying mechanism is unclear. In particular these studies demonstrate that a single low-dose intravenous infusion of KET alleviates depressive symptoms within hours with effects sustained for approximately 7 days, unlike traditional antidepressants which take weeks to reach efficacy [77,78].

Precisely those effects that limited its clinical use made the drug appealing to recreational drug users. The first reports of KET abuse began to appear soon after its

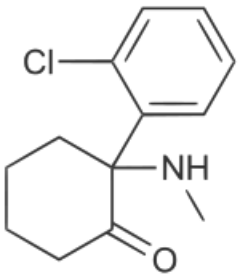
introduction into clinical practice, but use remained rare in Europe until the 1990s [71,72]. KET is primarily 'snorted' intranasally in a powder form, thus leading to a relatively rapid (~5 minutes) onset of effects on the brain. It can also be injected intramuscularly or occasionally intravenously; less frequently it is taken orally in tablet form, often mixed with other drugs of abuse [71,73,79]. Recreational doses are highly variable depending on the route of ingestion, the desired effect and the degree of tolerance [79]. At low doses KET induces distortion of time and space, hallucinations and mild dissociative effects. At large doses, it causes a more severe dissociation commonly referred to as a 'K-hole', wherein the user perceptions are completely separated from reality [71]. Risks associated with KET use include accidental injury or even death due largely to the dissociative and analgesic effects; intense abdominal pain ('K-cramps') resulting from prolonged abuse; KET-induced ulcerative cystitis, which appears more common in those using the drug on a frequent, often daily basis; cognitive impairment and possible development of psychotic disorders [71,73,79].

KET pharmacological effects are mainly attributed to its activity at the N-methyl -d-aspartate (NMDA) receptors, where the drug acts as a non-competitive antagonist. Nevertheless, KET also shows lower affinity for other receptor sites: it is reported to be a weak agonist of μ-opioid receptors, to blocks muscarinic acetylcholine receptors and to act as a weak $GABA_A$ receptor agonist [71,72,79]. KET is extensively N-demethylated by the cytochrome P450 system into the active metabolite norketamine (N-KET, **Fig. 1**) [72]. Other biotransformations include hydroxylation of the cyclohexone ring and conjugation with glucuronic acid [74].

Seeing that the recreational use of this drug has increased over recent years, it is becoming more important to assess KET recent intake. Moreover, it is necessary to conduct pharmacokinetics studies to further investigate KET analgesic and antidepressant properties. To these aims, blood (plasma, serum or whole blood) is the most appropriate matrix. However, its use is limited by some drawbacks, such as the invasiveness of the sample collection and the constant refrigeration required for storage and transportation. Dried blood spots (DBSs) represent an attractive alternative to conventional blood collection. They are simply obtained from a finger prick spotting the

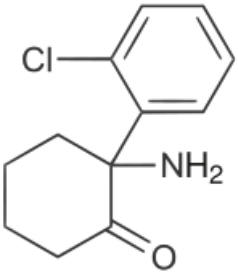
resulting drops of blood onto filter paper and they offer a number of advantages. First, the sampling method is less invasive and involves minimum discomfort to the patient, second, storage and transfer are easier because no refrigeration is required, finally, the dried matrix stabilizes many analytes and reduces biohazard risk [50]. All these characteristics make DBS a simple and convenient collection technique.

Several analytical methods dealing with the determination of KET in biological samples (urine [80-89], plasma [90-97], serum [98], blood [99], hair [100-106] and oral fluid [90,107-109]) can be found in the literature. KET, with or without its metabolites and/or other drugs of abuse, has mostly been analyzed by high performance liquid chromatography (HPLC) [80-84,90,91,98-102,107,109] or gas chromatography (GC) [85-89,103-106,108] both coupled with mass spectrometry (MS), but also a few HPLC-UV methods have been reported [92-97]. Sample pre-treatment has been carried out by solid phase extraction (SPE) [80,82-85,87,89,91,92,96,98-100,104,108], liquid-liquid extraction [88,93-95,97,101,102,105], headspace solid phase microextraction [86,103,106] and micro-solid phase extraction [109], being SPE the most common approach. To the best of knowledge, no analytical method has been published for the determination of KET and N-KET in DBSs. Therefore, the aim of this work was to develop an HPLC method for the quantitation of KET and its main active metabolite, N-KET, in DBSs. Sample pre-treatment was carried out by means of microextraction by packed sorbent (MEPS), a recent technique that allows the reduction of the amount of sample and solvents needed for the procedure. To exploit the convenience of MEPS also for the analysis of plasma specimens, the method was optimized and validated for plasma samples as well.



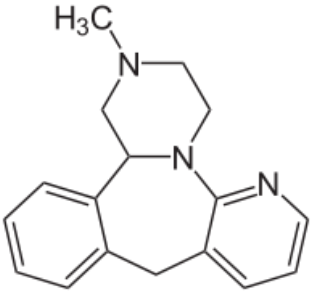
Ketamine

(KET)



Norketamine

(N-KET)



Mirtazapine

(IS)

Fig. 1

Chemical structures of ketamine, norketamine, and mirtazapine, used as the internal standard (IS).

5.2. Experimental

Chemicals

Methanolic stock solutions of KTM and N-KTM (1 mg mL^{-1}) were purchased from LGC Standards (Teddington, UK). Mirtazapine, used as the internal standard (IS, **Fig.1**), was obtained from Sigma Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile and methanol and potassium phosphate monobasic were also purchased from Sigma Aldrich. Sodium hydroxide solution (2N) was prepared from sodium hydroxide pellets (Sigma Aldrich). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA).

Preparation of stock and working solutions

IS stock solutions were prepared at a concentration of 1 mg mL^{-1} by dissolving the appropriate amount of pure substance in methanol. Stock solutions were stable for at least 3 months when stored at -20°C (as assessed by HPLC assays).

Working standard solutions were prepared fresh every day by diluting stock solutions with the mobile phase.

Sample collection

DBSs from healthy volunteers were collected by puncturing the subjects on a finger with a single-use safety lancet; the first drop of blood was wiped away, then the blood drops were directly applied within the pre-marked circles on a 903 Protein Saver Card. The blood spots thus obtained were left to dry for 2 h in the dark at room temperature and then stored in a sealed plastic bag containing a suitable desiccant (i.e. silica gel).

Blank plasma was obtained by drawing venous blood from healthy volunteers into tubes containing ethylenediaminetetraacetic acid (EDTA) as the anticoagulant. The blood samples were then centrifuged at 4000 rpm for 15 min at 5°C and the supernatant plasma was transferred into polypropylene vials and stored at -20°C.

Equipment

HPLC analyses were carried out on an Agilent (Santa Clara, CA, USA) 1100 series chromatographic system equipped with a diode-array detector (DAD). Sample pre-treatment was performed by means of MEPS technique using a BIN (Barrel Insert and Needle Assembly) containing 4 mg of solid phase inserted in a 250 µL gas-tight syringe from SGE Analytical Science (Melbourne, VIC, Australia). A Crison (Barcelona, Spain) MicropH 2000 pHmeter, a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge, a Branson (Danbury, CT, USA) 1510 ultrasonic bath and an IKA (Staufen, Germany) RV 10 rotary evaporator were also used. DBS samples were collected on Whatman (Maidstone, UK) 903 Protein Saver Cards.

Data were handled by means of ChemStation (Rev. A.09.01) software (Agilent, Santa Clara, CA, USA).

Chromatographic conditions

The chromatographic separation was achieved on an Agilent (Santa Clara, CA, USA) Poroshell 120 EC-C8 reversed-phase column (4.6 x 100 mm, 2.7 µm) kept at room temperature (25 ± 3°C). The mobile phase was composed of acetonitrile and 40 mM potassium phosphate buffer (21:79, v/v); the pH of the buffer solution was adjusted to 6.0 in order to obtain an apparent pH value of 6.5 in the mobile phase mixture. The flow rate was kept constant at 1.0 mL min^{-1} and the injections were made by means of a 50 µL loop. Prior to use, the mobile phase was filtered through Varian nylon filters (47 mm diameter, 0.2 µm pore size) and degassed by sonication. The diode-array detector was set at $\lambda = 210$ nm.

Extraction from DBS

A 10 µL aliquot of IS working solution was added to a 50 µL DBS and let to dry for at least 1 h. After drying, the DBS was cut out of the card and placed into a polypropylene tube. A 300 µL aliquot of a mixture of water and methanol (10:90, v/v) was added and the tube was sonicated for 10 min. The extract was dried under vacuum

using a rotary evaporator, redissolved in 160 µL of phosphate buffer (50 mM, pH 7.4) and subjected to the MEPS procedure reported in the section below.

The unknown volume of the DBS samples was determined by interpolation on a calibration curve constructed as follows: known volumes of blood (from 5 to 60 µL) were applied onto the 903 Protein Saver Card with a micropipette, the diameter of the resulting spots was measured and plotted against the corresponding volume of blood.

