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A rapid UPLC–MS/MS method for simultaneous determination of flunitrazepam, 7-aminoflunitrazepam, methadone and EDDP in human, rat and rabbit plasma

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ABSTRACT

A simple, high-throughput, sensitive LC–ESI-MS/MS method is presented for the simultaneous determination of methadone (MET), flunitrazepam (FNZ) and their major metabolites, EDDP (2-ethilidene-1,5-dimethyl-3,3-diphenylpyrrolidone) and 7-aminoflunitrazepam (7-AFNZ), respectively, in human, rat and rabbit plasma. The isolation of the selected compounds involved a liquid–liquid extraction with ethyl acetate at a basic pH. Good chromatographic separation was achieved on a HSS T3 column (1.8 μm particle size), with a 3 min gradient elution using a mixture of acetonitrile with 0.1% formic acid (solvent A) and 5 mM ammonium acetate (solvent B) as the mobile phase. The tandem mass spectrometric detection was performed in multiple reaction monitoring (MRM) mode with ionization of the analytes in positive mode. The assay was fully validated according to current acceptance criteria for bioanalytical methods validation. It was proved to be linear in the range of 0.5–250 ng/mL, with adequate accuracy and precision over this range. Based on accuracy and CV% values the LOQ and ULOQ values were set at 0.509 ng/mL and 2036 ng/mL for MET, 0.520 ng/mL and 2080 ng/mL for EDDP, 0.524 ng/mL and 2096 ng/mL for FNZ and 0.528 ng/mL and 2114 ng/mL for 7-AFNZ, respectively. The method was tested for potential matrix effects, without observing significant ion suppression. The investigated compounds stability was examined in plasma at room temperature and after three freeze–thaw cycles and in the final extract when maintained at 4 °C in the autosampler. Potential stability issues were observed only for FNZ at room temperature. The method was successfully applied to quantify the selected compounds in human, rat and rabbit plasma samples, after exposure to FNZ or simultaneous exposure to FNZ and MET.

1. Introduction

Poly-substance abuse is becoming a major issue to address in toxicology in the 21st century. The use of prescription drugs for a purpose other than the medical one has been reported frequently for opioid analgesics, sedatives/tranquilizers and stimulant therapeutic agents [1]. There are reports suggesting the co-occurrence of nonmedical benzodiazepine use with nonmedical use of prescription opiates [2].

Methadone (MET) represents the most often used drug worldwide in opioid substitution treatment, but has been used also for the relief of moderate-to-severe pain [3–9]. MET appears also as a street drug and, recreationally, is abused for its sedative and analgesic effects. The increased use and availability of the drug might explain the more frequent MET overdose cases in children (unintentional MET ingestion) and adults [10–12]. Most fatalities linked to MET occurred due to an overdose or were the result of the concurrent consumption of prescription or illegal drugs, such as benzodiazepines [4]. These types of concomitant exposures were associated with severe sedation, impaired motor and cognitive performance, respiratory depression and even death [13,14]. Therefore, it is of great importance to determine the concentration of MET and also the other drugs which might be co-ingested [15].

The metabolism of MET is mediated mainly by cytochrome P450 [16,17]. The major metabolite, 2-ethilidene-1,5-dimethyl-3,3-diphenylpyrrolidone (EDDP), is inactive and is the only metabolite of MET found to any significant extent in blood [9,18]. Considerable inter-individual variations in the serum concentrations of MET produced by the same dose have been reported [9], an individualization of the doses being necessary in order to achieve optimum treatment [19]. Pharmacokinetic factors might explain, at least in part, the great inter-individual variability in MET blood levels for a certain dose, and in sensitivity to respiratory depression following intake of a high dose of MET in tolerant patients [20–22].

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Another possible explanation could be the simultaneous intake of other central respiratory depressing agents such as benzodiazepines [20]. Several studies showed high rates of illicit drug use and poly-substance use in groups of patients on chronic MET therapy [23,24], benzodiazepines being frequently detected in biological samples coming from these individuals [2,23].

Benzodiazepines are prescribed on a regular basis for sleep and anxiety disorders in patients under MET treatment [13,17,25,26]. Benzodiazepines are often co-abused with opioid drugs, particularly with MET, due to their capacity to "boost" the subjective effects of opiates or to enhance their high [17,27–30]. In many cases of MET-related deaths, the simultaneous exposure to at least one benzodiazepine was reported [17,26,28,31]. Respiratory depression and coma induced by MET may be aggravated by benzodiazepines [32]. However, only few studies have examined the interaction between MET and benzodiazepines [13,33].

Flunitrazepam (FNZ) is a benzodiazepine more potent than diazepam, used mainly as a hypnotic and preanesthetic in Europe, Mexico and South America [28,34,35]. It has also received attention as a drug of abuse, its misuse being associated with risky behavior and altered decision making (e.g. theft, violence, drug facilitated sexual assaults and intoxication related car accidents) [34–38]. Existing studies showed greater abuse liability of FNZ when compared to other benzodiazepines (e.g. triazolam) [29,37,39] which is consistent with reports of exceptionally high abuse rates of FNZ relative to other benzodiazepines worldwide [39,40]. According to several studies FNZ is the most [30,41,42] or at least one of the most frequently abused benzodiazepines by patients enrolled in MET maintenance treatment [17,28]. The rapid absorption, high liposolubility and rapid onset of effects are important factors which could explain the high abuse liability of the drug [43]. The euphoria induced by FNZ in MET-maintained patients could be another reasonable explanation of its abuse and relative preference over other benzodiazepines [29]. Existing studies suggested that MET may augment reinforcing effects of low FNZ doses [28]. Existing studies showed potentially dangerous interactions between FNZ and selected opioids, interactions which may result from pharmacokinetic or pharmacodynamic mechanisms [13,14,16,33,44,45]. A study conducted by Borron et al. showed that high-dose FNZ augments lethality two fold in MET-treated rats and significantly decrease MET's median lethal dose and time to death [32]. Given that the toxicity mechanisms of the MET–FNZ association as well as the pharmacological and metabolic interactions between these drugs are only poorly understood, further studies are needed.

FNZ is extensively metabolized in the body, by reduction to 7-AFNZ as the main metabolite, but also by hydroxylation and demethylation (oxidative metabolic pathways involving CYP450) [43,46–51]. After ingestion of FNZ, the parent compound and 7-AFNZ (active metabolite) are the main components found in plasma.

Due to the great inter-individual variability regarding the response to MET, the high prevalence of benzodiazepine use and abuse in case of patients treated with MET and the high risk of clinically important interactions associated with this co-ingestion, a regular patient monitoring is highly recommended [16,17,52]. Due to the associated overdose risk, potential for abuse and links with criminal activity, the analysis of MET, FNZ and their metabolites became a major target in certain areas of toxicology (post-mortem and forensic). Elaborating a sensitive and selective analytical method for the simultaneous analysis of these compounds and their metabolites from plasma could prove very useful in a monitoring program of patients enrolled in a MET maintenance treatment, it will make possible to detect cases of co-abuse of the two drug (with high risk of respiratory depression and CNS related problems) and could be used also in animal or human studies to investigate potential pharmacokinetic interactions between MET and FNZ.

The common doses for FNZ and MET are between 1 and 2 mg and 10 and 120 mg (most frequently between 60 and 80 mg/d), respectively [4,12,21,53]. The range of therapeutic concentrations in plasma for MET and FNZ is in general between 100 and 500 ng/mL and 5 and 15 ng/mL, while the usual blood levels reported in postmortem cases are around 50 ng/mL and 1000 ng/mL, respectively [53–57]. In a bioavailability study after oral, rectal and i.v. administration of MET (10 mg dose) the maximum plasma concentrations reported were between 20 and 129 ng/mL [58]. The serum concentrations found by He et al. in patients after a single oral dose of MET (45–90 mg/dose) were between 20 and 50 ng/mL for the two MET enantiomers, and 15–30 ng/mL for both enantiomers of EDDP [59]. Bogusz [15] reported concentrations of MET, FNZ and 7-AFNZ in whole blood samples taken from living subjects (road traffic violators and other offenders) between 25 and 671 ng/mL (with most of them below 400 ng/mL), 4–48 ng/mL and 4–36 ng/mL, respectively. The plasma level of 7-AFNZ reported after a 2 mg oral dose of FNZ was 4.6 ng/mL [43].

There are only few chromatographic methods published in the literature regarding the simultaneous quantification of FNZ, MET and their major metabolites in biological fluids. Viette et al. elaborated an automated SPE method followed by LC–MS/MS analysis on a <2 μm column for screening of 97 drugs/drugs of abuse and metabolites, including FNZ, MET and EDDP in human plasma. The limit of detection achieved in case of FNZ was 10 ng/mL in human plasma, while in case of MET and EDDP the LOD was set at 50 ng/mL [54]. Doherty et al. studied the analytical behavior of 26 psycho-active drug, including MET and FNZ, using ESI-MS^n, HPLC–ESI-MS, GC-FID and polarography, but only qualitative data were obtained [60]. Eichorst et al. elaborated a UPLC (ultra-performance liquid chromatography)–MS/MS (ESI(+)) screening method for 40 drugs of abuse and metabolites in urine samples, including MET, EDDP, FNZ and 7-AFNZ. The criteria for setting the LOQ values were $<20\%$ CV (inter-assay precision) and S/N ratio >10, but the exact values are not reported for all compounds. They performed either screening or semi-quantitative analysis based on a 3 point calibration curve. Quantification was performed only over a narrow concentration range within a window of the cut-off value. The selected cut-off value for MET, EDDP, FNZ and 7-AFNZ screening was 100 ng/mL. LOQ for most compounds was set at 30% of the cut-off value or less. For 7-AFNZ 100 ng/mL could be considered the LOQ, since the % CV was 18.4% [61]. Reubsaet and Pederson-Bjergaard performed screening for more than 70 CNS-stimulating drugs in human plasma by HPLC-MS (ESI(+)). The LOD was set at a S/N ratio >3. The LOD value was 0.5 ng/mL and 5 ng/mL in case of MET and FNZ, respectively [55]. Souverain et al. investigated the efficiency of different protein precipitation procedures for the analysis of a MET, EDDP, FNZ, or FNZ, fluoxetine and norfluoxetine mixture from human plasma, at a concentration of 500 ng/mL in case of each analyte, without evaluating the linearity range and the LOQ of the selected capillary LC–ESI-MS/UV method. The MS detection was performed in SIM (single ion monitoring) mode. The chromatographic run time was 6 min [62]. He et al. compared the efficiency of LLE and SPE using reversed-phase silica sorbents with that of polymeric cartridges for the isolation of MET, EDDP and some benzodiazepines (including FNZ) from human serum and urine. They concluded that in general it is very difficult and time-consuming to achieve high, reproducible recoveries for a mixture of drugs and their metabolites, which are more polar than the parent compounds [59]. The LC–UV analysis required two separate injections with very long chromatographic run times, one on a chiral column for MET and EDDP (run time of approx. 20 min) and a second one on a RP column for the selected benzodiazepines (run time of approx. 17 min). The LOD values were 1 ng/mL for both enantiomers of MET and for the selected benzodiazepines, and 2 ng/mL in case of EDDP [59]. Dresen et al. elaborated a LC–MS/MS

