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Liquid chromatographic–electrospray ionization mass spectrometric quantitative analysis of buprenorphine, norbuprenorphine, nordiazepam and oxazepam in rat plasma

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Abstract

A liquid chromatographic–mass spectrometric method with electrospray ionization is presented for the simultaneous determination of buprenorphine, nordiazepam and their pharmacologically active metabolites, norbuprenorphine and oxazepam, in rat plasma. The drugs were extracted from plasma by liquid–liquid extraction and chromatographically separated using a gradient elution of aqueous ammonium formate and acetonitrile. Following electrospray ionization, the analytes were quantified in the single ion storage mode. The assay was validated according to current acceptance criteria for bioanalytical method validation. It was proved to be linear from 0.7 to 200 ng/ml plasma for buprenorphine, 1.0 to 200 ng/ml for norbuprenorphine, 2.0 to 200 ng/ml for nordiazepam, and from 5.0 to 200 ng/ml for oxazepam. The average recoveries of buprenorphine, norbuprenorphine, nordiazepam and oxazepam were 89, 39, 88 and 82%, respectively, with average coefficients of variation ranging from 1.8 to 14.3%. The limits of quantitation for these drugs were 0.7, 1.0, 2.0 and 5.0 ng/ml, respectively, with associated precisions within 17% and accuracies within ±18% of the nominal values. Both the intra- and inter-assay precision values did not exceed 11.3% for the four analytes. Intra- and inter-assay accuracies lay within ±15% of the nominal values. The validated method was applied to the determination of buprenorphine, norbuprenorphine, nordiazepam and oxazepam in plasma samples collected from rats at various times after intravenous administration of buprenorphine and nordiazepam.
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1. Introduction

Buprenorphine is a semi-synthetic opioid analgesic with mixed agonist–antagonist activity, which is used for the substitutive management of opioid-dependent persons as an alternative to methadone [1,2]. High dosage formulation in doses up to 8 mg of buprenorphine per tablet for sublingual use has been available in France since 1996 [3,4] and has recently been approved by the United States Food and Drug Administration for outpatient dosing in the treatment of opioid dependence. Buprenorphine at high dosage has been shown to substantially reduce the use of illicit opioids [2,5]. Moreover, maximal effects on respiratory depression have been observed after administration of 4–8 mg of sublingual buprenorphine doses, with no increase at doses up to 32 mg [6,7]. This “ceiling effect” is of utmost importance regarding the safety of buprenorphine for use in substitution treatment.

However, deaths have been reported among users of buprenorphine. Deaths may result from either overdose with substitution treatment or misuse, i.e. intravenous injection of crushed tablets, but in many cases benzodiazepines are

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implicated as co-intoxicants [4,8]. Heroin addicts and substitute heroin addicts frequently abuse benzodiazepines [9–11], though not all benzodiazepines at the same rate [4,8,12]. Benzodiazepines are considered as relatively safe drugs and deaths caused by benzodiazepines alone, in the absence of other pathologies, are uncommon [4]. However, their combination with substitution products (buprenorphine or methadone) is suspected to be a major risk factor of lethal overdose. This idea is reinforced by experimental studies. While high doses of buprenorphine and midazolam alone have limited effects on arterial blood gases in rats, there is a real potential for severe respiratory depression when these drugs are used concurrently, especially acutely [13,14]. Moreover, flunitrazepam increases lethality in buprenorphine-treated rats to a far greater extent than in methadone-treated rats, and had no significant effects on morphine lethality [15].

The toxicity mechanisms of buprenorphine and benzodiazepine association remain to be established. Certain arguments would tend to implicate pharmacodynamic mechanisms [16–18], while others would support pharmacokinetic processes [14]. Very little work in exploring the pharmacokinetic hypothesis has been conducted, mainly because diagnostic means of study and exploration were not available. We have recently described a gas chromatography–mass spectrometry (GC–MS) method allowing the simultaneous determination of buprenorphine, flunitrazepam and the active metabolites of these drugs in rat plasma [19]. This method has been successfully applied to their pharmacokinetic study after administration of toxic doses of buprenorphine and flunitrazepam to rats and the results have suggested metabolic interactions between these drugs [S. Pirnay, unpublished results]. However, the GC–MS method was difficult to adapt in the assessment of drug plasma kinetics when lower doses of buprenorphine and flunitrazepam came into question. In this case, the sensitivity was too low to determine the metabolites of these drugs.