Sample pre-treatment: microextraction by packed sorbent

The preparation of the samples was carried out by means of a MEPS procedure using a BIN containing 4 mg of C8 solid phase. The sorbent was activated with 3 x 100 µL of methanol and then conditioned with 3 x 100 µL of water. The volumes of methanol and water were drawn up and then discarded every time at a flow rate of 10 µL sec^{-1}. A 50 µL volume of plasma was added with 10 µL of IS and 100 µL of phosphate buffer (50 mM, pH 7.4). An aliquot of 100 µL of this mixture or of the extract from DBS (see the section above) was drawn up and down through the syringe 10 times at a flow rate of 5 µL sec^{-1} without discarding it. The sorbent was then washed once with 100 µL of phosphate buffer (50 mM, pH 7.4) and once with 100 µL of methanol/phosphate buffer (5:95, v/v) to remove biological interference (flow rate 10 µL sec^{-1}). The analytes were eluted with 250 µL of methanol (flow rate 5 µL sec^{-1}) and the eluate was dried under vacuum (rotary evaporator), redissolved in 100 µL of mobile phase and injected into the HPLC system. After each extraction, 3×100 µL of methanol followed by 3×100 µL of water were passed through the sorbent in order to clean it and to avoid carry-over. These steps also acted as the conditioning step for the following extraction of the analytes. One packing bed was used for about 60 extractions; then it was discarded due to low analyte extraction yields and sorbent clogging.

Method validation

The method was validated following official guidelines (USP XXXII [37] and "Crystal City" guidelines [38]).

- *Linearity, limit of quantitation, limit of detection*

Aliquots of 10 µL of analyte standard solutions at seven different concentrations (in order to obtain on-column concentrations over the 10-250 ng mL^{-1} range), containing the IS at a constant concentration (on-column concentration 100 ng mL^{-1}), were added to DBSs, paying attention not to go outside the blood spot, and were left to dry for at least 1 h. A 10 µL volume of analyte standard solutions at nine different concentrations (in order to obtain on-column concentrations over the 10-500 ng mL^{-1} range), containing the IS at a constant concentration (on-column concentration 100 ng mL^{-1}), was added to 50 µL of blank plasma. The resulting fortified DBS or plasma samples were subjected to the previously described specimen preparation and then injected into the HPLC system. This procedure was done in triplicate for each point. The analyte/IS peak-area ratios were plotted against the corresponding concentrations of the analytes (expressed as ng mL^{-1}) and the calibration curves were constructed by means of the least-square method.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as the analyte concentrations which give rise to peaks whose heights are 3 and 10 times the baseline noise, respectively.

- *Extraction yield, precision and accuracy*

A 10 µL volume of analyte standard solutions at three different concentrations, corresponding to the lowest, the intermediate and the highest point of the calibration curve (on-column concentrations 10, 100, 250 ng mL^{-1}, respectively), containing the IS at a constant concentration (on-column concentration 100 ng mL^{-1}), was added to DBSs and left to dry for at least 1 h. Aliquots of 10 µL of analyte standard solutions at three different concentrations (in order to obtain on-column concentrations of 10, 200 and 500 ng mL^{-1}), containing the IS at a constant concentration (on-column concentration 100 ng mL^{-1}), were added to 50 µL of blank plasma. The resulting spiked DBS or plasma samples were subjected to the previously described specimen preparation procedure and finally injected into the HPLC system. The analyte peak areas were

compared to those obtained injecting standard solutions at the same theoretical concentrations and the extraction yields were calculated and expressed as percentage value.

The assays described above were repeated six times within the same day to obtain intraday accuracy and precision and six times over six different days to obtain interday accuracy and precision. Intra- and interday precision were both expressed as percentage relative standard deviation (RSD%). Intra- and interday accuracy were calculated by dividing the mean measured concentrations of the analytes by the theoretical concentrations and were both expressed as percentage value.

- *Selectivity*

Blank DBS or plasma samples from six different healthy volunteers were subjected to the sample pre-treatment procedure and injected into the HPLC system. The resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was no interfering peak higher than an analyte peak corresponding to its LOD. Furthermore, standard solutions of several drugs of abuse and of antidepressant drugs were injected into the HPLC system. A substance was classified as interfering when it gave rise to a peak that was not baseline separated from those of the analytes or the IS.

- *Stability*

Stability assays were carried out in DBSs (n=3) spiked with standard solutions of the analytes and the IS and stored in the dark at room temperature (25 ± 3°C) over a period of 20 days. The analytes were considered stable if the concentrations found in the stored DBSs did not differ from the theoretical concentrations by more than ±15%.

5.3. Results and discussion

Development of the chromatographic conditions

A C8 column based on the core-shell particle technology was chosen due to its higher efficiency, better peak shape and increased sensitivity compared to the totally porous C8 and C18 columns tested. Preliminary assays were carried out using a mixture of acetonitrile and phosphate buffer (20:80, v/v). The pH of the mobile phase was studied in the 3.5-7.0 range showing that small changes in the pH value significantly affected retention times and resolution. (**Fig. 2**). Moreover, an interesting inversion in the elution order of KET and N-KET was noticed between pH 5.5 and pH 6.5, probably due to the pKa values of the two analytes (7.5 and 6.7 for KET and N-KET, respectively). Eventually, pH 6.5 was selected as it provided good resolution in a reasonable time. The optimization of the composition of the mobile phase took into account the chromatographic behaviour not only of the analytes but also of the IS. In fact, among all molecules tested as possible IS, MRT was chosen as it gave the best results in terms of extraction yield and position in the chromatogram. However, MRT lengthened the total run time; therefore it was decided to increase the amount of acetonitrile in the mobile phase. The increment was tested in the range from 20% to 25%, resulting in 21% acetonitrile being the most suitable amount to shorten the run time to acceptable values while maintaining good retention of the analytes.

A diode-array detector was employed due to the advantage it offers of recording the UV/Visible absorption spectra of the compounds eluting from a column, thus providing additional information useful for the confirmation of peak identity. To reach satisfactory sensitivity values the detector was set at a wavelength of 210 nm that corresponds to the maximum of absorbance of both KET and N-KET.

The chromatogram of a standard solution of the analytes and the IS is reported in **Fig. 3**. As can be seen, the peaks are symmetrical and well resolved and the total run time is less than 14 min.

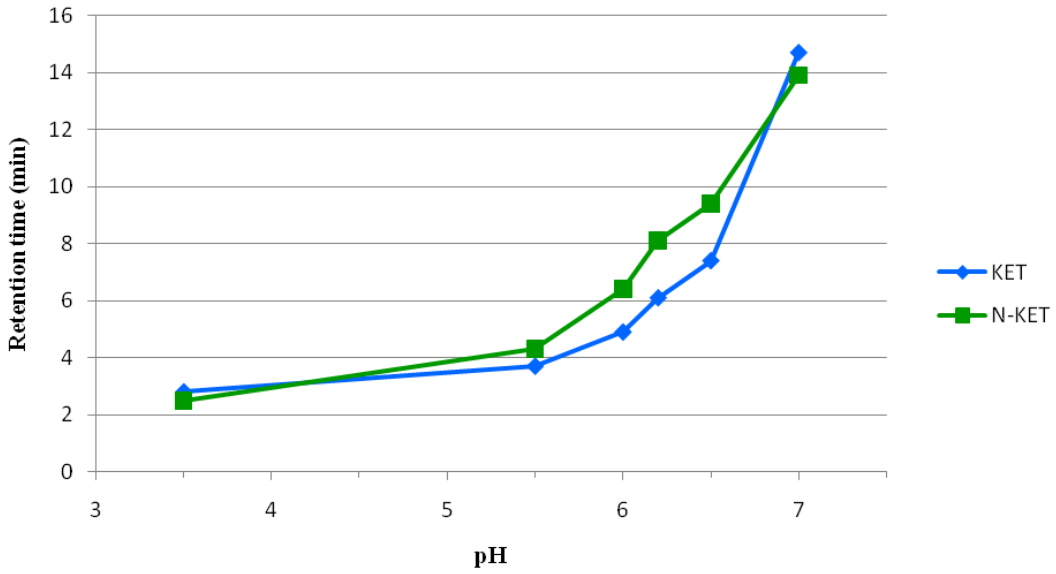


Fig. 2

Effect of the apparent pH value of the mobile phase on the retention of the analytes.

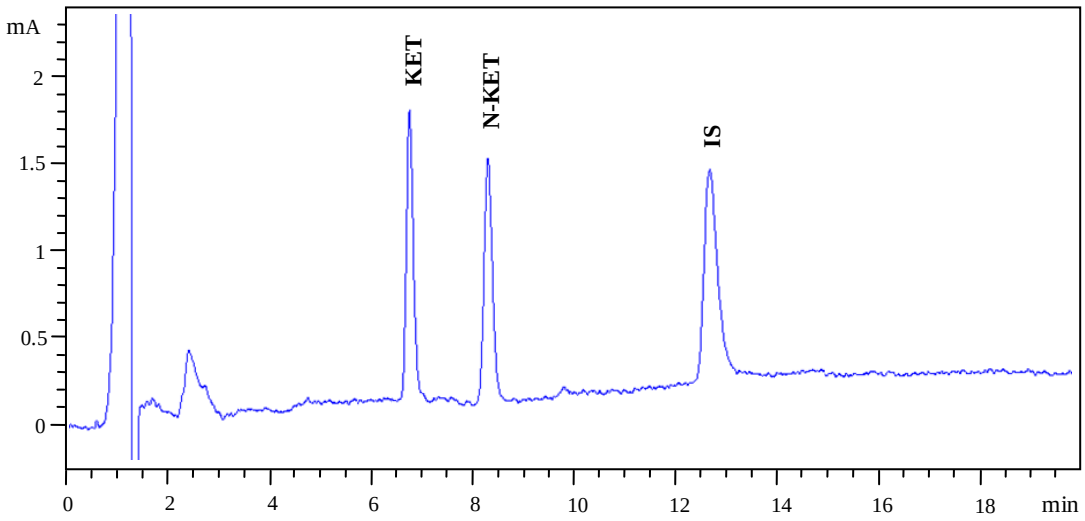


Fig. 3

Chromatogram of a standard solution containing 100 ng mL^{-1} of KET, N-KET and IS.