(ESI (+)) method for detection and identification of 700 drugs by multi-target screening (including FNZ, 7-AFNZ, MET and EDDP) in human plasma after LLE and in urine after a simple dilution of the biological fluid. The total run time was 17.5 min. LOD and LOQ values are not available. The authors evaluated the sensitivity of the method by analyzing serum spiked with a mixture of 12 benzodiazepines, 7-AFNZ and zolpidem. Calibration curves were constructed for all benzodiazepines but good linearity was obtained only for diazepam. The method allowed semi-quantitative analysis with the condition that deuterated analogs to be used as internal standard for each analyte, in order to compensate for matrix effects [63]. Adamowicz and Kała [64] and Song et al. [65] elaborated GC–MS methods for screening of drugs of abuse, date-rape drugs and metabolites in human urine [64] or whole blood [65] with [64] or without [65] a derivatization step included before the chromatographic separation. The LOQ values reported by Adamowicz and Kała were 10 μg/mL, 0.05 μg/mL, 1 μg/mL and 1.6 μg/mL for EDDP, MET, FNZ and 7-AFNZ, respectively [64]. The method elaborated by Song et al. offered only qualitative information, with MET being detected at 500 ng/mL, while FNZ and 7-AFNZ could not be detected at 100 ng/mL and 500 ng/mL, respectively [65]. Bogusz et al. elaborated a HPLC-APCI-MS method (chromatographic run time of 8 min) for the quantification of MET, FNZ, 7-AFNZ, N-desmethylFNZ and 3-hydroxyFNZ in human serum, blood and urine after a previous solid phase extraction [15]. The LOD values were 0.2 ng/mL for MET, FNZ and 7-AFNZ, 1 ng/mL for N-desmethylFNZ and 2 ng/mL in case of 3-hydroxyFNZ. Cheng et al. performed automated SPE followed by LC–ESI-MS/MS analysis of several opiates, MET, amphetamines, benzodiazepines and their metabolites (including 7-AFNZ), ketamine from human urine samples. FNZ and EDDP were not included on the list of monitored analytes. After validation by using at least 4 calibration levels (selected calibration range: 60–2200 ng/mL for MET and 60–500 ng/mL for 7-AFNZ), the method was used for semi-quantification based on a single point calibration at the level of the cut off concentration which was set at 300 ng/mL and 60 ng/mL in case of MET and 7-AFNZ, respectively [66]. Oiestad et al. reported a LC–ESI-MS/MS method for quantification of 32 drugs and metabolites (including MET, FNZ and 7-AFNZ) in oral fluid after a LLE step [67]. They elaborated an UPLC–ESI-MS/MS assay also for 28 drugs and metabolites in whole blood with a chromatographic run time of 9 min. MET and FNZ were included on the list of analytes, but without their major metabolites [68].

As it can be seen, most of these methods were elaborated mainly for qualitative purposes, multiple drug screening in clinical or forensic applications. The sensitivity showed by some of these methods is not good enough to allow the quantification of the selected analytes in real samples. Especially FNZ analysis in blood samples is very difficult due to the low concentrations of the drug, which can be explained by the low dosage, the high volume of distribution, but also by the rapid and extensive metabolism of the parent compound [56,69,70]. Another issue can be the long time delays between the assault and sampling time in case of sexual assault victims [71]. All these justify the need for very sensitive analytical tools. Many of the existing methods have no calibration data associated with or can be applied to obtain semi-quantitative information at the most.

The aim of this work was to elaborate a fast, highly sensitive and selective assay for FNZ, 7-AFNZ, MET and EDDP in human, rat and rabbit plasma samples. There was more than one objective when elaborating the method described in the manuscript, including the possibility to evaluate a potential pharmacokinetic interaction between flunitrazepam and methadone, by the means of the quantification of methadone, flunitrazepam and their major metabolites in animal plasma samples (the study was/is performed in rats and rabbits), the possibility to quantify the selected drugs and metabolites in human plasma samples in forensic cases (drug abuse, sexual assault) or in bioequivalence studies. The double objective justifies the need for a sensitive method for the simultaneous quantification of FNZ, MET, 7-AFNZ and EDDP in human and animal plasma over a wide concentration range (very small concentrations to be quantified in pharmacokinetic interaction studies and very high concentrations to be expected sometimes in forensic cases). The validated method was applied successfully to quantify the selected analytes from human, rat and rabbit plasma samples in pharmacotoxicological studies.

2. Material and methods

2.1. Chemicals and reagents

Flunitrazepam, 7-aminoflunitrazepam, methadone, EDDP (2-ethilidene-1,5-dimethyl-3,3-diphenylpyrrolidone) and bromazepam (BRO, internal standard, I.S.) standards were purchased from Lipomed (Arlesheim, Switzerland). Acetonitrile (LC-MS grade), formic acid, ammonium acetate (MS grade) and ethyl acetate (HPLC grade) were purchased from Sigma Aldrich (Steinheim, Germany). Analytical grade 25% ammonium hydroxide was obtained from Fluka (Buchs, Switzerland). Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water purification system. Drug free human plasma was supplied by the Local Bleeding Centre Cluj-Napoca, Romania. Drug free rat (male Wistar rats) and rabbit (male white rabbits from the Californian breed) plasma were obtained from the Practical Skills and Experimental Medicine Centre of the "Iuliu Hatieganu" University of Medicine and Pharmacy. Blood was collected from rats and rabbits, from the retro-orbital sinus in the presence of heparin as anticoagulant. Blank plasma samples were obtained from whole blood after centrifugation at 2000*g* for 10 min at 4 °C.

2.2. Calibration standards and quality control samples

Primary stock solutions of FNZ (0.524 mg/mL), 7-AFNZ (0.264 mg/mL), MET (0.509 mg/mL), EDDP (0.520 mg/mL) and BRO (0.545 mg/mL) were prepared by dissolving accurately weighed quantity of FNZ, 7-AFNZ, MET and EDDP in acetonitrile (weighed on a Discovery Analytical balance from Ohaus (Ohaus Corp., Pine Brook, USA)). Working solutions of FNZ (5.24, 0.524 and 0.0524 μg/mL), 7-AFNZ (5.28, 0.528 and 0.0528 μg/mL) MET (5.09, 0.509 and 0.0509 μg/mL) and EDDP (5.20, 0.520 and 0.052 μg/mL) were obtained by diluting specific volumes of stock solution with blank plasma. These working solutions were used to spike different volumes of blank human plasma, providing finally ten calibration standards containing a mixture of all analytes with the concentrations ranging from 0.524 to 262.00 ng/mL (FNZ), 0.528–264.25 ng/mL (7-AFNZ), 0.509–254.50 ng/mL (MET) and 0.520–260.00 ng/mL (EDDP).

Quality control (QC) samples (QC1, QC2, QC3, QC4) containing all four analytes at 1.57, 7.34, 62.88 and 104.80 ng/mL for FNZ, 1.59, 7.40, 63.60 and 105.70 ng/mL for 7-AFNZ, 1.53, 7.13, 61.08 and 203.60 ng/mL for MET and at 1.56, 7.28, 62.40 and 208.00 ng/mL for EDDP were prepared by diluting specific volumes of the working standards with blank human plasma.

For the evaluation of the ULOQ (upper limit of quantification) QC samples over the calibration range (2096 ng/mL for FNZ, 2114 ng/mL for 7-AFNZ, 2036 ng/mL for MET and 2080 ng/mL for EDDP) were prepared by dilutions of the working standards with blank human plasma.

For the optimization process, individual solutions and mixtures of the selected analytes and I.S. were prepared in different mobile phases (Table 1) with the concentrations as follows:

Table 1
Results obtained during method optimization (signal intensity) with direct infusion of a mixture of the selected analytes in different mobile phases (2.55 μg/mL MET, 2.60 μg/mL EDDP, 2.62 μg/mL FNZ, 2.64 μg/mL 7-AFNZ, 2.73 μg/mL BRO).

Mobile phase composition (solvent A/solvent B (50/50, v/v))			Signal intensity (arbitrary units)				
No.	*Solvent A*	*Solvent B*	*MET*	*EDDP*	*FNZ*	*7-AFNZ*	*BRO*
S1	**MeCN**	**0.1% Formic acid (v/v)**	$\mathbf{2.52\times10^7}$	$\mathbf{2.17\times10^7}$	$\mathbf{1.07\times10^7}$	$\mathbf{1.02\times10^7}$	$\mathbf{1.93\times10^6}$
S2	MeCN	0.1% Acetic acid (v/v)	1.19×10^7	1.66×10^7	3.54×10^6	4.98×10^6	1.23×10^6
S3	MeCN	0.2% Formic acid (v/v)	2.79×10^7	1.64×10^7	5.74×10^6	3.94×10^6	1.77×10^7
S4	MeCN	0.2% Acetic acid (v/v)	1.89×10^7	1.68×10^7	2.03×10^6	5.39×10^6	8.46×10^5
S5	MeCN	1.5 mM NH_4Ac	1.50×10^7	2.61×10^7	3.42×10^6	3.36×10^6	8.86×10^5
S6	MeCN	5 mM NH_4Ac	1.96×10^7	2.26×10^7	5.68×10^6	3.71×10^6	1.11×10^6
S7	**0.1% Formic acid in MeCN**	**5 mM NH_4Ac**	$\mathbf{2.37\times10^7}$	$\mathbf{2.37\times10^7}$	$\mathbf{7.8\times10^6}$	$\mathbf{1.43\times10^7}$	$\mathbf{1.14\times10^6}$
S8	0.1% Acetic acid in MeCN	5 mM NH_4Ac	6.22×10^6	1.30×10^7	3.33×10^6	3.16×10^6	6.31×10^5

MeCN=Acetonitrile; NH_4Ac=Ammonium acetate.

2.55 μg/mL MET, 2.60 μg/mL EDDP, 2.62 μg/mL FNZ, 2.64 μg/mL 7-AFNZ, 2.73 μg/mL BRO.