This drawback can be overcome by liquid chromatography–mass spectrometry (LC–MS) coupling which combines the advantages of a separation method with the high sensitivity and selectivity of mass spectrometry when applying thereto soft ionization techniques like electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Several methods based on the LC–MS technique have been developed for the determination of buprenorphine and norbuprenorphine in plasma or serum [20–23], whole blood [24] and urine [25]. In parallel, the recent developments of LC–MS have shown promising results for the analysis of benzodiazepines and their metabolites in biological samples [26–31]. Methods based on the LC–MS technique do not suffer from the limitations of GC separation, e.g. required derivatization of the analytes prior to instrumental analysis and associated problems of reproducibility [32] and thermal degradation of unstable analytes [33]. Moreover, the LC–MS technique is generally characterized by a short analysis time, making it suitable for studies including multiple sample analysis, such as pharmacokinetic studies. While LC–MS has become a very powerful and flexible method for the analysis of many substances of forensic interest, their metabolites and combinations of some of these substances [20,34,35], it has not yet been used for the simultaneous determination of buprenorphine and benzodiazepines.

This study reports an analytical method for the simultaneous measurement of buprenorphine and nordiazepam and their pharmacologically active metabolites, norbuprenorphine and oxazepam, in rat plasma using LC–ESI-MS after sample preparation by liquid–liquid extraction. The method was set up and adapted for the analysis of the small plasma samples taken from rats. It was validated according to current acceptance criteria for bioanalytical method validation. Finally, the analytical method was applied to the determination of buprenorphine, norbuprenorphine, nordiazepam and oxazepam in rat plasma, at different times after administration of buprenorphine and nordiazepam. We chose to test the combination of buprenorphine and nordiazepam because nordiazepam is a pharmacologically active metabolite common to several benzodiazepines [36], and because of the prevalent use of nordiazepam and oxazepam by heroin addicts [4,8,12].

2. Experimental

2.1. Chemicals

Buprenorphine (BUP), buprenorphine-d4 (BUP-d4), norbuprenorphine (NBUP), nordiazepam (NDD), nordiazepam-d5 (NDD-d5), oxazepam (OXA) and oxazepam-d5 (OXA-d5) in acetonitrile solutions (each 100 μg/ml) were purchased from Cerilliant (Austin, TX, USA). Buprenorphine HCl and nordiazepam were kindly supplied by Schering-Plough (Levallois-Perret, France) and Pfizer (Paris, France), respectively. Ammonium formate (analytical grade), methanol and acetonitrile (both HPLC grade) were obtained from Merck (Darmstadt, Germany). Water was deionized to >18.2 MΩ with a Milli-Q ultrapure water system (Millipore Corp., Woburn, MA, USA). Toxitubes A for liquid–liquid partition were obtained from Toxi-Lab Ansys Diagnostic (Lake Forest, CA, USA). Nitrogen gas generator from Domnick Hunter (Villefranche-sur-Saône, France) was used in electrospray ionization MS.

2.2. Procedures

2.2.1. Preparation of stock solutions

The acetonitrile stock solutions of the analytes and internal standards (100 μg/ml) were stored at −20 °C. Combined stock solutions of BUP, NBUP, OXA and NDD were prepared by dilution of the stock solutions in acetonitrile at concentrations of 0.01, 1 and 10 μg/ml, respectively. The combined stock solutions were used as needed for the preparation of calibration and quality control samples; they were stored at −20 °C between experiments. The stock solutions of internal standards (ISTDs), i.e. BUP-d4, OXA-d5 and NDD-d5, were diluted in acetonitrile to give working solutions at 1 μg/ml; they were stored at −20 °C. The stability of the analytes stored at −20 °C was demonstrated over a 4-month period.

2.2.2. Plasma sample collection

Male Sprague-Dawley rats (OFA strain, 250–300 g, 8–10 weeks) were obtained from Charles River Laboratories (L'Arbresle, France). Animals were maintained under standard conditions of temperature and lighting for 8 days with ad libitum access to food and water. Rats were anesthetized with ketamine (Panpharma, Fougères, France) 70 mg/kg, and xylazine (Bayer Pharma, Puteaux, France) 10 mg/kg intraperitoneally. All experiments complied with the ethical guidelines established by the French Minister of Agriculture for experimentation with laboratory animals (law no. 87–848).