Development of the extraction from DBS

In the first place, DBS samples pre-treatment requires the extraction of the analytes from the blood spot. This can be accomplished with pure organic or aqueous solvents or mixtures of both. In order to optimize the extraction performance, different solvents and mixtures were tested (methanol, acetonitrile, buffers, organic solvents mixed with water or acidic, neutral or basic buffers). Pure methanol or acetonitrile were not appropriate to remove the analytes from the paper (extraction yields < 50%), while treatment with phosphate buffer (pH 7.4) released the whole matrix into solution, thus giving dirtier extracts. The best results in terms of extraction yields (higher than 80%) and sample cleaning were provided by a water/methanol solution (10:90, v/v), that was therefore selected for the procedure. Further experiments were carried out to evaluate whether it was more suitable to use sonication or microwave-assisted extraction to facilitate the removal of the analytes from the filter paper; the latter gave inconsistent results, so sonication was employed. Finally, the influence of the increase of the extraction time from 5 to 15 min on the recovery of the analytes was investigated; sonication for 10 min provided the same extraction yield as 15 min and was less time consuming.

Even if the extraction step was optimized to reduce the release of the matrix components from the paper, the extracts needed an additional pre-treatment to further reduce endogenous interference. Thus they were subjected to the MEPS procedure before being injected into the HPLC-DAD system.

Development of the MEPS procedure

The development of an appropriate sample pre-treatment procedure is of paramount importance for the analysis of complex biological matrices, such as DBS and plasma. Sample clean-up should be selective, reliable, fast, cheap and easy to perform. Microextraction by packed sorbent satisfies all these requirements. In fact, it has the same selectivity as SPE while offering some notable advantages: it reduces the amount of organic solvents used in the procedure from millilitres to microlitres, it is faster than SPE (average time needed to perform a MEPS is 10-15 min. vs 30-45 min. for a SPE)

and, having a lower amount of sorbent (4 mg vs 30-100 mg of a SPE cartridge), it retains less interference, thus giving cleaner extracts. Therefore, MEPS technique was chosen to purify DBS and plasma samples before HPLC injection.

A C8 sorbent was employed and the steps affecting the overall performance of the procedure (i.e. loading, washing and elution) were carefully optimized. The loading and washing steps were carried out using pH 7.4 phosphate buffer to maximize the retention of the analytes on the sorbent. The speed and the number of the loading cycles needed to be thoroughly investigated as they exert a strong influence on the extraction efficiency; 10 loading cycles performed at 5 µL s^{-1} proved to give the best recovery of the analytes. After a first wash with phosphate buffer a second wash containing an aliquot of organic solvent was necessary to improve sample clean-up. Different organic solvents (methanol, isopropanol and acetonitrile) added in variable amount (5%-10%) were tested. Eventually, a mixture of methanol and phosphate buffer (5:95, v/v) was selected as it gave cleaner extracts without significantly reducing the extraction yields of the analytes. Methanol turned out to be the best solvent for elution. The influence of the amount of the elution solvent on the absolute recovery was investigated in a 100-250 µL range; a 250 µL volume gave extraction yields > 90% for both analytes.

As can be seen in the chromatograms reported in **Fig.4** and **Fig. 5**, the developed MEPS procedure effectively cleaned both matrices from endogenous interference and gave good extraction yields for the analytes and the IS.

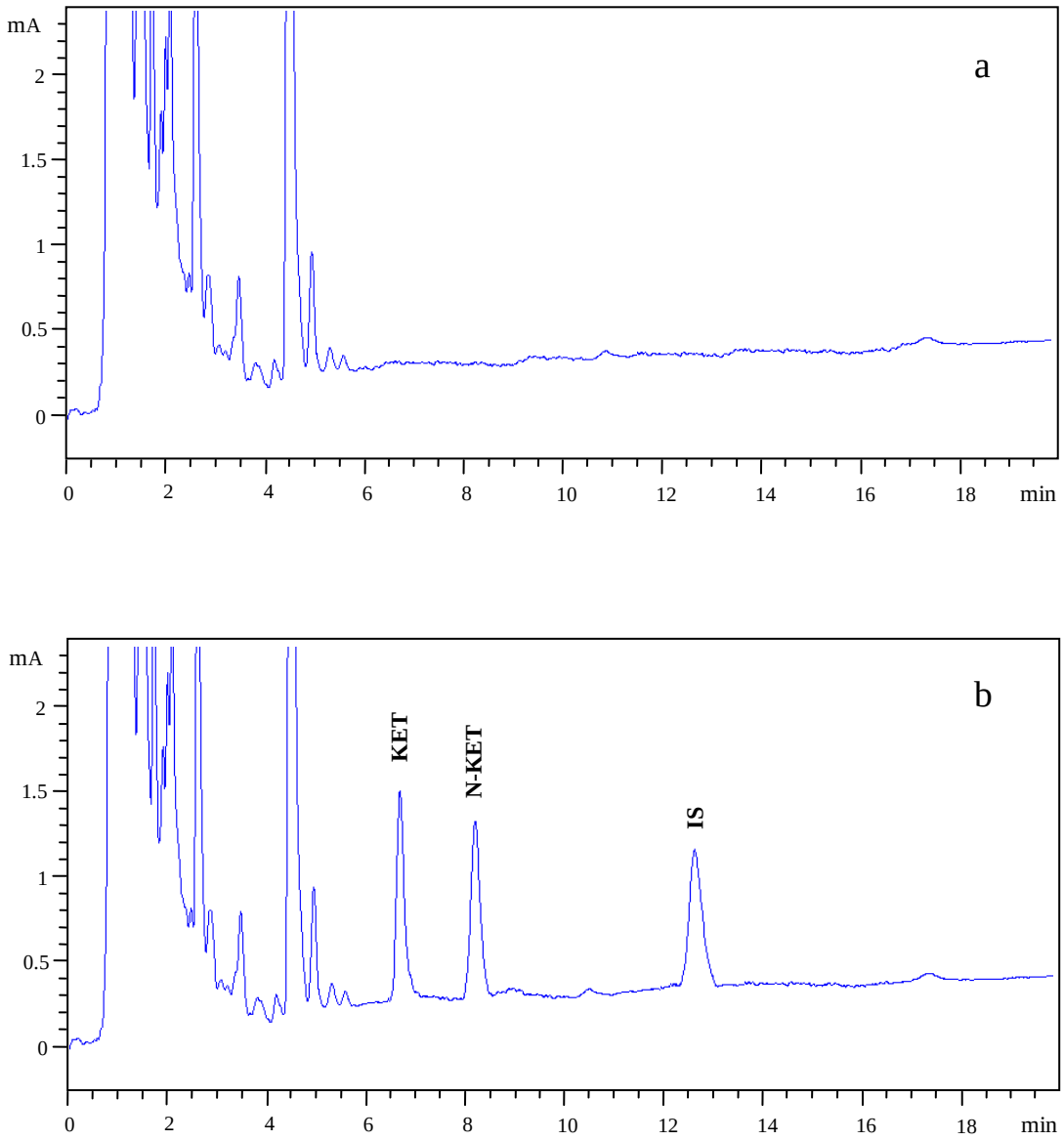


Fig. 4

Chromatograms of a blank DBS sample (a) and of the same sample spiked with a known amount of KET, N-KET and IS (on-column concentrations 100 ng mL^{-1}) (b).