The stock solution of the internal standard was diluted in deionised water to give a spiking solution 4.36 μg/mL. 50 μL from this solution was used to spike plasma samples (final concentration 218.00 ng bromazepam/mL plasma).

2.3. Chromatographic and mass spectrometry conditions

A Waters Acquity liquid chromatography system coupled with a Waters TQD triple quadrupole mass spectrometer was used (Waters, Milford, USA). The UPLC system included a binary pump, degasser, autosampler with an injection loop of 10 μL and a column heater-cooler. Chromatographic separation was carried out using a HSS T3 column (50 mm × 2.1 mm i.d., 1.8 μm) from Waters (Waters, Milford, USA) preceded by a 0.2 μm online filter. The UPLC column was held at 60 °C, while the sample compartment was maintained at 4 °C. The mobile phase consisted of acetonitrile with 0.1% formic acid (solvent A) and 5 mM ammonium acetate (solvent B). A 3 min gradient elution at a constant flow rate of 3 mL/min was performed as follows: an increase from 15% (at 0 min) to 45% solvent A (at 1.2 min) according to gradient curve no. 2, followed by a quick increase to 95% solvent A in 0.1 min, held at 95% A until 1.8 min, followed by return to 15% solvent A in 0.1 min and re-equilibration until 3 min.

Mass spectrometric detection was carried out using an electrospray interface (ESI) operated in the positive ionization mode with multiple reaction monitoring (MRM) for all analytes. Nitrogen was used as desolvation gas at 600 L/h flow rate, with desolvation temperature set at 350 °C and the source temperature at 150 °C. The collision gas (argon) flow was set at 0.1 mL/min. The capillary voltage was set at 4 kV. The MS analyzer parameters were as follows: LM1 and HM1 Resolution, 12; ion energy 1, 2.20 V; LM2 and HM2 Resolution, 9.0 and 7.0, respectively, ion energy 2, 0.22 V, dwell time, 5 ms. The cone voltage and collision energy were optimized in case of each analyte so as to maximize the signal corresponding to the major transition observed in the MS/MS spectra, following the fragmentation of the $[M+H]^+$ ions corresponding to the selected compounds.

The MassLynx software (Version 4.1, SCN 714) was used to control the LC–MS/MS system as well for data acquisition and processing.

2.4. Sample preparation

Aliquots of 1 mL plasma were spiked with 50 μL of I.S. working solution (corresponding to a final concentration of 218 ng/mL bromazepam). After vortex-mixing (10 s), the samples were treated with 50 μL of 10% ammonium hydroxide solution, followed by the addition of 6 mL ethyl acetate. The samples were submitted to liquid–liquid extraction by vortexing thoroughly for 5 min with a MultiPulse vortexer (Glas Col, Terre Haute, USA). The extraction step was followed by centrifugation for 5 min at 2000*g* (Sigma 2-16, Osterode am Harz, Germany). The organic phase was transferred to a clean tube and evaporated to dryness under a gentle stream of nitrogen, at 40 °C. Extracts were reconstituted with 150 μL of a mixture of acetonitrile with 0.1% formic acid/5 mM ammonium acetate (50/50, v/v). After vortex-mixing (30 s), 5 μL volumes (in partial loop with needle overfill mode) of the extracts were subjected to LC–MS/MS analysis.

2.5. Ion suppression testing

The evaluation of the matrix effect was performed by comparing the analytical response for a mixture of FNZ, 7-AFNZ, MET, EDDP and BRO at all four QC levels prepared and injected directly in a mixture of acetonitrile with 0.1% formic acid/5 mM ammonium acetate (50/50, v/v) with the response of the same concentrations of analytes added to pre-extracted blank samples [72].

2.6. Assay validation

The assay was validated in accordance to the industrial guidance for the bioanalytical methods validation [73–75].

Selectivity was checked by comparing several different plasma blanks ($n=6$ for human plasma blank, $n=5$ for rat plasma blank and $n=3$ for rabbit plasma blank, respectively), with the corresponding spiked plasma samples for interference of endogenous compounds with the analytes. Except selectivity, the other parameters evaluated during the validation process were determined only for human plasma.

Linearity was studied by analyzing singlicate calibration standards at 10 concentration levels for each analyte. The concentration of analyte was determined automatically by the instrument data system using the internal standard method. Linearity was determined by checking three calibration curves on three different working days. A least squares linear regression analysis was performed to determine slope, intercept and coefficient of correlation. The applied calibration model for all curves was $y=ax+b$ (weighting $1/y$), where y=peak area ratio analyte/I.S., x=concentration of the analyte in plasma, a=slope of the curve and b=intercept. The calibration model was accepted if the residuals were within $\pm20\%$ at the lower limit of quantification (LOQ) and within $\pm15\%$ at all other calibration levels.

The lower limit of quantitation (LOQ) was defined as the lowest concentration with a precision and accuracy below 20%.

Since the aim of this work was to elaborate a LC–MS/MS assay useful in toxicokinetic studies (toxicokinetic studies of the selected analytes or pharmacokinetic interaction studies between them), but also in clinical and forensic cases, an important issue to

be considered was the ability to quantify the selected compounds over a wide concentration range. As a consequence, the upper limit of quantitation (ULOQ) was evaluated also, by analyzing QC samples (spiked plasma samples) with concentration levels over the highest calibration standard. These QC samples were analyzed after a tenfold dilution with blank plasma. The ULOQ was set at the highest concentration with a precision and accuracy below 15%.

Within-run accuracy and precision were determined by analysis on the same day of five different samples (plasma spiked with all four analytes) at each QC level. The between-run accuracy and precision were determined at the same concentration levels, but on five different experimental days. Accuracy was calculated as the percentage difference between the concentration of analyte calculated from calibration curve and the nominal concentration. Precision was expressed as coefficient of variation (CV%).

The mean absolute recoveries of the selected analytes were determined at the same concentrations as those used to study the intra- and inter-day accuracy and precision, by comparing the mean area (response) of treated plasma samples (three replicates at each calibration level) with the area of freshly prepared un-extracted standards in a mixture of acetonitrile with 0.1% formic acid/5 mM ammonium acetate (50/50, v/v) with the same concentration of FNZ, 7-AFNZ, MET, EDDP and I.S. as in the final extracts obtained from the plasma calibration standards.

The stability study of FNZ, 7-AFNZ, MET and EDDP in human plasma included the evaluation of room-temperature stability, freeze–thaw stability and post-preparative stability in the autosampler. The evaluation of stability was performed at two QC levels (QC2 and QC3) (7.34 and 62.88 ng/mL for FNZ, 7.40 and 63.60 ng/mL for 7-AFNZ, 7.13 and 61.08 ng/mL for MET and at 7.28 and 62.4 ng/mL for EDDP). Samples (analytes) were considered to be stable when concentrations of stability test samples fell within $\pm 15\%$ of the nominal value.

2.7. Clinical application and in-study validation

The validated method was applied for the determination of FNZ and 7-AFNZ, in plasma sample collected from a human volunteer 10 h after a single oral dose of 1 mg flunitrazepam (Rohypnol, 1 mg flunitrazepam/tablet).

The selected analytes (MET, EDDP, FNZ and 7-AFNZ) were determined in animal plasma samples also, obtained from rats ($n=5$) and a rabbit, after simultaneous exposure by gastric intubation to methadone and flunitrazepam (doses of 5 mg methadone/kgbw and 0.5 mg flunitrazepam/kgbw in case of rats and 5 mg methadone/kgbw and 1 mg flunitrazepam/kgbw in case of the rabbit). Plasma samples were collected at 1 and 3 h after exposure in case of rats and at 1, 2, 4 and 24 h after exposure in case of rabbits. Animals were maintained under standard conditions of temperature and lighting with ad libitum access to food and water throughout the experiment. The experimental protocol was in compliance with the institutional and national guidelines for experimentation with laboratory animals and it was approved by the bioethics commission (Reg. no. 365/18.07.2011). Plasma samples from the human volunteer and from the animals exposed to MET or FNZ were stored at $-80\,^{\circ}C$ until analysis. In case of the human volunteer and for the rabbit plasma samples were analyzed in triplicate. In case of rats each sample was analyzed in singlicate.

The accuracy and precision of the validated method was monitored to ensure that it continued to perform satisfactorily during analysis of volunteer and animal samples. To achieve this objective, a number of QC samples prepared in duplicate at three