Blank plasma samples were obtained from anesthetized rats by carotid bleeding, after centrifugation at 2000 × *g* for 10 min at 5 °C. Harvested plasma samples were mixed to obtain a homogeneous pool of blank plasma which was stored at −20 °C until use.

Real blood samples were obtained from a second set of rats treated with BUP and NDD. Drug solutions were freshly prepared by separate weighings, at a concentration of 18.2 mg/ml in Tween 20 (Sigma–Aldrich, St-Quentin Fallavier, France) for BUP, and at 6 mg/ml in polyethyleneglycol-400 (Merck) for NDD. These solutions were diluted with sterile water to adjust the doses of BUP and NDD to the weight of each rat. On the day before treatment, animals were anesthetized as described above and the femoral vein and artery were catheterized with silastic tubes (30 cm long, 0.51 mm i.d., 0.94 mm e.d.) from Dow Corning (Midland, MI, USA). The catheters were then tunneled subcutaneously and fixed at the back of the neck. The rats were given a 24 h recovery period to allow for washout of anesthesia. On the study day rats were placed in a restraining chamber (20 cm long, 6.5 cm i.d.) from Harvard Apparatus (Holliston, MA, USA). They received a 30 mg/kg dose of BUP in a volume of 1.3 ml, by intravenous injection via femoral vein cannulation. Injection was performed over 3 min at a constant rate of 433 μl/min, using a perfusion pump (PHD 2000) from Harvard Instruments (Holliston, MA, USA). Immediately after, the rats were given a dose of 10 mg/kg NDD, also in a volume of 1.3 ml, by intravenous perfusion over 30 min and at a rate of 43.3 μl/min. The study involved serial arterial blood sampling (∼250 μl) with five samples obtained from each animal at the following time points: after BUP perfusion (−30 min) and after NDD perfusion (0, 5, 20 and 60 min). Blood was collected into heparinized microtubes and centrifuged at 2000 × *g* for 10 min at 5 °C. The plasma (∼110–120 μl) was separated and frozen immediately at −20 °C until analysis by the procedure described below. No major problems were encountered during catheterization, drug administration or collection of arterial blood samples.

2.2.3. Sample preparation

Previous studies of our laboratory were used as a starting point for the extraction of the compounds under study [19,37,38]. The real plasma samples from BUP/NDD-treated rats (100 μl) and the pooled blank plasma samples (100 μl) fortified with BUP, NBUP, NDD and OXA were both spiked with 80 μl of each internal standard at 1 μg/ml (i.e. BUP-d4, OXA-d5 and NDD-d5). The sample volume was adjusted to 1.0 ml with deionized water. After vortex mixing (15 s), the samples were submitted to liquid–liquid extraction using Toxitubes A. The Toxitubes A contain a mixture of dichloromethane, 1,2-dichloroethane, heptane and isopropanol, and are suitable for the extraction of neutral and basic drugs and unconjugated metabolites. The samples were poured into Toxitubes A into which 2 ml of deionized water had been previously added, in order to keep the sample volume in the tube between 2 and 5 ml, as recommended by the manufacturer. The loaded Toxitubes A were automatically agitated for 5 min at ambient temperature before being centrifuged at 100 × *g* for 5 min. Following centrifugation, 0.9 ml out of the 1 ml of organic phase was transferred to a clean tube and evaporated to dryness under a gentle stream of nitrogen at ambient temperature. Extracts were reconstituted with 40 μl of a water–methanol mixture (50:50 v/v). After vortex mixing (30 s), 10 μl-volumes of the extracts were submitted to instrumental analysis.

2.3. Analytical method

2.3.1. Instrumentation

A ThermoFinnigan "Surveyor" high performance liquid chromatograph (HPLC) coupled with a LCQ Advantage ion trap mass spectrometer was used (ThermoFinnigan, Massy, France). The HPLC system included a degasser, a binary pump and an autosampler with an injection loop of 20 μl. Ionization of the analytes was performed in the ESI mode. The operating parameters are described in Section 2.3.3. The Finnigan™ Xcalibur® software for LCQ Advantage LC–MS (Version 1.0/1.2) was used for quantitation of the analytes.