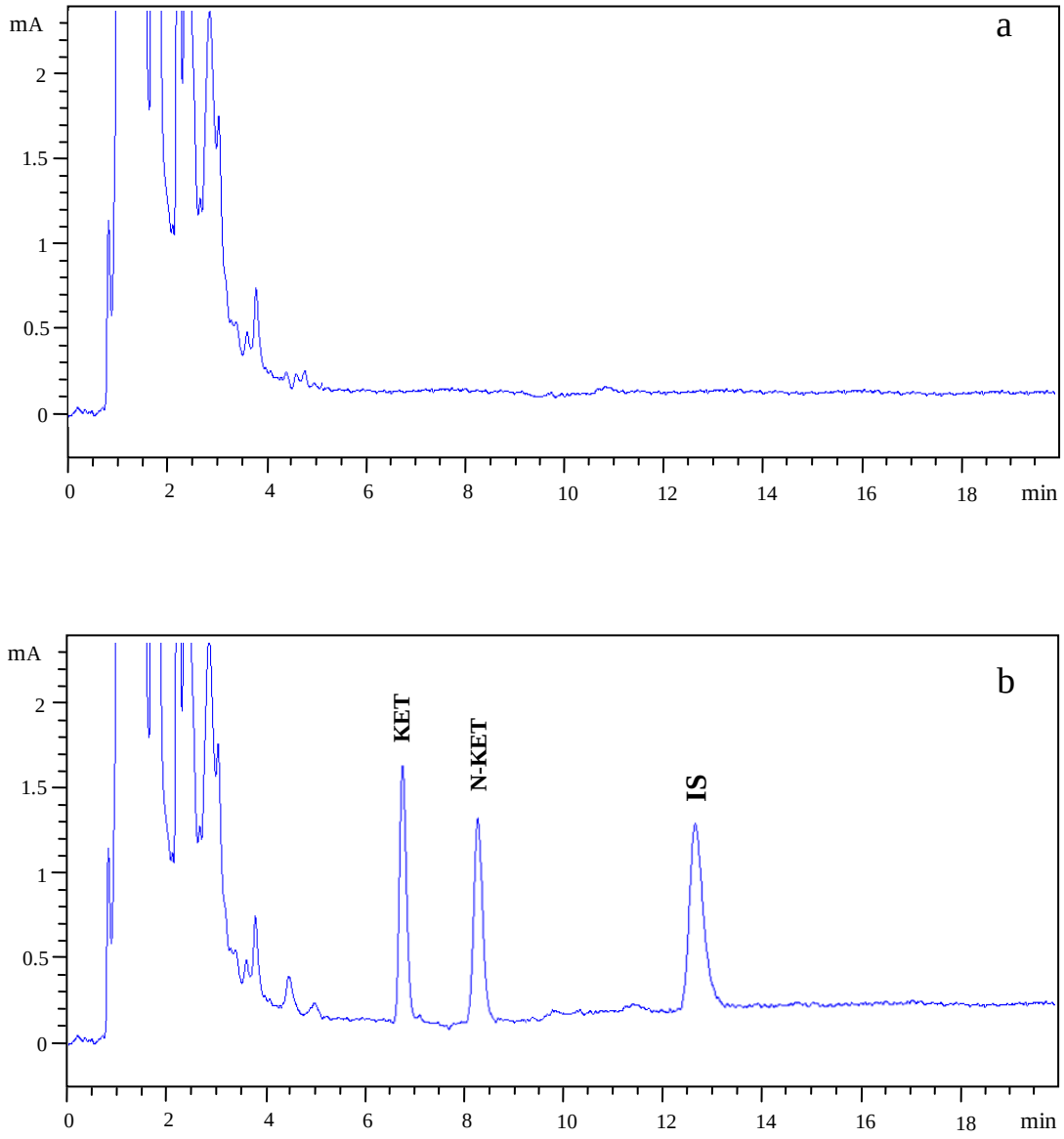


Fig. 5

Chromatograms of a blank plasma samples (a) and of the same sample spiked with a known amount of KET, N-KET and IS (on-column concentrations 100 ng mL^{-1}) (b).

Method Validation

Calibration curves were set up on blank matrices fortified with different concentrations of the analytes and a constant concentration of the IS. Good linearity (r^2 > 0.9992) was obtained over the studied concentration ranges (**Table 1**). The LOD and LOQ values were 3 and 10 ng mL^{-1} (on column concentrations), respectively, for both of the analytes.

Extraction yield, precision and accuracy assays were carried out on blank DBS and plasma spiked with analyte concentrations corresponding to the lowest, the intermediate and the highest point of each calibration curve (**Table 2**). As can be noted, extraction yield values were satisfactory, ranging between 83% and 88% in the DBS samples and between 90% and 95% in the plasma specimens. The mean extraction yields of the IS were 86% and 92% in the DBS and plasma samples, respectively. Precision was also good, with RSD values always lower than 5.7%; accuracy ranged between 89.7% and 105.2%.

- *Selectivity*

Selectivity was evaluated with respect to potential interference from endogenous and exogenous compounds. The analysis of blank DBS and plasma samples from six healthy volunteers showed no evidence of unacceptable endogenous interference at the retention times of the analytes and the IS. Moreover, standard solutions of several antidepressants (i.e. citalopram, lamotrigine, agomelatine, fluvoxamine, risperidone and amisulpride) and drugs of abuse (namely morphine, heroin, amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), cocaine and some of its metabolites (benzoylecgonine, norcocaine and ecgonine methyl ester)) were injected into the HPLC system at concentrations higher than the upper limit of the calibration curves. None of them gave rise to peaks that could interfere with the determination of the analytes.

- *Stability*

Stability evaluation was carried out in fortified DBSs stored in the dark at room temperature for 20 days. The amounts of KTM and N-KTM determined after storage were compared to the respective theoretical concentrations. A mean difference of -5% and -8% was observed for KTM and N-KTM, respectively. These values were deemed not significant, thus the analytes were considered stable in DBS under the tested storage conditions.

Analyte	Matrix	Linearity range (ng mL^{-1}) [a]	Equation coefficients (y = ax + b) [b]		r^2 [c]
			a	b	
KTM	DBS	10-250	0.0079	-0.0461	0.9993
	Plasma	10-500	0.0059	0.0117	0.9992
N-KTM	DBS	10-250	0.0062	0.0201	0.9994
	Plasma	10-500	0.0063	-0.0010	0.9997

[a] on-column concentrations
[b] y = analyte/IS peak-area ratio; x = analyte concentration (ng mL^{-1})
[c] r^2 = correlation coefficient

Table 1

Linearity parameters.

Analyte	Matrix	Concentration (ng mL^{-1})[a]	Extraction yield (%)[b]	Intraday precision (RSD%)[b]	Interday precision (RSD%)[b]	Intraday accuracy (%)[b]	Interday accuracy (%)[b]
KTM	DBS	10	88	4.9	5.7	92.9	91.8
		100	85	4.3	4.9	95.6	94.3
		250	84	3.7	4.4	102.1	105.2
	Plasma	10	95	4.4	5.2	91.8	90.6
		200	93	3.9	4.3	96.9	95.1
		500	93	3.5	3.9	99.2	101.2
N-KTM	DBS	10	87	4.7	5.3	89.7	91.9
		100	85	4.0	4.8	103.1	98.6
		250	83	3.4	3.9	101.4	100.1
	Plasma	10	93	4.3	5.5	90.2	89.8
		200	91	3.7	4.6	98.7	97.5
		500	90	3.1	3.7	100.3	103.1

[a] on-column concentration
[b] n = 6

Table 2

Validation parameters: extraction yield, precision and accuracy data.

5.4. Conclusions

An analytical method based on the use of HPLC coupled with diode array detection has been developed for the simultaneous determination of KET and its main active metabolite, N-KET, in DBS and human plasma. Sample pre-treatment was carried out by means of an original MEPS procedure which followed a solvent extraction of the analytes in case of DBS specimens.

The main novelty of this work consists in the use of DBS as an alternative to conventional venous blood collection. DBS technique offers many advantages (e.g. minimal invasiveness and ease of the sampling method, simplicity and cheapness of transport and storage), resulting in a significant simplification of blood sample collection and handling and in improved patient comfort.

Another important innovation is the employment of MEPS technique for sample clean-up. Compared to the more commonly used SPE and liquid-liquid extraction, MEPS requires a lower amount of sample, is faster and limits the consumption of organic solvents, thus reducing costs. The developed pre-treatment gave good purification of both matrices and satisfactory extraction yields (> 83% for DBS and > 90% for plasma samples). Good results were also obtained in terms of precision (RSD < 5.7%), accuracy (89.7%-105.2%) and selectivity.

The presented method could be used to assess KET recent intake for recreational purposes or to conduct pharmacokinetic studies valuable to further elucidate the analgesic and antidepressant activity of this drug.

6. DETERMINATION OF OXYCODONE AND ITS MAIN METABOLITES IN HUMAN PLASMA BY HPLC-MS/MS

6.1. Introduction

Oxycodone ((5R,9R,13S,14S)-4,5α-epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one, OXC, **Fig. 1**) is a semisynthetic μ-opioid receptor agonist widely used in the management of moderate to severe pain [110,111]. It is also reported to bind to k-opioid and δ-opioid receptors, but with a lower affinity than to μ-receptors [110]. OXC is rather well absorbed after oral administration with a bioavailability of approximately 60–80% [110]. It is extensively metabolized by the cytochrome P450 (CYP) system and less than 10% is excreted unchanged in the urine. The majority of OXC is *N*-demethylated to noroxycodone (NOXC) by CYP3A4/5, while a smaller fraction is *O*-demethylated to oxymorphone (OXM) by CYP2D6. Both noroxycodone and oxymorphone are then further metabolized to noroxymorphone (NOXM) by CYP3A and CYP2D6 (**Fig. 1**). OXC and its oxidative metabolites also undergo 6-keto reduction, but this represents just a minor metabolic pathway. Among phase II reactions, conjugation with glucuronic acid occurs, especially for OXMP, leading to the formation of oxymorphone-3-*β*-D-glucuronide (OXM-G, **Fig. 1**) [110,112,113]. NOXC and NOXM seem to scarcely contribute to the antinociceptive effect of OXC, while the role of OXM in the pharmacological action of OXC is still controversial. In fact, OXM has a potent analgesic activity, but its plasma concentrations following OXC administration have been reported to be very low [113,114].