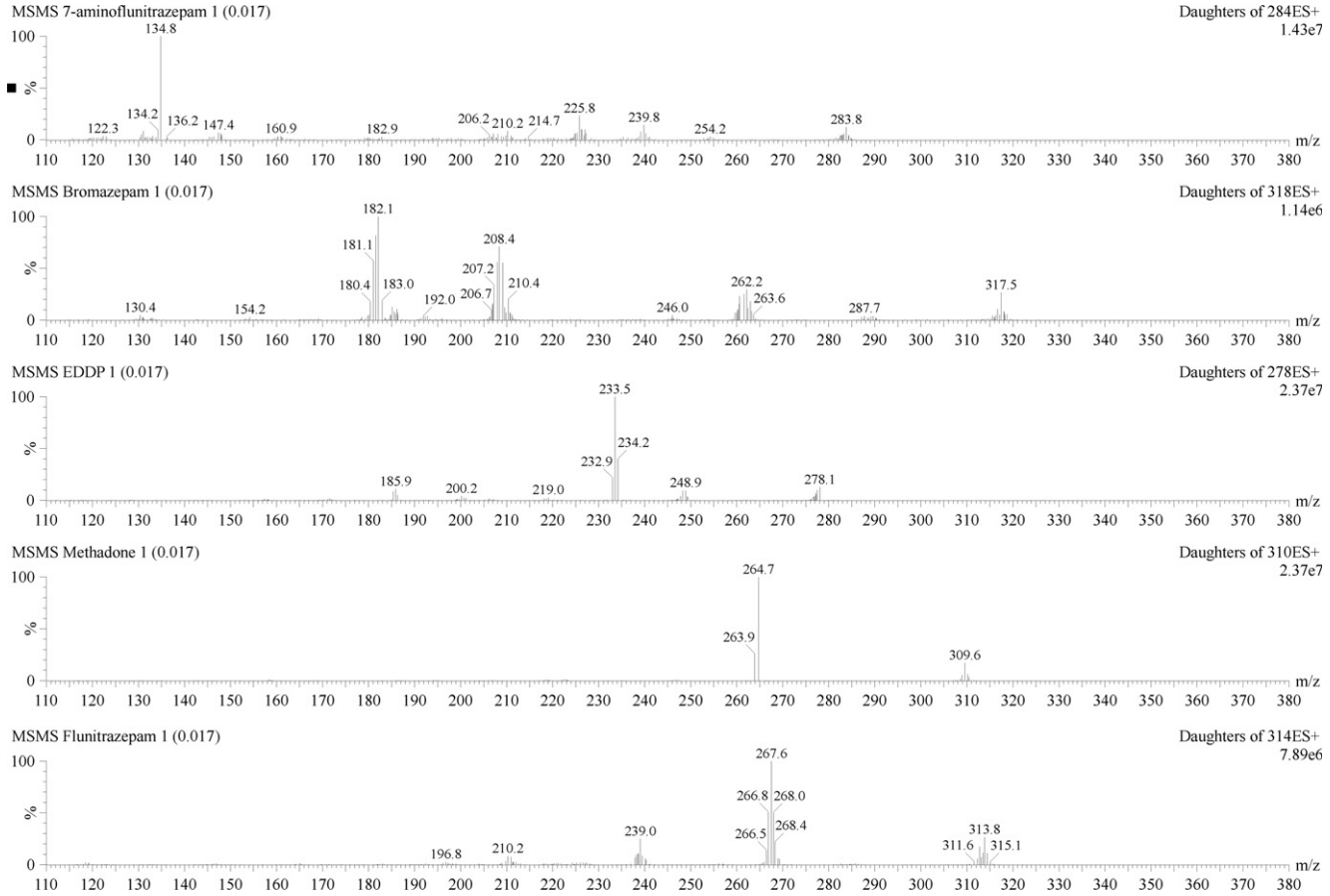

Fig. 1. MS/MS spectra of MET, EDDP, FNZ, 7-AFNZ and I.S., using the collision energy and cone voltage established following the tuning process.

concentration levels (QC2, QC3 and QC4) were analyzed in each assay run and the results compared with the corresponding calibration curve. At least 2/3 of the QC samples should be within 15% of their respective nominal values.

3. Results and discussion

3.1. Optimization of the LC–MS/MS method

Currently, LC–API-MS/MS can be considered a standard, robust and very reliable tool in toxicology for sensitive detection and/or quantification of drugs or drugs of abuse in complex matrices. This technique guarantees high sensitivity similar to that of GC–MS/MS methods, but without the need for the laborious derivatization step in case of polar metabolites like GC assays [15]. The aim of the present work was to elaborate a rapid simple, sensitive and selective method for the simultaneous quantification of MET, EDDP, FNZ and 7-AFNZ. The best option to do this is by LC–MS/MS. The MS/MS detection can guarantee the high selectivity and sensitivity needed. Instead of choosing classical HPLC for the separation of the selected compounds, in order to take full advantage of the benefits of the LC and MS/MS hyphenation, UPLC was coupled to the mass spectrometer. The recent development of ultra high pressure liquid chromatography, combining the use of small particle sizes ($<2\,\mu m$) and high pressure (>600 bar) allows the simultaneous analysis of several compounds with a shorter chromatographic run time and improved efficiency, resolution and increased signal to noise ratio when compared to conventional HPLC. The hyphenation of UPLC to MS/MS combines the high speed and efficiency of UPLC with the sensitivity and selectivity granted characteristic to MS/MS [54,76].

The first step in elaborating the UPLC–MS/MS method was to record the MS spectra of the selected compounds. ESI in positive ion mode was selected for the ionization of all analytes. The MS spectra recording and the optimization of the ESI-MS parameters was performed (with collision gas off) by continuous direct infusion of solutions containing the individual compounds (2.55 μg/mL MET, 2.60 μg/mL EDDP, 2.62 μg/mL FNZ, 2.64 μg/mL 7-AFNZ, 2.73 μg/mL BRO) in different mobile phases (Table 1) in the mass spectrometer ionization source (data not shown) at a flow rate of 20 μL/min. All analytes were successfully ionized by using positive ESI mode and the protonated molecular ion, $[M+H]^+$, was the base peak for all compounds in the MS spectrum. The protonated molecular ion for each analyte was chosen as parent ion and tuned for daughter ion scan automatically. The mass transition from the protonated molecular ion $[M+H]^+$ to the most abundant product ion was selected as quantifying ion transition for each compound (Fig. 1).

Table 2
The major ion transitions selected for quantification of the selected analytes and the corresponding optimum MS/MS parameters.

Analyte	MRM transition	Cone voltage (V)	Collision energy (eV)
MET	m/z 309.9→m/z 264.6	28.0	16.0
EDDP	m/z 277.9→m/z 233.7	50.0	32.0
FNZ	m/z 313.8→m/z 267.5	44.0	30.0
7-AFNZ	m/z 283.8→m/z 134.9	50.0	30.0
I.S.	m/z 317.5→m/z 181.9	40.0	38.0

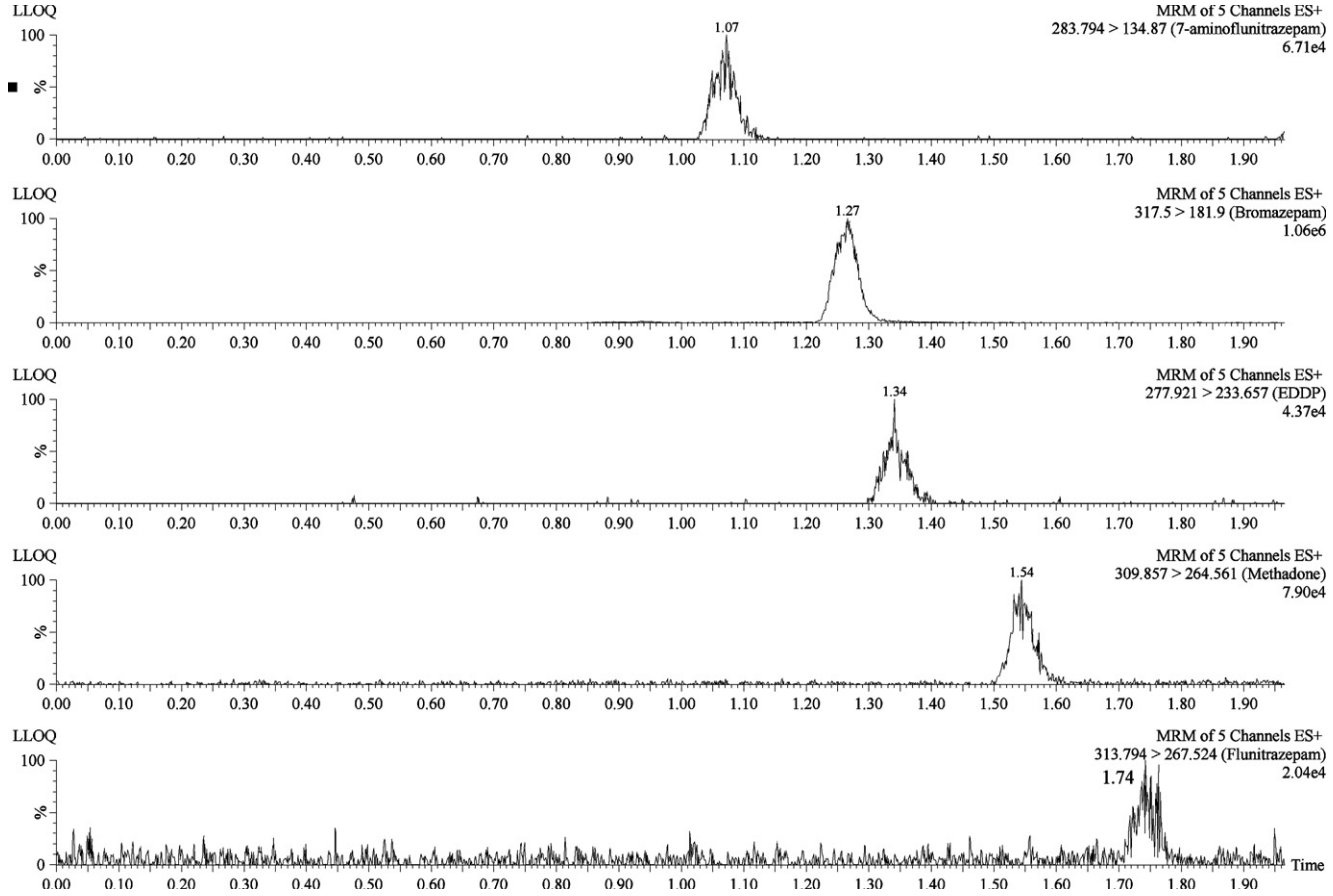

Fig. 2. Representative LC–ESI-MS/MS chromatogram of a human blank plasma spiked with the selected analytes at LOQ level (0.509 ng/mL MET, 0.520 ng/mL EDDP, 0.524 ng/mL FNZ and 0.528 ng/mL 7-AFNZ) and I.S. (218.00 ng/mL), aquired in MRM mode, by monitoring the selected ion transitions for each compound.

The major product ions from the MS/MS spectra of MET and EDDP result most likely due to the loss of the end nitrogen atom from the side chain of $[M+H]^+$ as the corresponding secondary amine $HN(CH_3)_2$ [77,78]. In case of FNZ the major transition in the MS/MS spectra is attributable to the loss of NO_2 [78–81]. In case of 7-AFNZ a more intense fragmentation of $[M+H]^+$ was observed, with the major product ion at m/z 134.9, a transition reported as the major one in ESI(+) mode by other researchers too [82].

Since the efficiency of the ionization process is highly dependent on the mobile phase composition, several solvent mixtures and mobile phase additives were tested and autotune was performed in MS/MS mode. This process involved adjusting the collision energy, cone voltage and capillary voltage, aiming this way to maximize the signal for both the precursor ions and the product ions generated in the MS/MS mode. The optimum conditions and mobile phase composition were selected based on the signal intensity obtained in each case for the selected ion transitions. The results of this optimization process are summarized in Table 1.