2.3.2. HPLC conditions

Chromatographic separation was carried out using a X-terra C18 column (150 mm × 2.1 mm i.d.; 5 μm particle size) from Waters (St-Quentin-en-Yvelines, France). The HPLC column was held at 30 °C. The mobile phase consisted of a mixed solvent system of 2 mM aqueous ammonium formate adjusted to pH 3.0 with formic acid (solvent A) and acetonitrile (solvent B). Both solvents were degassed in an ultrasonic bath for 30 min before use, and were degassed by the integrated surveyor degasser during use. The HPLC system was flushed with 70% solvent A until the beginning of the analysis. Gradient elution at a constant flow-rate of 100 μl/min was performed as follows: linear decrease from 70 to 30% solvent A in 7 min; linear increase from 30 to 70% solvent A in 8 min; 20 min hold at 70% solvent A for re-equilibration. The HPLC effluent entered the electrospray ionization chamber during the total run time (35 min).

2.3.3. MS conditions

Ionization of BUP, NBUP, NDD and OXA was operated in the ESI mode. ESI was preferred over APCI because the tested drugs are polar drugs. The following parameters of the electrospray interface were optimized to provide a maximum of $[M+\mathrm{H}]^+$ ions for all the analytes and internal standards: capillary temperature, 220 °C; capillary voltage, 10 V; spray voltage, 5200 V; spray current, 0.10 μA; nebulizer and auxiliary gas pressures (nitrogen in both cases), 80 and 10 psi, respectively. The analyzer temperature was 30 °C, and dynode and electron multiplier

voltages were set at −14,870 and −880 V, respectively. The automatic gain control (AGC) value was set at 2×10^7. Tuning of the mass spectrometer was performed with the help of the software autotune feature. The following ion optic settings were applied: tube lens, 30 V; multipole 1 offset, −5 V; lens voltage, −26 V; multipole 2 offset, −7 V; multiple RF amplitude, 400 V_{p-p}, sp.

For quantitation, the most abundant ions (*m/z*) of the compounds and deuterated analogs were used in the single ion storage (SIS) mode: *m/z* 414.4 for NBUP (time window 4.00–8.00 min); *m/z* 468.3 for BUP and *m/z* 472.3 for BUP-d4 (time window 8.00–11.15 min); *m/z* 287.1 for OXA and *m/z* 292.3 for OXA-d5 (time window 11.15–12.15 min); *m/z* 271.2 for NDD and *m/z* 276.2 for NDD-d5 (time window 12.15–18.00 min).

2.4. Method validation

The described procedure was validated according to internationally accepted recommendations [39]. Calibration standards and quality control (QC) samples were prepared from working solutions of stock sources on each validation day. The concentrations of analytes in QC samples were calculated via calibration curves prepared daily as described below.

2.4.1. Linearity of calibration

This was studied by analyzing aliquots of blank plasma (100 μl) spiked with different drug concentrations: 0.7, 1.0, 2.0, 5.0, 10, 20, 50, 100 and 200 ng/ml plasma for BUP, NBUP, OXA and NDD, respectively. The concentrations of the combined stock solutions used to do the spiking (0.01, 1 or 10 μg/ml in acetonitrile) were at least 10 times higher than those of the corresponding calibration standards, and the volumes added ranged between 5 and 100 μl. The calibration standards were also spiked with 80 μl of each ISTD solution to give concentrations of 0.8 μg/ml of plasma for BUP-d4, OXA-d5 and NDD-d5, respectively. Samples were extracted using Toxitubes A and analyzed by LC–ESI-MS as described above. Linearity was studied by checking five calibration curves on 5 working days (single measurement per level on each day). The linear regression model used for the calculation of all calibration curves was $y = a \times x$, where x is the concentration of analyte (ng/ml) in spiked plasma, y the peak area ratio between analyte and ISTD as recorded from the mass spectrometer, and a is the slope of the curve. This model was used because the calibration intercepts were not significantly different from zero ($p = 0.932$ for BUP, 0.102 for NBUP, 0.735 for NDD and 0.245 for OXA).