Because of the involvement of CYP450 enzymes, OXC metabolism is prone to a great interindividual variability due to drug-drug interactions and to genetic polymorphism [110,112]. Differences in drug metabolism can lead to severe toxicity or therapeutic failure by altering the relationship between the dose and steady-state blood concentration of the pharmacologically active drug [115]. Pharmacokinetic variability needs to be carefully evaluated especially in case of administration of drugs characterized by a narrow therapeutic index, like opioids [112]. In this case, unexpectedly increase in the plasmatic level of the active drug may lead to potentially fatal adverse effects, like respiratory depression. On the other hand, reduction of blood

concentration might result in loss of pain relief. Therefore, plasma levels of OXC and its metabolites should be carefully monitored and the dosage adjusted accordingly. Moreover, further investigation of the specific CYP isoforms implicated in OXC metabolism and of their degree of involvement is needed to better predict potential drug-drug interactions and metabolic differences arising from genetic polymorphism. In fact, *in vitro* data have suggested that CYP enzymes other than CYP2D6 and CYP3A may also be involved and the impact of CYP2D6 and CYP3A activity modulation on the pharmacokinetics of OXC remains poorly explored [112,116]. In addition, besides CYP450, other enzymes contribute to OXC metabolism, namely the uridine diphosphate glucuronosyltransferase (UGT) superfamily, that mediates OXM glucuronidation to OXM-G [115,117]. Similar to CYP450, UGT displays different isoforms which can be subjected to induction, inhibition and genetic polymorphism. Hence, investigation of the UGT forms implicated in OXC metabolism may also be clinically relevant [115,118].

OXC has a significant abuse potential and is one of the most commonly abused drugs in the USA, where non-medical use of prescription psychotherapeutics and in particular opioid analgesic is an increasing public health problem [2,119]. Data from the National Survey on Drug Use and Health indicate that in 2008 abuse of prescription psychoactive drugs was second just to marijuana and hashish use, and was more prevalent of that of cocaine and heroin, showing an increment from 1998 to 2008 of 151%.[120] When abused, OXC tablets are often crushed and snorted or injected intravenously, thereby increasing the risk for serious medical complications, including overdose [2,119]. Monitoring of OXC therapy may also have the added benefit of discouraging improper use and of providing information on patient compliance and diversion.

Several methods can be found in the literature for the detection of OXC together with other opioids, sometimes including OXM, in different biological matrices (blood [121-126], urine [124,125,127,128], hair [129,130], oral fluid [130,131], meconium [125,132]). These methods are based on gas chromatography (GC) [121,122,127,129-131] or liquid chromatography (LC) [123-126,128,132] coupled with mass spectrometry (MS). Few papers deal with the determination of OXC and two of its metabolites (OXM

and NOXC) in urine by GC-MS [133] or in rat plasma by LC-MS/MS [134,135]. Two LC-MS/MS methods [136,137] have been proposed for the quantification of OXC, NOXC, OXM and NOXM in human plasma, but they do not include OXM-G and they present some drawbacks, like long total run time and employment of a column-switching apparatus. The determination of OXM-G has been recently reported in human urine together with OXC, OXM and other opiods using hydrophilic interaction LC tandem MS [138]. Biological sample pre-treatment has been carried out mainly by solid phase extraction (SPE) [122,125,126,129-133,135,137], but liquid-liquid extraction [121,123,124,127] and protein precipitation [134,136] have also been reported. To the best of knowledge, no analytical method is currently available for the simultaneous determination of OXC, NOXC, OXM, NOXM and OXM-G. The analysis of OXM-G along with the other metabolites allows to gain a deeper understanding of OXC pharmacokinetics evaluating also the involvement of different UGT isoforms. Hence, the aim of the present study was to develop a LC-MS/MS method for the simultaneous quantification of OXC and its main metabolites (NOXC, NOXM, OXM and OXM-G) in human plasma. An original SPE procedure has been used for sample clean-up.

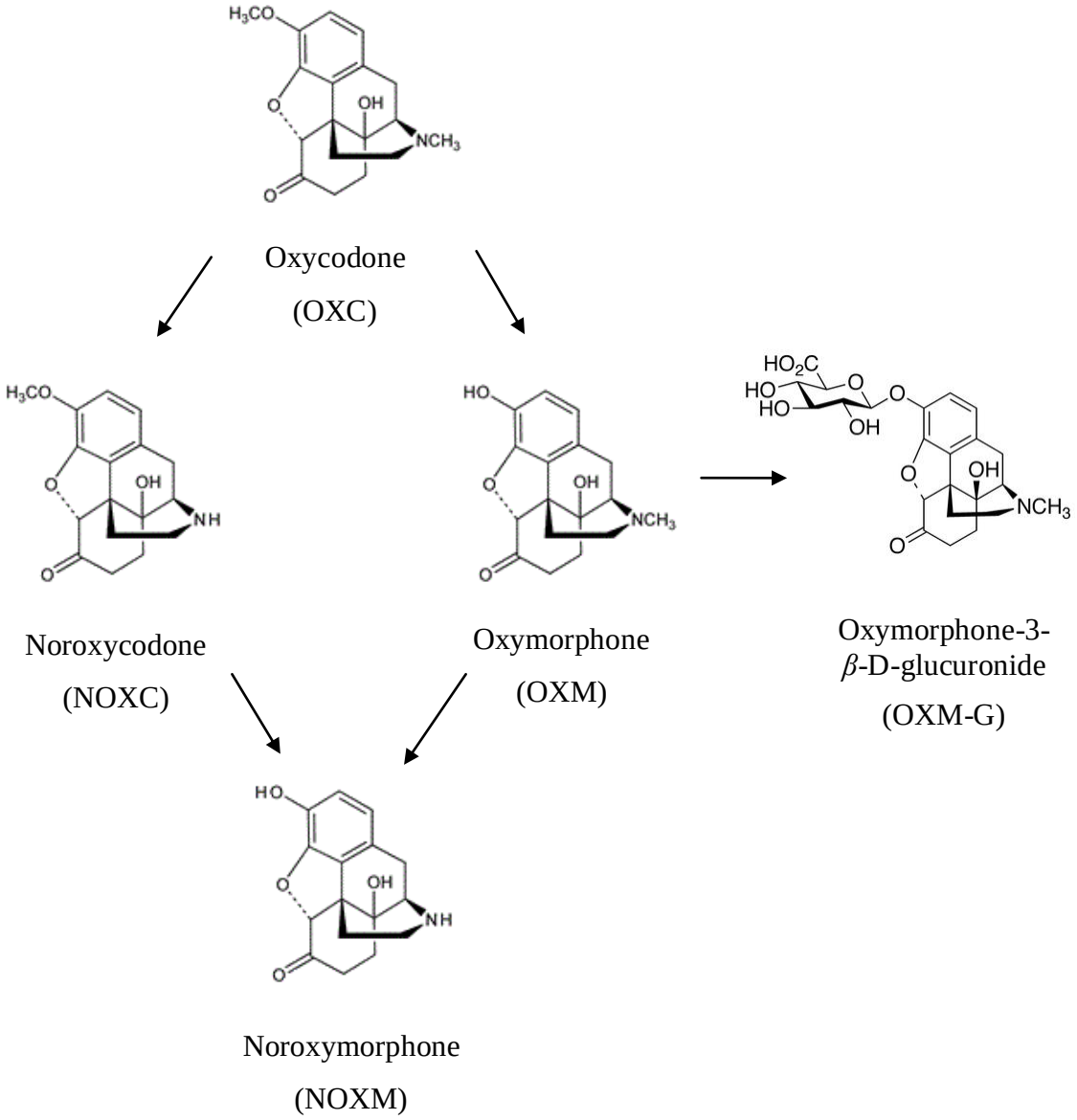


Fig. 1

Metabolic pathway of oxycodone and chemical structures of oxycodone, noroxycodone, oxymorphone, noroxymorphone and oxymorphone-3-*β*-D-glucuronide.

6.2. Experimental

Chemicals

Standard stock solutions of OXC (1 mg mL^{-1}), OXC-d_6 (100 µg mL^{-1}), NOXC (1 mg mL^{-1}), NOXC-d_3 (100 µg mL^{-1}), OXM (1 mg mL^{-1}), OXM-d_3 (100 µg mL^{-1}), OXM-G (100 µg mL^{-1}), OXM-G-d_3 (100 µg mL^{-1}) and NOXM (100 µg mL^{-1}) were purchased from Cerilliant (Round Rock, TX, USA). All standards were methanolic solutions, except OXM-G and its deuterated internal standard (IS) that were dissolved in methanol/water (1:1). Ammonium acetate was obtained from Sigma Aldrich (St. Louis, MO, USA). Ammonium hydroxide and concentrated formic acid were from Fisher Scientific (Pittsburgh, PA, USA). HPLC grade methanol was purchased from J.T. Baker (Phillipsburg, NJ, USA). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA). Human blank plasma was from the University of Utah blood bank.

Preparation of working solutions

Calibrator working solutions containing all analytes were prepared by diluting standard stock solutions with methanol/water (50:50, v/v). Separate working solutions were prepared in the same way for QC samples. A deuterated internal standard (IS) working solution was prepared in methanol/water (50:50, v/v) at a concentration of 0.1 µg mL^{-1}. All working solutions were stored at -20°C. Stock solutions were stable for at least 4 months when stored at -20 °C (as assessed by HPLC assays).