The optimum results in terms of signal intensity were obtained with mobile phases S1 and S7, with S1 showing higher signal intensity for MET and FNZ, while S7 showed better results in case of EDDP and 7-AFNZ. These two solvent mixtures were tested as potential mobile phases for the chromatographic separation of the selected analytes. For these tests, 5 μL of a standard mixture of MET (63.75 ng/mL), EDDP (65.5 ng/mL), FNZ (65.5 ng/mL), 7-AFNZ (66.0 ng/mL) and I.S. (68.3 ng/mL) was injected to the chromatographic column. Following autotuning of each analyte and I.S., the optimized parameters were used to construct the MS/MS method, which was then used to acquire data in the MRM (multiple reaction monitoring) mode. S7 guaranteed a better separation of the analytes than S1 (in case of S1 the peaks corresponding to EDDP and I.S. were partially overlapping) (results not shown here). All these taken together (signal intensity, peak separation) indicated S7 as the optimum mobile phase composition for the LC–MS/MS analysis of the selected compounds. The final MS/MS optimization process was performed with combined flow, by continuous post-column infusion (20 μL/min) of a mixture of the selected analytes (containing 2.55 μg/mL MET, 2.60 μg/mL EDDP, 2.62 μg/mL FNZ, 2.64 μg/mL 7-AFNZ, 2.73 μg/mL BRO in a mixture of acetonitrile with 0.1% formic acid/5 mM ammonium acetate) combined with the LC flow coming from the chromatographic column, consisting of the same mixture of acetonitrile with 0.1% formic acid/5 mM ammonium acetate, delivered at a flow rate of 0.3 mL/min. The major ion transitions selected for MRM, together with the final optimum collision energies and cone voltages providing maximum signal intensity, are summarized in Table 2.

Using the selected mixture with gradient elution according to the gradient program presented in Section 2.3, an adequate chromatographic separation was achieved with a total run time of 3 min. Methadone was eluted at 1.54 min, EDDP at 1.35 min,

Table 3
Recovery and ion suppression for MET, EDDP, FNZ and 7-AFNZ.

Analyte	Absolute recovery % (average ± SD)	Ion suppression (average ± SD)
MET	73.79 ± 4.86	−6.75 ± 2.52
EDDP	61.91 ± 6.77	−2.43 ± 1.40
FNZ	80.30 ± 5.00	−4.35 ± 1.38
7-AFNZ	80.41 ± 6.29	−8.25 ± 5.61
I.S.	81.24 ± 5.94	−4.29 ± 4.75

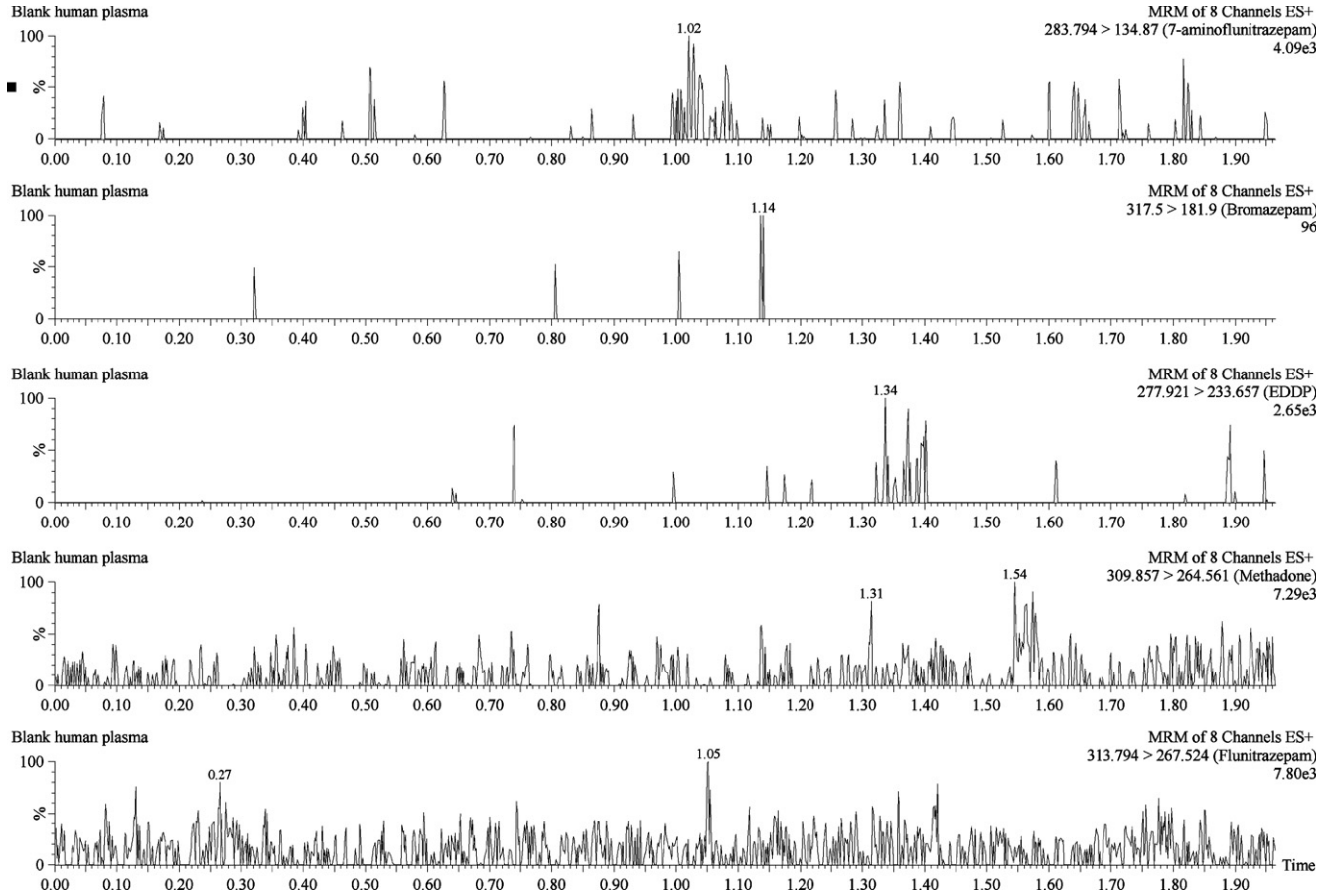

Fig. 3. LC–ESI-MS/MS chromatogram of an extract from blank human plasma.

Table 4
Linearity data for MET, EDDP, FNZ and 7-AFNZ.

Analyte	Concentration range (ng/mL)	Slope (average ± SD)	Intercept (average ± SD)	r^2 (average ± SD)
MET	0.51–254.50	25.6243 ± 0.9862	−0.4988 ± 1.6297	0.9948 ± 0.0035
EDDP	0.52–260.00	11.2692 ± 0,1588	0.3073 ± 0.0718	0.9954 ± 0.0037
FNZ	0.52–262.00	5.1485 ± 0.3534	0.9455 ± 0.3024	0.9994 ± 0.0004
7-AFNZ	0.53–264.25	17.8039 ± 1.2244	3.4912 ± 0.0855	0.9965 ± 0.0003

SD—standard deviation.

Table 5
Within-run precision and accuracy for MET, EDDP, FNZ and 7-AFNZ.

Analyte	$c_{nominal}$ (ng/mL)	$c_{measured}$ (mean ± SD)	Precision (CV%)	Inaccuracy %
MET	0.51 (LOQ)	0.55 ± 0.01	1.53	7.66
	1.53	1.49 ± 0.03	1.92	−2.48
	7.13	6.98 ± 0.36	5.15	−2.05
	61.08	68.22 ± 1.14	1,67	11.69
	203.60	199.90 ± 8.06	4.03	−1.82
	2036.00 (ULOQ)	2286.43 ± 33.98	1.49	12.30
EDDP	0.52 (LOQ)	0.55 ± 0.03	5.60	5.77
	1.56	1.52 ± 0.06	4.03	−2.44
	7.28	6.91 ± 0.53	7.67	−5.10
	62.40	64.11 ± 3.02	4.71	2.73
	208.00	196.44 ± 7.37	3.75	−5.56
	2080.00 (ULOQ)	2345.16 ± 26.02	1.11	12.75
FNZ	0.52 (LOQ)	0.55 ± 0.03	5.34	5.34
	1.57	1.51 ± 0.06	3.90	−3.57
	7.34	7.52 ± 0.51	6.78	2.45
	62.88	63.04 ± 3.91	6.20	0.25
	104.80	99.22 ± 3.21	3.24	−5.33
	2096.00 (ULOQ)	2305.29 ± 43.20	1.87	9.99
7-AFNZ	0.53(LOQ)	0.49 ± 0.03	7.16	−7.58
	1.59	1.60 ± 0.07	4.54	0.50
	7.40	7.62 ± 0.56	7.35	3.02
	63.60	61.99 ± 3.31	5.34	−2.53
	105.70	100.87 ± 7.43	7.37	−4.57
	2114.00 (ULOQ)	2104.58 ± 31.98	1.52	−0.45

$c_{nominal}$—nominal concentration, $c_{measured}$—measured concentration, SD—standard deviation, CV—coefficient of variation.

Table 6
Between-run precision and accuracy for MET, EDDP, FNZ and 7-AFNZ.

Analyte	$c_{nominal}$ (ng/mL)	$c_{measured}$ (mean ± SD)	Precision (CV%)	Inaccuracy %
MET	0.51 (LOQ)	0.55 ± 0.03	4.95	7.32
	1.53	1.51 ± 0.06	4.06	−1.31
	7.13	7.07 ± 0.53	7.49	−0.82
	61.08	67.37 ± 1.50	2.23	10.30
	203.60	201.57 ± 8.42	4.18	−1.00
	2036.00 (ULOQ)	2265.34 ± 53.63	2.37	11.26
EDDP	0.52 (LOQ)	0.54 ± 0.03	6.35	3.13
	1.56	1.48 ± 0.08	5.64	−5.45
	7.28	7.22 ± 0.58	8.03	−0.83
	62.40	67.26 ± 3.36	5.00	7.79
	208.00	195.00 ± 9.46	4.85	−6.25
	2080.00 (ULOQ)	2252.76 ± 161.53	7.17	8.31
FNZ	0.52 (LOQ)	0.50 ± 0.07	14.64	−5.18
	1.57	1.51 ± 0.07	4.43	−3.89
	7.34	7.35 ± 0.51	6.94	0.14
	62.88	60.26 ± 5.35	8.88	−4.17
	104.80	100.20 ± 3.33	3.32	−4.39
	2096.00 (ULOQ)	2308.50 ± 52.98	2.30	10.14
7-AFNZ	0.53(LOQ)	0.47 ± 0.04	8.26	−10.27
	1.59	1.60 ± 0.09	5.78	0.82
	7.40	7.31 ± 0.64	8.76	−1.24
	63.60	62.02 ± 3.15	5.08	−2.48
	105.70	99.75 ± 9.69	9.71	−5.62
	2114.00 (ULOQ)	2036.93 ± 82.14	4.03	−3.65

FNZ at 1.75 min, 7-AFNZ at 1.08 min, and I.S. at 1.27 min, as shown in a representative chromatogram in Fig. 2.