2.4.2. Limits of quantitation and detection

Four groups of 10 replicates of blank plasma samples (100 μl) spiked with 0.7, 1.0, 2.0 and 5.0 ng/ml of BUP, NBUP, NDD and OXA, respectively, were used to determine the lower limit of quantitation (LLOQ), i.e. the lowest concentration of the calibration curve which can be measured with acceptable accuracy and precision [39]. All samples were also spiked with 80 ng of each ISTD. Extraction of samples was followed by LC–ESI-MS analysis as described above. The limits of detection were estimated using a signal-to-noise ratio of 3:1 as a criterion. The noise data was taken from the analysis of pooled blank plasma for peaks interfering with the detection of the analytes or the internal standards.

2.4.3. Intra-assay and inter-assay variations

The QC samples for intra-assay and inter-assay variations were prepared by spiking 100 μl aliquots of pooled blank plasma with 80 ng of each internal standard and the tested concentrations of the analytes, followed by extraction and analysis as described. Ten replicates at four different concentrations of the drugs (5, 20, 100 and 200 ng/ml plasma) were used for the determination of intra-assay precision and accuracy. Inter-assay precision and accuracy were determined within a 2-week period (five separate analytical sessions with a single measurement per level on each session). Precision is expressed as a coefficient of variation (CV%) for specific added target concentrations, and accuracy as a percentage error (error%) of the determined concentration as compared with target added concentrations.

2.4.4. Recoveries

Absolute recoveries of the analytes were measured by spiking pooled blank plasma samples (100 μl) with BUP, NBUP, NDD and OXA at four different concentrations: 5, 20, 100 and 200 ng/ml of plasma (five replicates each), followed by extraction using Toxitubes A as described in Section 2.2.3. The internal standards (80 ng of BUP-d4, NDD-d5 and OXA-d5 in acetonitrile, respectively) were added to the collected extract from Toxitubes A just before evaporation of the organic phase. As controls, a set of samples were prepared by adding the same amounts of reference substances and internal standards to acetonitrile (1.0 ml), and then evaporated to dryness. In all cases, the dry residues were dissolved in 40 μl of a mixture of water–methanol (50:50 v/v). After LC–ESI-MS analysis, the absolute recovery was obtained by comparing the ratio of the peak areas analyte/internal standard measured in the spiked plasma samples and controls.

2.5. Determination of BUP, NDD and their metabolites in real rat plasma samples

Real plasma samples from BUP/NDD-treated rats were assayed with the described LC–ESI-MS method. The plasma levels of BUP, NBUP, NDD and OXA were quantified by the peak area ratios between analytes and respective internal standards. With these ratios, the drug concentrations in the plasma specimen were computed on the basis of calibration curves prepared as described above. QC samples (20 and 100 ng drug/ml plasma) were included in each analytical batch to check calibration, accuracy and precision.

3. Results and discussion

3.1. LC–ESI-MS analysis

Our first attempts to develop a MS–MS method showed no significant fragmentation of the precursor ions of BUP and

NBUP at collision energies below 30–40 eV. The use of higher collision energies resulted in extensive ion fragmentation with an overall significant loss of sensitivity. These observations were in accordance with those from previous reports on the LC–ESI-MS–MS [21,22] and LC–APCI-MS–MS analysis of BUP and NBUP [23]. Quantitation of the analytes and deuterated analogs was thus performed in the SIS mode to achieve a maximum of sensitivity, using the most abundant $[M+H]^+$ ion of each analyte: the structures and ESI mass spectra of the studied analytes and internal standards are presented in Fig. 1.