Preparation of calibration standards and quality control samples

Calibration standards were prepared daily by adding the appropriate aliquot of calibrator working solutions and 30 µL of deuterated IS working solution to 1 mL of blank plasma. Calibrators were at the following plasma concentrations: 0.2, 0.5, 1.0, 5.0, 20.0, 50.0, 75.0 and 100.0 ng mL^{-1} for OXM, NOXC and OXC and 0.5, 1.0, 5.0, 20.0, 50.0, 75.0 and 100.0 ng mL^{-1} for OXM-G and NOXM. In the same way, quality

control (QC) samples were prepared by adding the appropriate amount of QC working solutions and 30 µL of deuterated IS working solution to 1 mL of blank plasma. QC samples were at three concentration levels: low QC contained 0.6 ng mL^{-1} of OXM, NOXC and OXC and 1.5 ng mL^{-1} of OXM-G and NOXM; medium and high QC were at a concentration of 10.0 and 85.0 ng mL^{-1}, respectively, for all analytes.

Equipment

LC–MS/MS analyses were performed using an ACQUITY UPLC® system (Waters, Milford, MA, USA) coupled to a Quattro Premier XE™ triple quadrupole mass spectrometer (Waters). Solid phase extraction (SPE) was carried out on Oasis MCX cartridges (60 mg, 3 mL) (Waters) using a Vac Elut manifold. A Thermo Scientific (Waltham, MA, USA) IEC FL40 floor centrifuge and a Zymark (Hopkinton, MA, USA) TurboVap® evaporator were also used.

Data were handled with MassLynx™ (v 4.1) software (Waters).

LC-MS/MS conditions

The chromatographic separation was achieved on a Supelco (Bellefonte, PA, USA) Discovery® HS F5 column (2.1 x 50 mm, 3 µm) held at 40°C. The mobile phase consisted of 10 mM ammonium acetate containing 0.1% formic acid (A) and methanol (B). The flow rate was kept constant at 0.25 mL min^{-1} and a gradient program was run. Initial mobile phase conditions were 90% A and 10% B; B was increased linearly from 10 to 70% in 6 min, held at 70 % for 0.50 min, decreased back to the initial mobile phase condition of 10% B in 0.30 min and finally held at 10% for 4.0 min to re-equilibrate the column (total chromatographic run time was 10.80 min). Injections were performed with an autosampler maintained at 4°C; injection volume was 12 µL.

The mass spectrometer was operated in electrospray positive ionization (ESI+) mode and performed multiple reaction monitoring (MRM). Capillary voltage was set at 3.00 kV and source and desolvation temperature were set at 100°C and 350°C, respectively. Nitrogen was used as the desolvation gas at a flow rate of 800 L h^{-1} while argon was

used for collision. Cone voltage and collision energy were optimized for each analyte and are listed in **Table 1** along with the selected MRM transitions. Two product ions were monitored for each analyte; the most abundant one was used for quantification while the other one was used as qualifier. Dwell time was 200 ms for OXM-G and OXM-G-d_3 ion transitions and 100 ms for all the other MRM transitions monitored.

Analyte	MRM transitions* (m/z)	Cone voltage (V)	Collision energy (eV)
OXM-G	478.1 → 284.4	40	32
	478.1 → 227.3	40	50
OXM-G-d_3	481.1 → 287.4	40	32
	481.1 → 230.3	40	50
NOXM	288.3 → 270.4	35	18
	288.3 → 213.3	35	30
OXM	302.3 → 284.3	35	20
	302.3 → 227.4	35	26
OXM- d_3	305.3 → 287.4	35	20
	305.3 → 230.4	35	26
NOXC	302.3 → 284.3	30	16
	302.3 → 187.3	30	26
NOXC- d_3	305.3 → 287.3	30	16
	305.3 → 190.3	30	26
OXC	316.3 → 298.3	30	20
	316.3 → 241.3	30	28
OXC- d_6	322.3 → 304.3	30	20
	322.3 → 247.3	30	28

*The quantification transition for each analyte is given in the upper row.

Table 1

MRM transitions, cone voltage and collision energy selected for each analyte.

Sample pre-treatment: solid phase extraction

Sample pre-treatment was carried out by SPE on mixed-mode cation-exchange cartridges conditioned with 2 mL of methanol followed by 2 mL of water. A 1-mL aliquot of plasma fortified with the analytes was spiked with 30 µL of deuterated IS working solution (0.1 µg mL^{-1}) and acidified with 1 mL of ammonium acetate (100 mM, pH 5.0). The resulting mixture was briefly vortexed and centrifuged at 2800 rpm for 10 min to remove any particulate material. The supernatant was loaded onto previously conditioned cartridges that were then washed with 2 mL of ammonium acetate (100 mM, pH 5.0) and 2 mL of methanol. Analytes were eluted with 2 mL of 10% ammonium hydroxide in methanol and the eluate was evaporated to dryness under air at 40°C. Finally, the residues obtained were reconstituted in 75 µL of 0.1% formic acid and transferred into autosampler vials.

Method validation

The method was validated following the U.S. Food and Drug Administration (FDA) guidelines for bioanalytical method validation [139].

- *Linearity and lower limit of quantitation*

Linearity was evaluated over the 0.2-100 ng mL^{-1} concentration range for OXM, NOXC and OXC and over the 0.5-100 ng mL^{-1} concentration range for OXM-G and NOXM by analyzing in duplicate the calibration standards subjected to the previously described sample preparation procedure. The analyte/IS peak-area ratios were plotted against the corresponding concentrations of the analytes (expressed as ng mL^{-1}) and the calibration curves were constructed by means of the least-square method.

The lower limit of quantitation (LLOQ) was defined as the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. It was assessed by analyzing six fortified samples (each prepared from a different plasma source) and determining precision (expressed as percentage relative standard deviation (RSD%)) and accuracy (expressed as percentage of the nominal concentration). The

samples were accepted as LLOQ if RSD% was ≤ 20% and if the mean measured concentration was within ± 20% of the theoretical concentration.

- *Extraction yield, precision and accuracy*

Extraction yield was determined for each analyte at the low, medium, and high QC concentrations (5 replicate each). QC samples were subjected to the previously described pre-treatment procedure and analyzed along with unextracted standard solutions prepared at the same theoretical concentrations. The average analyte peak areas of the extracted samples were compared to those of the unextracted standard solutions and the extraction yields were calculated and expressed as percentage value. The data obtained are reflective of the combination of extraction recovery of the analytes and matrix effect and they can also be referred to as overall process efficiency, according to the definition of Matuszewski et al. [140].

Intraday accuracy and precision were obtained by analysing low, medium and high QC samples five times within the same day; interday accuracy and precision were evaluated by analysing the three QC levels five times over three different days for a total of fifteen results per concentration. Intra- and interday precision were both expressed as percentage relative standard deviation (RSD%). Intra- and interday accuracy were calculated by dividing the mean measured concentrations of the analytes by the theoretical concentrations and were expressed as percentage value.

- *Selectivity*

Selectivity was assessed by the analysis of plasma samples from six different healthy donors. Each sample was extracted and analysed to determine if any potential interference from endogenous components was present at the retention time of the analytes and the ISs. In addition, three replicates for each plasma source were spiked with the ISs and one replicate was spiked with the ISs and the analytes at a concentration corresponding to the LLOQ. To be acceptable, the mean peak-area ratio of any signal at the retention time of the analytes to the corresponding IS must not exceed 20% of the mean analyte/IS peak-area ratio at LLOQ. Moreover, the area of any

peak at the retention time of the ISs must be less than 5% of the mean peak area of the ISs in the LLOQ samples.

- *Stability*

Stability experiments were performed with low and high QC samples (three replicates for each concentration level) stored under various conditions. The stability of the analytes in plasma samples was evaluated after three freeze-thaw cycles and at room temperature. For the assessment of freeze-thaw stability, frozen QC samples were allowed to completely thaw unassisted at room temperature and then were frozen again for at least 12 h. This was repeated for a total of three times. Room temperature stability was evaluated on QC samples left at room temperature for 24 h prior to analysis. Stability was also tested in processed samples stored on the autosampler (+4°C) for 5 days and at -20°C for 7 days. The analytes were considered stable under the tested conditions if the mean concentrations found in the stored samples did not differ from the theoretical concentrations by more than ±15%.

6.3. Results and discussion

Development of the chromatographic conditions

The investigated analytes are all quite polar molecules, characterized by a logP < 1.2 [141]. OXM-G is much more polar than the other compounds as a result of the presence of the glucuronic acid moiety. The polarity difference between OXM-G and all the other analytes presented a great challenge throughout the method development process. The first difficulty was encountered during the selection of the stationary phase. Preliminary tests were carried out on a C18 column specifically designed for polar compounds. However, OXM-G was too poorly retained; this affected ionization efficiency resulting in a great loss of sensitivity. A HILIC stationary phase was also tried, but no significant improvements were obtained. Eventually, a pentafluorophenylpropyl (HS F5) column was tested. Thanks to its unique chemical properties, HS F5 bonded phase provided stronger retention of all analytes than C18 column, resulting in a satisfactory separation of OXM-G from the void peak. Furthermore, HS F5 column gave better peak shape and increased sensitivity compared to the other columns tested. A gradient program was applied to optimize resolution of the analytes and total run time. The developed conditions allow the complete chromatographic separation of OXM and NOXC, which is essential for their identification as they show common MRM transitions.