In general the analysis of biological samples through LC–ESI-MS/MS requires an effective sample preparation in order to remove interfering compounds and to avoid matrix effects, improving this way the sensitivity [81]. The risk of interferences decreased by selecting data acquisition in MRM mode, but a minimum sample treatment was still needed in order to eliminate, at least in part, some of the endogenous compounds responsible for ion suppression. In this case the sample preparation step consisted in a simple liquid–liquid extraction with ethyl acetate (a slightly modified version of the extraction procedure elaborated earlier by the authors for the isolation of FNZ or FNZ and 7-AFNZ from human plasma [83,84]). Taking into account the pK_a values of the analytes (pK_a=8.3 for MET [85]; 7.71 for EDDP [86]; pK_{a1}=1.4–1.8 and pK_{a2}=11.18 for FNZ; 2.16 and 12.12 for 7-AFNZ; 2.16 and 11.0 for I.S., respectively [87]), the extraction was performed at basic pH in order to increase the recovery.

3.2. Method validation

The final analytical method (liquid–liquid extraction followed by LC–ESI-MS/MS analysis) was validated according to international guidelines and recommendations.

Human, rat and rabbit blank plasma samples were analyzed in order to see whether these matrices contained interfering endogenous components. The selectivity study showed that there are no significant interferences from endogenous compounds at the retention times of the analytes (Fig. 3).

No significant ion suppression was observed for the selected analytes. The results of the matrix effect evaluation are summarized in Table 3.

The elaborated method guaranteed similar recovery levels of FNZ, 7-AFNZ and I.S. (Table 3). Even if the recovery was somehow lower for MET and EDDP, the validation data were acceptable for these analytes also. The results obtained at the different concentration levels showed that the recovery was not concentration dependent.

A linear relationship was found when plotting peak area ratios (analyte/I.S.) against analyte concentration, by using a weighting factor of $1/y$. The assay was found to be linear for all analytes under investigation over a wide concentration range. The mean values for the regression parameters and the linearity range of the different analytes are listed in Table 4.

The multi-methods (involving the analysis of tens of compounds) are of great use in forensic toxicology but in general they are screening methods, not focusing necessarily on quantitative analysis and on achieving very low LOQ values. For achieving all our objectives we needed a very sensitive method, validated over

a very wide concentration range, allowing the simultaneous quantification of the four selected analytes. The sensitivity of the method is superior to other previously published methods for the simultaneous analysis of MET, EDDP, FNZ and 7-AFNZ in plasma samples [54,55,59,65]. The LOQ of the assay was set at the level of the lowest calibrator for each analyte. The elaborated LC–ESI-MS/MS method allowed the quantification of MET, FNZ and their major metabolites down to approximately 0.5 ng/mL plasma (LOQ=0.509 ng/mL plasma for MET, 0.520 ng/mL plasma for EDDP, 0.524 ng/mL plasma for FNZ and 0.528 ng/mL plasma for 7-AFNZ) (Fig. 2), with inaccuracy and precision below 20% (Tables 5 and 6). Oiestad et al. and Bogusz et al. published a UPLC–ESI-MS/MS and a LC–APCI-MS method, respectively, with similar sensitivity, but the former one included only MET and FNZ on the list of analytes, without the possibility to quantify their major metabolites, while the second assay did not included EDDP [15,68]. The need for a highly sensitive assay is justified for example in forensic cases (drug-facilitated sexual assault), because often there is a long time delay between the assault and sampling [71]. The pharmacokinetic, metabolic interaction or bioequivalence studies necessitate also a very low quantification limit, due to the need to quantify the drugs not only at the time corresponding to the maximum plasma level, but also shortly after administration and/or at a relatively long time after the exposure.

Given that in some cases investigated in clinical or forensic toxicology laboratories the concentrations of the selected analytes could be over the evaluated linearity range (a situation occurring mostly for MET and EDDP, since the therapeutic concentrations in maintenance treatment are over 100 ng/mL and in post-mortem cases concentrations of up to 1000 ng/mL were reported) [53–55], the upper limit of quantification was evaluated also. These samples were analyzed according to the elaborated protocol, after a 1:10 dilution with blank human plasma. The assay allowed the accurate quantification of the selected analytes at concentrations as high as 2096 ng/mL plasma for FNZ, 2114 ng/mL plasma for 7-AFNZ, 2036 ng/mL plasma for MET and 2080 ng/mL plasma for EDDP (Tables 5 and 6).

The within-assay and between-assay precision and accuracy determined in human plasma at four levels of concentration of MET, EDDP, FNZ and 7-AFNZ (QC1–QC4) are summarized in Tables 5 and 6. The method performed well in terms of accuracy and precision over the selected concentration range, with all results being within the appropriate range of % CV and accuracy (%).

The results of stability study showed that ambient temperature storage of QC samples for up to 24 h prior to liquid–liquid extraction has little effect on the quantitation results of MET, EDDP and 7-AFNZ (inaccuracy between 0.80% and 12.53%; CV% below 9.74%). However, a potential instability of FNZ in plasma was suggested in these storage conditions, since inaccuracy values at QC2 and QC3 for this compound were of −14.86% and −14.40%, respectively. These results suggest the importance of rapid analysis of samples after blood sampling or, if this is not possible, samples should be stored in appropriate conditions until the analysis. Instability issues were reported earlier for FNZ in plasma samples. Robertson and Drummer suggested that FNZ is stable at −20 °C. However, they found 7-AFNZ to be relatively unstable at that temperature. This justifies the need for plasma samples to be conserved at −80 °C until the analysis [53,88]. The post-preparative stability study showed the stability of the

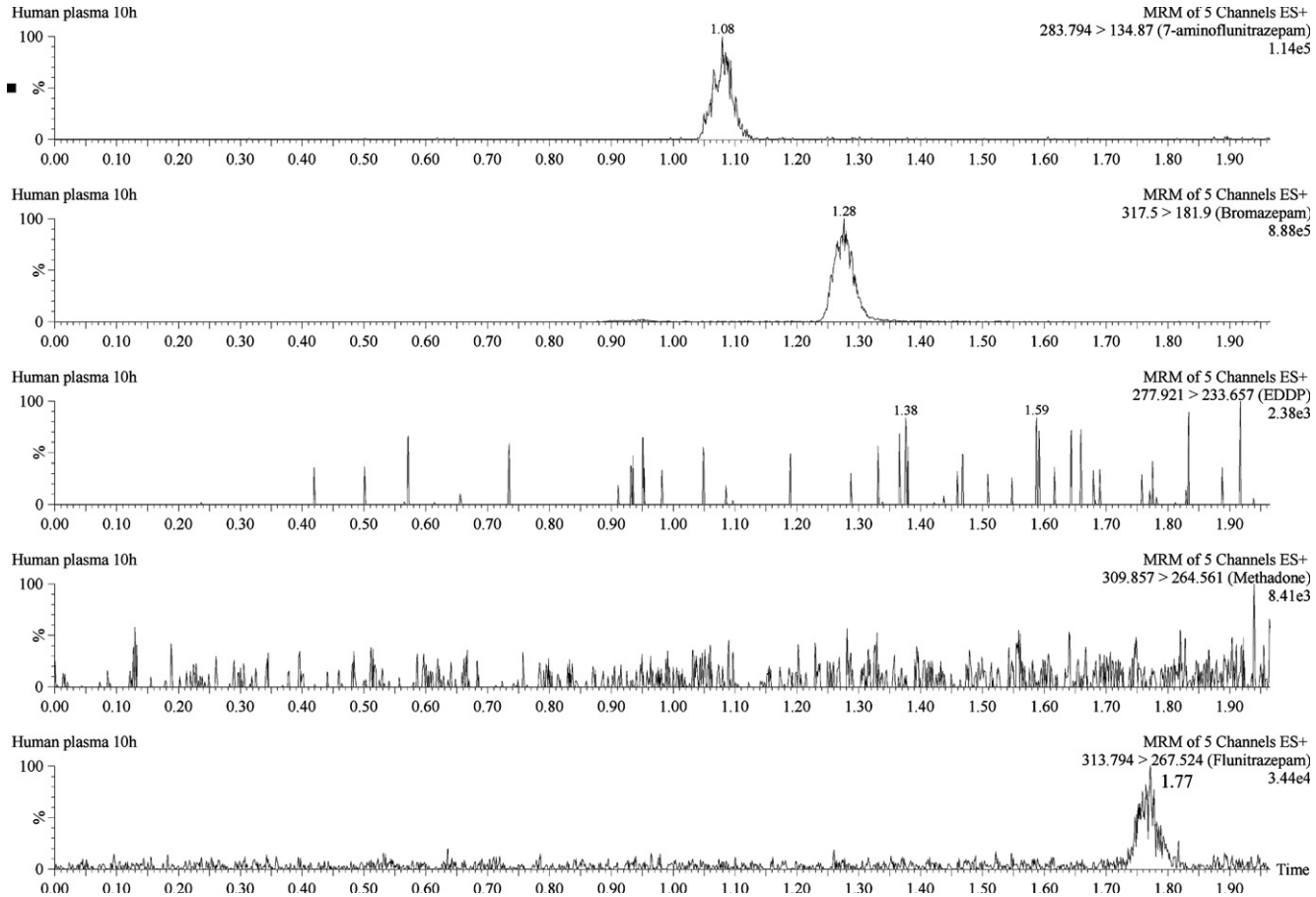

Fig. 4. LC–ESI-MS/MS chromatogram of an extract of real human plasma sample collected from a female volunteer at 10 h after single oral administration of a 1 mg flunitrazepam tablet (measured concentrations: 1.50 ng/mL plasma for FNZ and 1.60 ng/mL plasma for 7-AFNZ).

selected compounds in the final extracts maintained at 4 °C in the autosampler for at least 24 h (inaccuracy values between −2.66% and 9.55%; CV% up to 10.73%). The mean changes in MET, EDDP, FNZ and 7-AFNZ concentration after three freeze–thaw cycles indicated no stability problems under these conditions (inaccuracy values between −9.33% and 11.54%; CV% up to 9.61%).

3.3. Clinical application in healthy Subjects

The validated method was successfully applied to the assay of FNZ and 7-AFNZ in plasma sample from a healthy human subject (body weight of 50 kg) who received a single oral dose of 1 mg flunitrazepam (Fig. 4). The proposed method was used also for the simultaneous quantification of MET, EDDP, FNZ and 7-AFNZ in rat (simultaneously exposed to 5 mg methadone/kgbw and 0.5 mg flunitrazepam/kgbw) and rabbit (simultaneously exposed to 5 mg methadone/kgbw and 1 mg flunitrazepam/kgbw) plasma samples (Fig. 5). The results of the quantitative analysis performed on real plasma samples of human or animal origin are presented in Table 7. The method continued to perform in terms of accuracy, in each analytical run, not more than two out of six QC samples being outside of ±15% nominal value, but not all two at the same concentration.