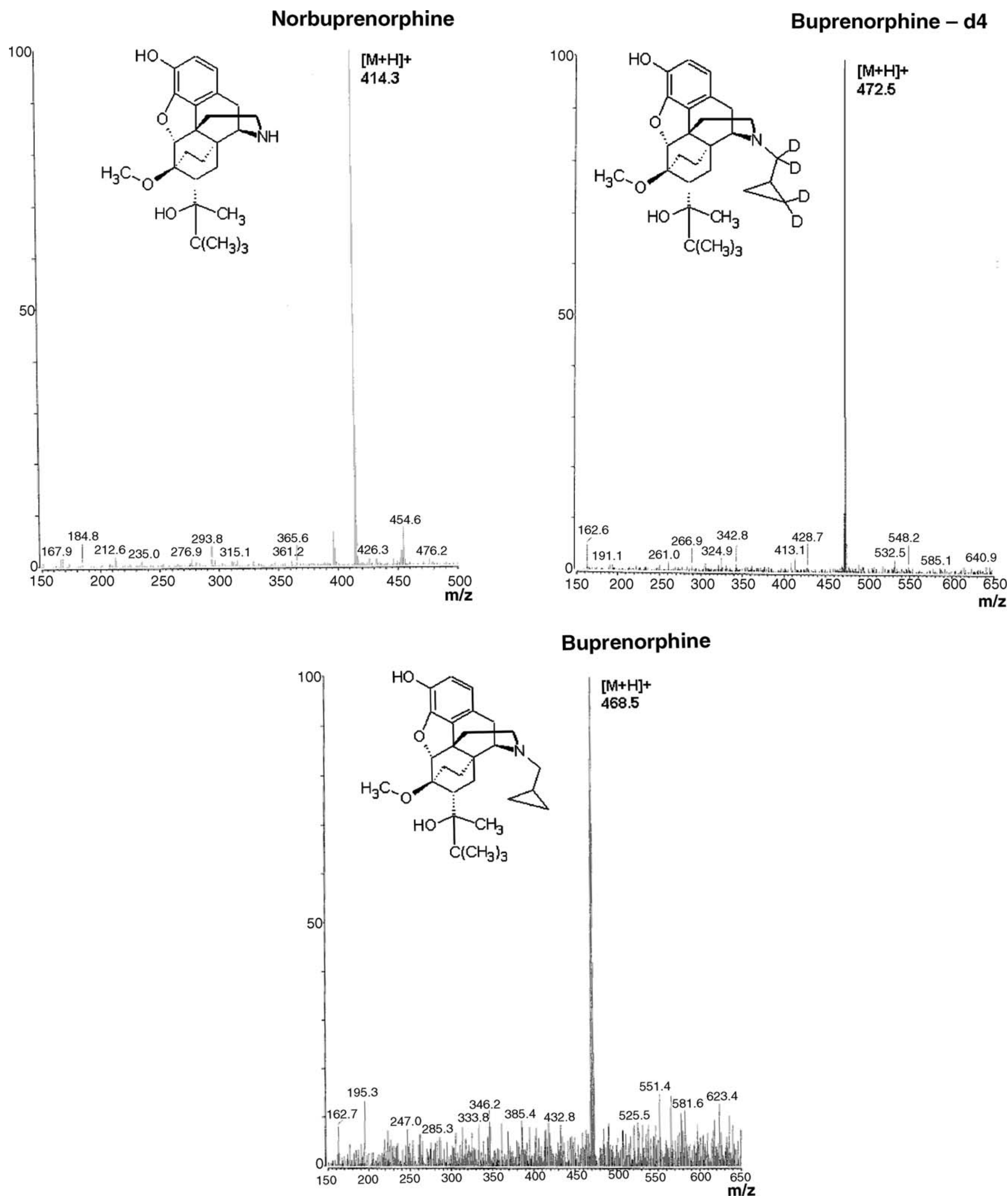

Fig. 1. Chemical structures and ESI mass spectra of NBUP, BUP-d4, BUP, OXA-d5, OXA, NDD-d5 and NDD. The concentration of each analyte was 100 ng/ml in water–methanol (50:50 v/v). In the spectra, the abscissa represents the *m/z* value and the ordinate the relative abundances of the ions.