MS/MS parameters were optimized by infusing standard solutions of each analyte directly into the source along with the mobile phase at initial composition. As the most abundant fragment ion corresponded to the loss of water for all analytes except OXM-G, which loses the glucuronic acid moiety as well, two parent-product ion transitions were monitored for each compound to improve method selectivity; the most intense transition was used for quantification and the other one was employed to confirm peak identity. Since NOXM deuterated analogue is not commercially available, OXM-d_3 was used as the IS for NOXM, as it was the closest eluting peak; for all other analytes the corresponding deuterated analogue was employed.

The representative chromatograms of a standard solution of the analytes and the ISs are reported in **Fig. 2**.

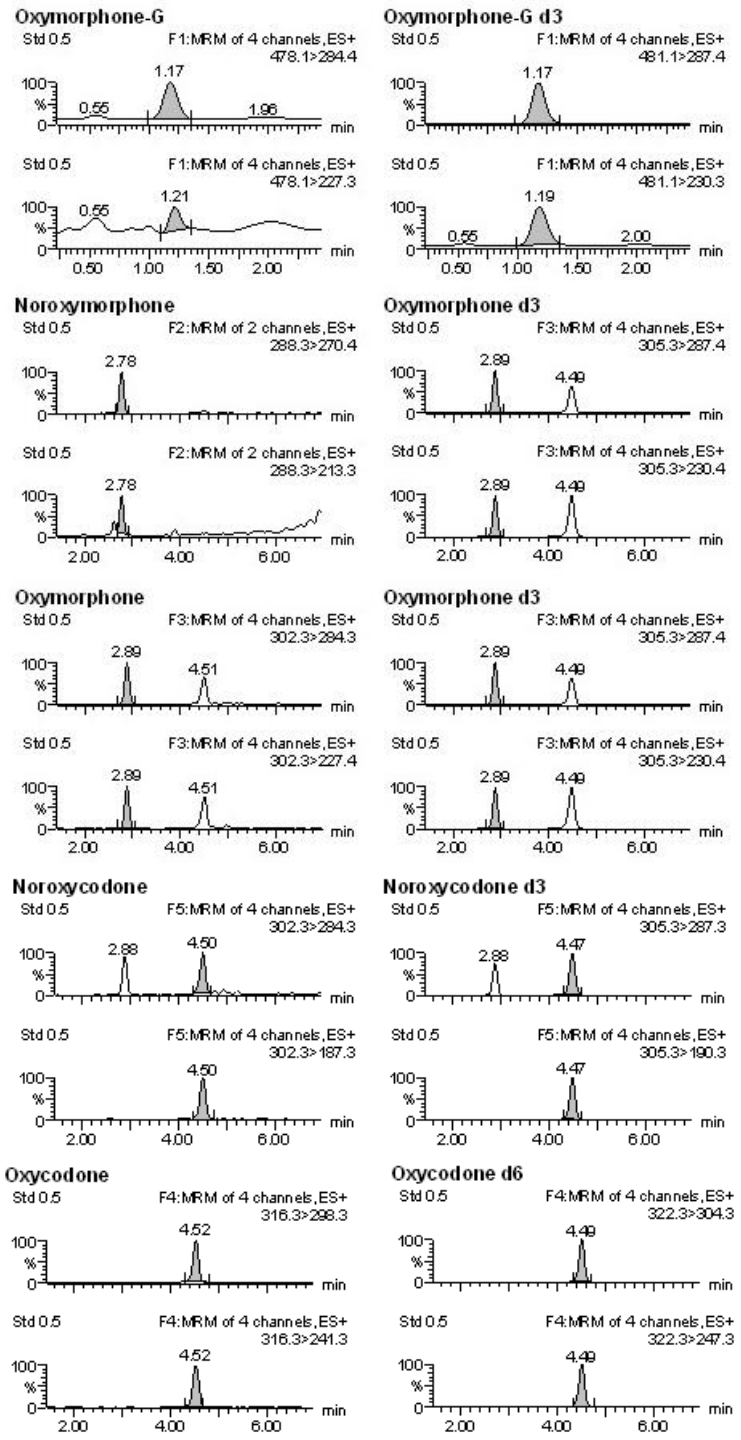


Fig. 2

Chromatograms of a standard solution of the analytes (0.5 ng mL^{-1}) and the ISs. For each analyte both MRM transitions are shown (quantification transition is provided first).

Development of the SPE procedure

As for chromatographic method development, the hydrophilicity of the analytes and the polarity difference between OXM-G and the other compounds challenged the optimization of the extraction conditions. Various SPE cartridges and different procedures were tested. C18 sorbent gave low recovery (< 30%) even though the samples were basified before loading to stabilize the analytes in their deprotonated, more lipophilic, form. Moderately hydrophobic cartridges (C2) could not sufficiently retain OXM-G that eluted during the washing step. Better results in terms of retention of the compounds were obtained on C8 and hydrophilic-lipophilic balance (HLB) cartridges (loading the former in basic conditions). However, none of the elution solvents tested (methanol, acetonitrile, acidified methanol) gave satisfactory extraction yields that were especially low for OXM-G. Eventually, mixed-mode cation-exchange (MCX) polymeric sorbent proved to be suitable for the extraction of the analytes from the matrix. The compounds of interest in their protonated form were highly retained by ionic interaction and the elution with basified methanol gave encouraging results. Nonetheless, it was necessary to carry out some tests to optimize the recovery of OXM-G. Among the solutions investigated, 10% ammonium hydroxide in methanol gave extraction yields higher than 70% for all the analytes. In addition, thanks to the retention mechanism, it was possible to wash the cartridge with 100% organic solvent without affecting the recovery, thus removing also hydrophobic interferences and obtaining cleaner extracts.

The chromatograms of a blank plasma sample subjected to the SPE procedure (**Fig. 3**) show that no significant peaks are present at the retention times of the analytes. The representative chromatograms of a plasma sample fortified with the analytes (low QC) and subjected to the SPE pre-treatment is reported in **Fig 4**.

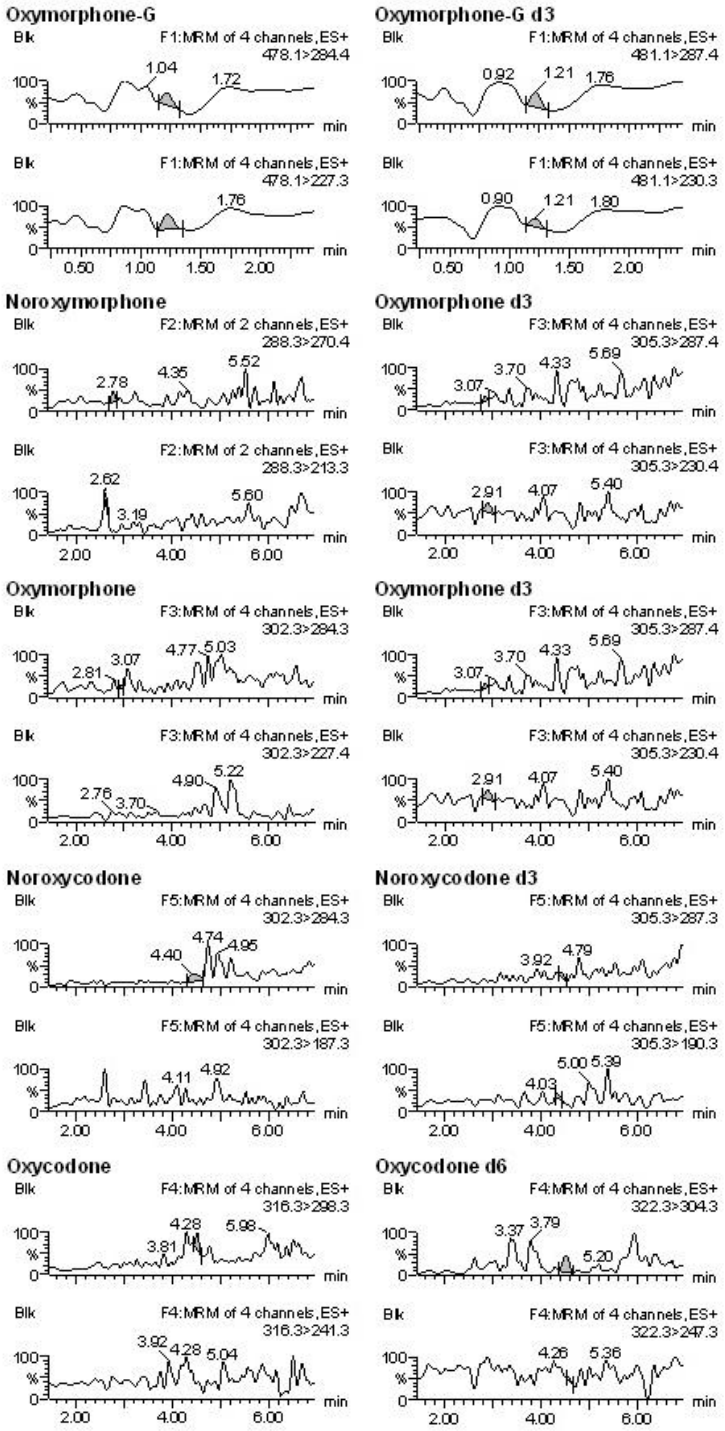


Fig. 3

Chromatograms of a blank plasma sample. Both MRM transitions are shown for the analytes and the ISs (quantification transition is reported first).