Fig. 5 shows representative LC–MS/MS chromatograms corresponding to plasma samples collected from the human volunteer at 10 h post-administration and from rabbit at 2 h after oral administration.

4. Conclusions

The aim of this work was to develop a high-throughput, selective and sensitive method for the simultaneous determination of MET, FNZ and their major metabolites, EDDP and 7-AFNZ in human, rat and rabbit plasma. Analytes were extracted by liquid–liquid extraction and separated by LC on a 1.8 μm column. The hyphenation of UPLC with the ESI-MS/MS detection guaranteed the high speed, sensitivity and selectivity of the assay. The validation confirmed the linearity, acceptable accuracy and precision over a wide concentration range (0.5–250 ng/mL) and the high sensitivity (LOQ at approximately 0.5 ng/mL for each analyte) of the assay. The working range is even wider, with ULOQ set around 2000 ng/mL based on

Table 7
Quantification results for MET, EDDP, FNZ and 7-AFNZ in real plasma samples.

Subject	Sampling time after exposure (h)	$c_{measured}$ (mean ± SD)			
		MET	EDDP	FNZ	7-AFNZ
Human	10	–	–	1.45 ± 0.06	1.53 ± 0.10
Rabbit	1	45.10 ± 5.40	14.20 ± 1.55	16.02 ± 1.93	0.77 ± 0.15
	2	20.20 ± 2.26	9.85 ± 1.06	6.10 ± 0.85	0.60 ± 0.14
	4	10.40 ± 0.85	6.80 ± 0.34	2.63 ± 0.26	0.63 ± 0.05
	24	1.88 ± 0.10	1.55 ± 0.13	0.55 ± 0.07	< LOQ
Rat	1	2.55 ± 0.55	19.75 ± 2.59	3.57 ± 0.35	< LOQ
	3	7.40 ± 0.92	26.23 ± 4.26	3.25 ± 0.21	< LOQ

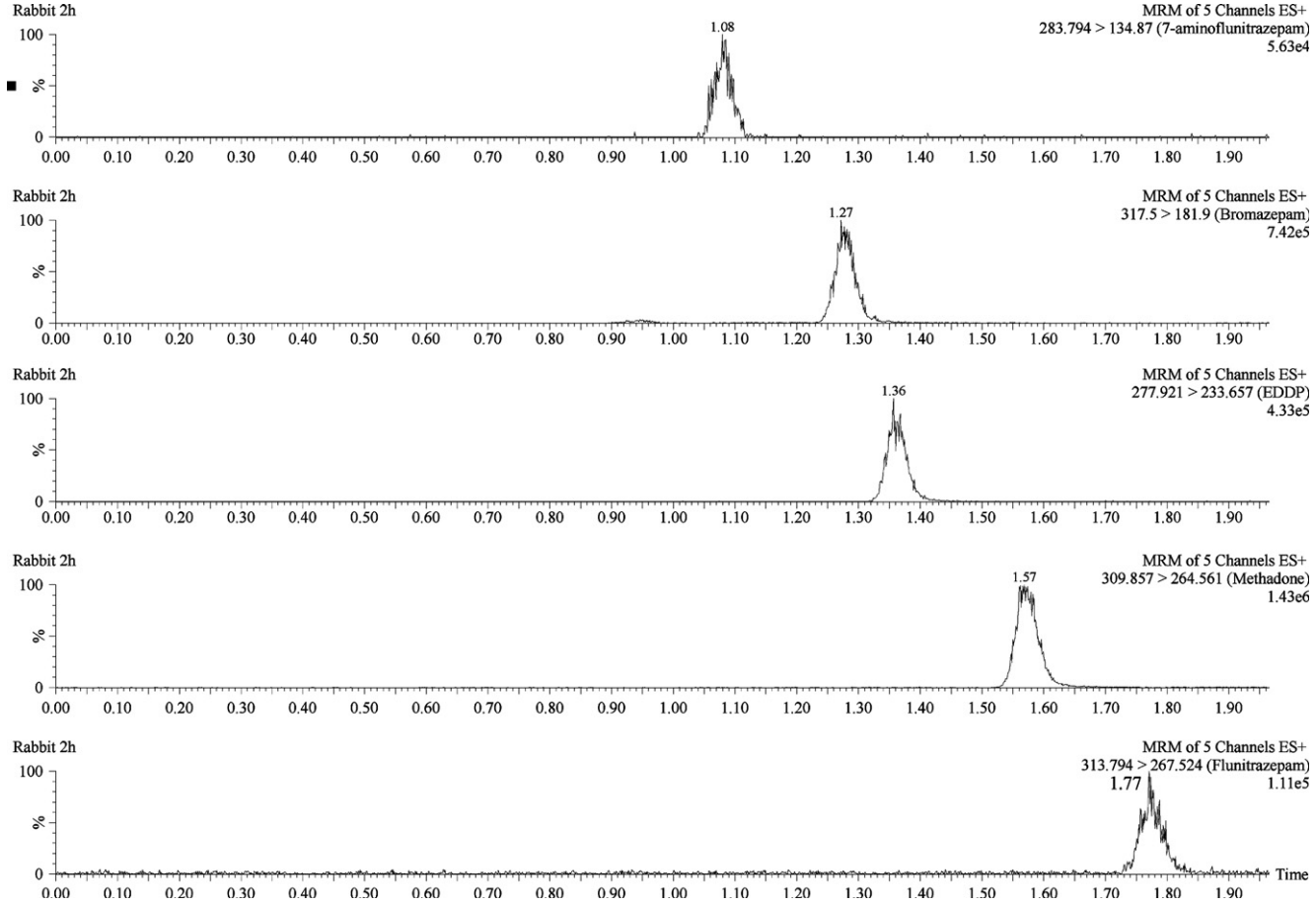

Fig. 5. LC–ESI-MS/MS chromatogram of an extract of real rabbit plasma sample collected at 2 h after single oral administration of a 5 mg methadone/kgbw and 1 mg flunitrazepam/kgbw (measured concentrations: 21.86 ng/mL plasma for MET, 10.6 ng/mL plasma for EDDP, 6.7 ng/mL plasma for FNZ and 0.7 ng/mL plasma for 7-AFNZ).

accuracy and CV% values. The method was applied to quantify the selected drugs and metabolites in human, rat or rabbit plasma samples, after exposure to single doses of MET and/or FNZ. Given the simple and easy sample preparation, the high throughput, sensitivity and selectivity, the elaborated LC–ESI-MS/MS method should be useful in the field of clinical or forensic toxicology, in pharmacokinetic studies, to explore potential metabolic interactions in case of MET–FNZ association but also in therapeutic drug monitoring.