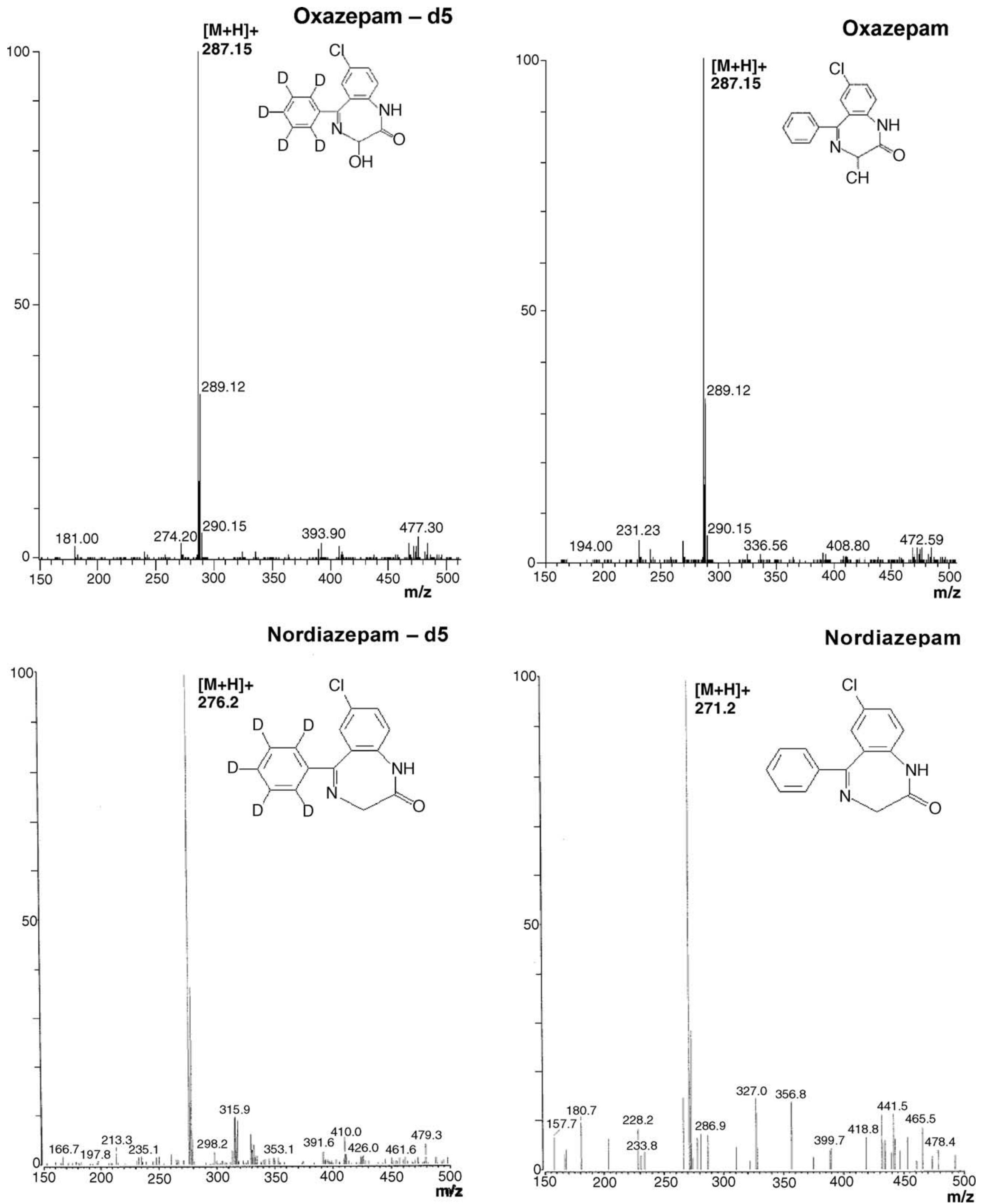

Fig. 1. (*Continued*).

Rat blank plasma samples were analyzed in order to see whether the matrix contained interfering endogenous components. Typical LC–ESI-MS chromatograms from an extract of blank plasma (100 μl) and an extract of blank plasma (100 μl) spiked with 5 ng/ml of each analyte and 0.8 μg/ml of each ISTD before extraction, are presented in Figs. 2 and 3, respectively. As shown in these figures, no endogenous source of interference was observed at the retention times of the analytes. Good chromatographic separation was achieved for all the analytes: NBUP, BUP, OXA and NDD were separated within less than 15 min