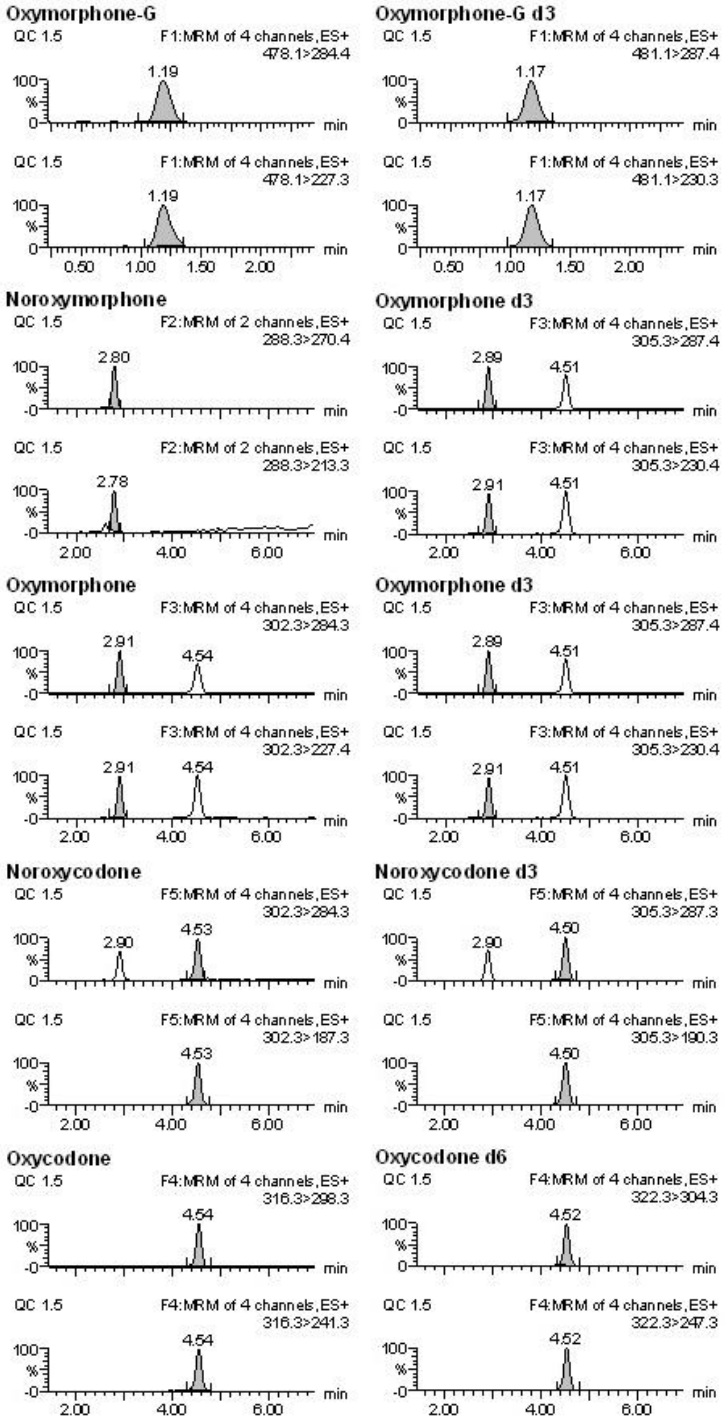


Fig. 4

Chromatograms of a plasma sample fortified with the analytes and the ISs. Concentrations correspond to low QC. Both MRM transitions are shown for each analyte (quantification transition is reported first).

Method Validation

Calibration curves were constructed by means of the least-square method and a 1/x weighting factor was applied. Good linearity ($r^2 > 0.995$) was obtained over the studied concentration ranges. The linearity range, slope, intercept and correlation coefficient for each analyte are summarized in **Table 2**. The LLOQ was 0.2 ng mL^{-1} for OXM, NOXC and OXC and 0.5 ng mL^{-1} for OXM-G and NOXM. Precision and accuracy at the LLOQ assessed in 6 fortified samples from different plasma sources satisfied the acceptance criteria (i.e. RSD% ≤ 20% and deviation from the theoretical concentration within ± 20%). Extraction yield, precision and accuracy assays were carried out for each analyte at the low, medium, and high QC concentrations (5 replicate each); mean results are provided in **Table 3**. Extraction yield values calculated herein are reflective of the combination of matrix effect and extraction recovery of the analytes. Results were satisfactory, being always higher than 70%. Precision and accuracy were also acceptable: RSD values were always lower than 8.8% and accuracy ranged between 87.0% and 105.4%.

- *Selectivity*

The analysis of plasma samples from six different healthy volunteers showed no evidence of unacceptable endogenous interference at the same MRM transitions and retention times of the analytes and the ISs, proving the selectivity of the method toward endogenous compounds.

- *Stability*

Stability of the analytes was assessed in plasma samples stored at room temperature for 24 h and after three freeze-thaw cycles. Stability was also evaluated in processed samples stored on the autosampler (+4°C) for 5 days and at -20°C for 7 days. The mean observed concentrations of the analytes in the stored samples were always within ±15% of the nominal concentrations, indicating that the analytes are stable under all tested conditions.

Analyte	Linearity range (ng mL^{-1})	Equation coefficients ($y = ax + b$) [a]		r^2 [b]
		a	b	
OXM-G	0.5-100.0	0.6660	-0.0404	0.995
NOXM	0.5-100.0	0.2930	-0.0399	0.996
OXM	0.2-100.0	0.9170	0.0847	0.997
NOXC	0.2-100.0	0.9400	0.0414	0.999
OXC	0.2-100.0	0.9550	0.0040	0.999

[a] y = analyte/IS peak-area ratio; x = analyte concentration (ng mL^{-1})
[b] r^2 = correlation coefficient

Table 2

Linearity parameters.

Analyte	Concentration (ng mL^{-1})	Extraction yield (%)[a]	Intraday precision (RSD%)[a]	Interday precision (RSD%)[a]	Intraday accuracy (%)[a]	Interday accuracy (%)[a]
OXM-G	1.5	70	8.3	8.6	98.7	100.2
	10.0	75	6.7	8.3	99.0	100.2
	85.0	72	7.6	8.8	102.1	102.7
NOXM	1.5	75	8.2	8.7	92.4	100.2
	10.0	77	7.9	8.7	87.0	90.2
	85.0	81	8.0	8.5	90.3	94.9
OXM	0.6	79	2.8	5.7	96.3	94.4
	10.0	86	1.5	2.9	103.0	104.0
	85.0	83	3.1	3.7	101.9	101.5
NOXC	0.6	80	3.5	5.9	99.3	101.0
	10.0	86	2.3	2.8	105.4	105.2
	85.0	88	2.0	2.5	100.4	100.8
OXC	0.6	89	2.3	3.3	104.3	101.7
	10.0	92	1.9	3.6	99.2	102.0
	85.0	91	1.1	3.6	102.4	104.7

[a] n = 5

Table 3

Validation parameters: extraction yield, precision and accuracy data.

6.4. Conclusions

An analytical method based on the use of liquid chromatography coupled with tandem mass spectrometry and on a solid phase extraction procedure for sample clean-up has been developed for the simultaneous determination of OXC, NOXC, OXM, NOXM and OXM-G in human plasma. Few other methods have been reported for the detection of some of the analytes (namely OXC, NOXC, OXM and NOXM) [136,137], but to the best of knowledge this is the first one that includes OXM-G in the assay.

The method has been fully validated according to the FDA guidelines with satisfactory results and it will be used to investigate the impact of the activity of the CYP450 and UGT isoforms on the pharmacokinetics of OXC. The developed method is also suitable for therapeutic drug monitoring (TDM) of OXC in patients treated for pain relief, allowing individualization of the therapy. Benefits of TDM include dose optimization and minimization of side effects, resulting in improved patient outcome and safety. Moreover, TDM provides information on patient compliance and may discourage OXC abuse.

6.5. Acknowledgements

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FOOTNOTES

Some of the analytical methods developed during the Ph.D. program and presented herein have been published in scientific journals:

- L. Somaini, M.A. Saracino, C. Marcheselli, S. Zanchini, G. Gerra, M.A. Raggi

  "Combined liquid chromatography-coulometric detection and microextraction by packed sorbent for the plasma analysis of long acting opioids in heroin addicted patients"

  Anal. Chim. Acta, 702 (**2011**) 280.

- M.A. Saracino, C. Marcheselli, L. Somaini, G. Gerra, F. De Stefano, M.C. Pieri, M.A. Raggi

  "Simultaneous determination of disulfiram and bupropion in human plasma of alcohol and nicotine abusers"

  Anal. Bioanal. Chem. 398 (**2010**) 2155.

Another analytical method resulted from the research performed during the three years of the Ph.D. program has been object of publication:

- F. Bugamelli, C. Marcheselli, E. Barba, M.A. Raggi

  "Determination of L-dopa, carbidopa, 3-O-methyldopa and entacapone in human plasma by HPLC-ED"

  J. Pharm. Biomed. Anal. 54 (**2011**) 562.

However, as it is not strictly related to the problem of drug abuse, it has not been included in the present thesis work.

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