References

[1] M.W. Compton, N.D. Volkow, Drug Alcohol Depend. 83S (2006) S4–S7.
[2] S.E. McCabe, Drug Alcohol Depend. 79 (1) (2005) 53–62.
[3] A. Verdejo, T. Inmaculada, C. Orozco, K.L. Puente, M. Perez-Garcia, Drug Alcohol Depend. 78 (3) (2005) 283–288.
[4] M. Connock, A. Juarez-Garcia, S. Jowett, E. Frew, Z. Liu, R.J. Taylor, A. Fry-Smith, E. Day, N. Lintzeris, T. Roberts, A. Burls, R.S. Taylor, Health Technol. Assess. 11 (9) (2007) 1–171.
[5] M.J. Kreek, K.S. LaForge, E. Butelman, Nat. Rev. Drug Discovery 1 (9) (2002) 710–726.
[6] H.D. Kleber, Am. J. Addict. 12 (S2) (2003) 5–18.
[7] K.A. Harris, J.H. Arnsten, H. Joseph, J. Hecht, I. Marion, P. Juliana, M.N. Gourevitch, J. Subst. Abuse Treat. 31 (2006) 433–438.
[8] H. Olsen, in: M.N.G. Dukes, J.K. Aronson (Eds.), Meyler's Side Effects of Drugs, 14th ed.,Elsevier, Amsterdam, 2000, pp. 198–230.
[9] J.P. Bernard, M.S. Opdal, R. Karinen, J. Morland, H.Z. Khiabani, Eur. J. Clin. Pharmacol. 63 (8) (2007) 777–782.
[10] S. Tiras, V. Haas, L. Chevret, M. Decobert, A. Buisine, D. Devictor, P. Durand, P. Tissieres, Ann. Emerg. Med. 48 (4) (2006) 448–451.
[11] K. Wolff, Ther. Drug Monit. 24 (4) (2002) 457–470.
[12] F.J. Couper, K. Chopra, M.L.Y. Pierre-Louis, Forensic Sci. Int. 153 (1) (2005) 71–73.
[13] N. Lintzeris, T.B. Mitchell, A.J. Bond, L. Nestor, J. Strang, Drug Alcohol Depend. 91 (2–3) (2007) 187–194.
[14] F.-J. Baud, Ann. Pharm. Fr. 67 (5) (2009) 353–359.
[15] M.J. Bogusz, J. Chromatogr. B Biomed. Sci. Appl. 748 (1) (2000) 3–19.
[16] A. Ferrari, C.P.R. Coccia, A. Bertolini, E. Sternieri, Pharmacol. Res. 50 (6) (2004) 551–559.
[17] N. Lintzeris, S. Nielsen, Am. J. Addict. 19 (1) (2010) 59–72.
[18] S. Bomsien, G. Skopp, Eur. J. Clin. Pharmacol. 63 (9) (2007) 821–827.
[19] Y.F. Cheng, U.D. Neue, L.L. Woods, J. Chromatogr. B Biomed. Sci. Appl. 729 (1–2) (1999) 19–31.
[20] S. Crettol, M. Monnat, C.B. Eap, Crit. Care 11 (1) (2007) 119.
[21] S. Crettol, J.-J. Deglon, J. Besson, M. Croquette-Krokkar, I. Gothuey, R. Hammig, M. Monnat, H. Huttemann, P. Baumann, Clin. Pharmacol. Ther. 78 (6) (2005) 593–604.
[22] J. Hanna, D.J.R. Foster, A. Salter, A.A. Somogyi, J.M. White, F. Bochner, Br. J. Clin. Pharmacol. 60 (4) (2005) 404–413.
[23] J.D. Raffa, J. Grebely, H. Tossonian, T. Wong, M. Viljoen, M. Khara, A. Mead, M. McLean, F. Duncan, A.J. Petkau, S. DeVlaming, B. Conway, Drug Alcohol Depend. 89 (2–3) (2007) 306–309.
[24] L. Pimentel, D. Mayo, J. Emerg. Med. 34 (3) (2008) 287–290.
[25] L. Xiao, Y. Tang, A.K. Smith, Y. Xiang, L. Sheng, Y. Chi, W. Du, S. Guo, Z. Jiang, G. Zhang, X. Luo, Psychiatry Res. 179 (1) (2010) 91–95.
[26] J.G. Bramness, H. Kornør, Drug Alcohol Depend. 90 (2–3) (2007) 203–209.
[27] S.C. Licata, J.K. Rowlett, Pharmacol. Biochem. Behav. 90 (1) (2008) 74–89.
[28] N.A. Ator, R.R. Griffiths, E.M. Weerts, Drug Alcohol Depend. 78 (2) (2005) 113–123.
[29] M. Farre, M.-T. Teran, P.N. Roset, M. Mas, M. Torrens, J. Cami, Psychopharmacology (Berlin) 140 (4) (1998) 486–495.
[30] M. Fatseas, E. Lavie, C. Denis, M. Auriacombe, J. Subst. Abuse Treat. 37 (4) (2009) 407–411.
[31] E. Peles, S. Schreiber, Y. Naumovsky, M. Adelson, J. Affect. Disord. 99 (1–3) (2007) 213–220.
[32] S.W. Borron, C. Monier, P. Risede, F.J. Baud, Hum. Exp. Toxicol. 21 (11) (2002) 599–605.
[33] N. Lintzeris, T.B. Mitchell, A. Bond, L. Nestor, J. Strang, J. Clin. Psychopharmacol. 26 (3) (2006) 274–283.
[34] H. Druid, P. Holmgren, J. Ahlner, Forensic Sci. Int. 122 (2–3) (2001) 136–141.
[35] L.K. Bechtel, C.P. Holstege, Emerg. Med. Clin. North Am. 25 (2) (2007) 499–525.
[36] S.D. Lane, D.R. Cherek, S.O. Nouvion, Psychopharmacology (Berlin) 196 (2) (2008) 177–188.
[37] A.M. Dåderman, G. Edman, Psychiatry Res. 103 (1) (2001) 27–42.
[38] T. Ohshima, J. Clin. Forensic Med. 13 (1) (2006) 44–45.
[39] M.Z. Mintzer, R.R. Griffiths, Drug Alcohol Depend. 53 (1) (1998) 49–66.
[40] V. Pauly, E. Frauger, F. Rouby, S. Sirere, S. Monier, C. Paulet, V. Gibaga, J. Micallef, X. Thirion, L'Encéphale 36 (2010) 122–131.
[41] M. Gelkopf, A. Bleich, R. Hayward, G. Bodner, M. Adelson, Drug Alcohol Depend. 55 (1–2) (1999) 63–68.
[42] X. Thirion, J. Micallef, K. Barrau, S. Djezzar, J.L. Sanmarco, G. Lagier, Eur. Addict. Res. 7 (1) (2001) 32–36.
[43] T. Kilicarslan, R.L. Haining, A.E. Rettie, U. Busto, R.F. Tyndale, E.M. Sellers, Drug Metab. Dispos. 29 (2001) 460–465.
[44] B. Megarbane, N. Deye, F. Baud, Reanimation 13 (2004) 334–342.
[45] Y. Chang, D.E. Moody, Eur. J. Clin. Pharmacol. 60 (12) (2005) 875–881.
[46] A. Galetin, C. Brown, D. Hallifax, K. Ito, J.B. Houston, Drug Metab. Dispos. 32 (12) (2004) 1411–1420.
[47] J.K. Coller, A.A. Somogyi, F. Bochner, Xenobiotica 29 (10) (1999) 973–986.
[48] I. Gafni, U.E. Busto, R.F. Tyndale, H.L. Kaplan, E.M. Sellers, J. Clin. Psychopharmacol. 23 (2) (2003) 169–175.
[49] L.M. Hesse, K. Venkatakrishnan, L.L. Von Moltke, R.I. Shader, D.J. Greenblatt, Drug Metab. Dispos. 29 (2) (2001) 133–140.
[50] J.K. Coller, A.A. Somogyi, F. Bochner, J. Chromatogr. B Biomed. Sci. Appl. 719 (1–2) (1998) 87–92.
[51] A. El Mahjoub, C. Staub, J. Chromatogr. B Biomed. Sci. Appl. 754 (2) (2001) 271–283.
[52] C.B. Eap, M. Bourquin, J.-L. Martin, J. Spagnoli, S. Livoti, K. Powell, P. Baumann, J.-J. Deglon, Drug Alcohol Depend. 61 (1) (2000) 47–54.
[53] O.H. Drummer, Forensic Sci. Int. 142 (2-3) (2004) 101–113.
[54] V. Viette, D. Guillarme, R. Mylonas, Y. Mauron, M. Fathi, S. Rudaz, D. Hochstrasser, J.L. Veuthey, Clin. Biochem. 44 (1) (2011) 32–44.
[55] J.L. Reubsaet, S. Pedersen-Bjergaard, J. Chromatogr. A 1031 (1–2) (2004) 203–211.
[56] M. Kollroser, C. Schober, J. Pharm. Biomed. Anal. 28 (6) (2002) 1173–1182.
[57] I. Marchi, J. Schappler, J.L. Veuthey, S. Rudaz, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 877 (23) (2009) 2275–2283.
[58] O. Dale, P. Sheffels, E.D. Kharasch, Br. J. Clin. Pharmacol. 58 (2) (2004) 156–162.
[59] H. He, C. Sun, X.R. Wang, C. Pham-Huy, N. Chikhi-Chorfi, H. Galons, M. Thevenin, J.R. Claude, J.M. Warnet, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 814 (2) (2005) 385–391.
[60] B. Doherty, F. O'Donnell, W.F. Smyth, J.C. Leslie, V.N. Ramachandran, N.S. Boyd, C.J. Hack, E. O'Kane, S. McClean, Talanta 72 (2) (2007) 755–761.
[61] J.C. Eichhorst, M.L. Etter, N. Rousseaux, D.C. Lehotay, Clin. Biochem. 42 (15) (2009) 1531–1542.
[62] S. Souverain, S. Rudaz, J.L. Veuthey, J. Pharm. Biomed. Anal. 35 (4) (2004) 913–920.
[63] S. Dresen, N. Ferreiros, H. Gnann, R. Zimmermann, W. Weinmann, Anal. Bioanal. Chem. 396 (7) (2010) 2425–2434.
[64] P. Adamowicz, M. Kała, Forensic Sci. Int. 198 (1–3) (2010) 39–45.
[65] S.M. Song, P. Marriott, A. Kotsos, O.H. Drummer, P. Wynne, Forensic Sci. Int. 143 (2–3) (2004) 87–101.
[66] W.C. Cheng, T.S. Yau, M.K. Wong, L.P. Chan, V.K. Mok, Forensic Sci. Int. 162 (1–3) (2006) 95–107.
[67] E.L. Øiestad, U. Johansen, A.S. Christophersen, Clin. Chem. 53 (2) (2007) 300–309.
[68] E.L. Øiestad, U. Johansen, A.M. Øiestad, A.S. Christophersen, J. Anal. Toxicol. 35 (5) (2011) 280–293.
[69] J. Hackett, A.A. Elian, Forensic Sci. Int. 157 (2–3) (2006) 156–162.
[70] B. Guthery, A. Bassindale, C.T. Pillinger, G.H. Morgan, Rapid Commun. Mass Spectrom. 23 (3) (2009) 340–348.
[71] M. Scott-Ham, F.C. Burton, J. Clin. Forensic Med. 12 (4) (2005) 175–186.
[72] T.M. Annesley, Clin. Chem. 49 (7) (2003) 1041–1044.
[73] U.S. Department of Health and Human Services, Guidance for Industry—Bioanalytical Method Validation, Food and Drug Administration, Center for Drug Evaluation and Research, May 2001, 〈http://www.fda.gov/cder/guidance/4252fnl.pdf〉.
[74] U.S. Department of Health and Human Services, Guidance for Industry: Bioavailability and Bioequivalence Studies for Orally Administered Drug Products—General Considerations, Food and Drug Administration, Center for Drug Evaluation and Research, Rockville, USA, March 2003, 〈http://www.fda.gov/cder/guidance/5356fnl.pdf〉.
[75] The European Agency for the Evaluation of Medicinal Products, Note for Guidance on the Investigation of Bioavailability and Bioequivalence, CPMP/EWP/QWP/1401/98, London, UK, July 2001, 〈http://www.emea.europa.eu/pdfs/human/qwp/140198enfin.pdf〉.
[76] S. Jacob, S. Jickells, K. Wolff, N. Smith, Drug Metab. Lett. 2 (4) (2008) 245–247.
[77] C. Joyce, W.F. Smyth, V.N. Ramachandran, E. O'Kane, D.J. Coulter, J. Pharm. Biomed. Anal. 36 (3) (2004) 465–476.
[78] W.F. Smyth, P. Brooks, Electrophoresis 25 (10–11) (2004) 1413–1446.
[79] W.F. Smyth, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 824 (1–2) (2005) 1–20.
[80] G. Vanhoenacker, F. de l'Escaille, D. De Keukeleire, P. Sandra, J. Pharm. Biomed. Anal. 34 (3) (2004) 595–606.
[81] M.R. Fuh, S.W. Lin, L.L. Chen, T.Y. Lin, Talanta 72 (4) (2007) 1329–1335.
[82] H.L. Lord, M. Rajabi, S. Safari, J. Pawliszyn, J. Pharm. Biomed. Anal. 40 (3) (2006) 769–780.
[83] B. Kiss, D.S. Popa, M. Bojita, F Loghin, Rev. Chim. (Bucharest) 59 (12) (2008) 1301–1304.
[84] B. Kiss, C. Bogdan, A. Pop, F. Loghin, J. Liq. Chromatogr. Relat. Technol. 33 (14) (2010) 1381–1392.
[85] A.C. Moffat, M.D. Osselton, B. Widdop (Eds.), Clarke's Analysis of Drugs and Poisons in Pharmaceuticals, Body Fluids and Postmortem Material, third ed.,Pharmaceutical Press, London, 2004.
[86] A. Maquille, D. Guillarme, S. Rudaz, J.L. Veuthey, Chromatographia 70 (2009) 1373–1380.
[87] S. Diallo, E. Bugni, F. Senhadj-Raoul, S. Gasdeblay, D. Marot, M.C. Dessalles, G. Mahuzier, Talanta 55 (4) (2001) 721–732.
[88] M.D. Robertson, O.H. Drummer, J. Forensic Sci. 43 (1) (1998) 5–8.