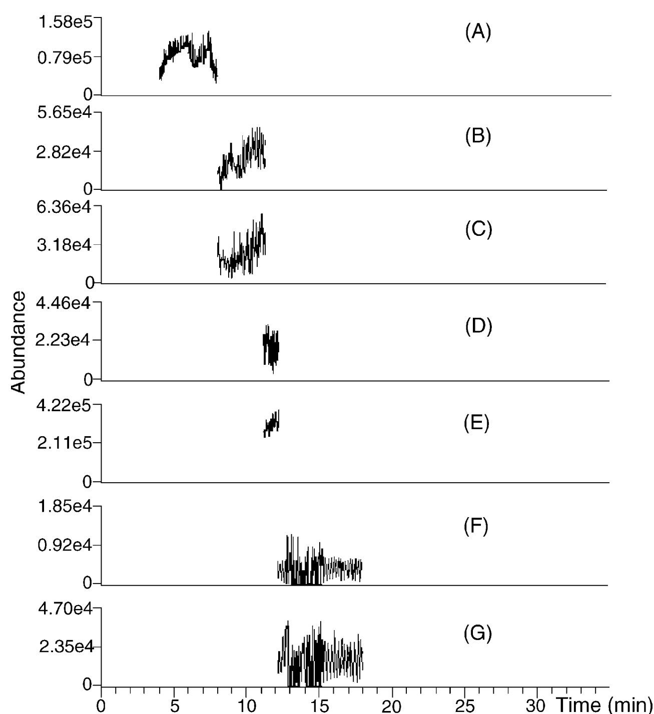

Fig. 2. LC–ESI-MS chromatograms of an extract of pooled rat blank plasma (100 μl).

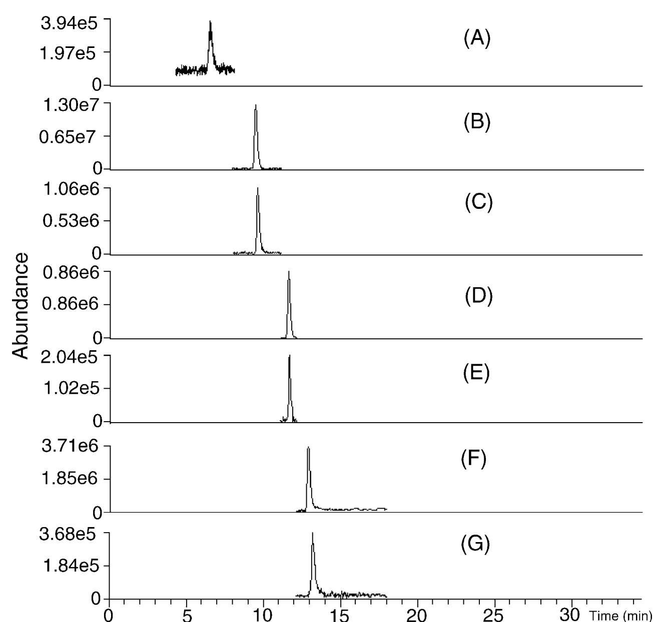

Fig. 3. LC–ESI-MS chromatograms of a blank plasma sample (100 μl) spiked with 5 ng/ml of each analyte and 0.8 μg/ml of each internal standard. The sample was extracted and analyzed as described in the text. The chromatograms for: (A) NBUP, (B) BUP-d4, (C) BUP, (D) OXA-d5, (E) OXA, (F) NDD-d5 and (G) NDD are shown. The *m/z* values of the pseudo-molecular ions of the analytes and ISTDs used for quantitation were *m/z* 414.4 for NBUP, *m/z* 472.3 for BUP-d4, *m/z* 468.3 for BUP, *m/z* 292.3 for OXA-d5, *m/z* 287.1 for OXA, *m/z* 276.2 for NDD-d5 and *m/z* 271.2 for NDD. The retention times of the analytes and ISTDs are given in the text.

and showed well defined peaks at retention times of 6.70, 9.49, 11.90 and 13.16 min, respectively (Fig. 3). Gradient elution was used to reduce retention time and avoid excessive broadening of the analytes' peaks. BUP-d4 was used as internal standard for BUP and NBUP, and NDD-d5 and OXA-d5 as internal standards for NDD and OXA, respectively. The internal standards BUP-d4, OXA-d5, and NDD-d5 had retention times of 9.45, 11.82 and 13.04 min, respectively, and were co-eluted with their non deuterated analogues (Fig. 3).

Previous authors have suggested that differences in retention time between analytes and internal standards, particularly under gradient elution, can be a source of imprecision in LC–MS or LC–MS–MS analysis, as compounds may variably respond to possible fluctuations of the parameters involved in ionization and/or collisionally activated dissociation [21]. As shown in Fig. 3, NBUP and BUP-d4 used as internal standard for NBUP have different retention times. With the aim of optimizing the conditions of the proposed assay, norbuprenorphine-d3 (NBUP-d3) was also tested as internal standard for NBUP. However, NBUP-d3 showed no major difference in terms of sensitivity and selectivity. Thus, for simplicity's sake and cost effectiveness, BUP-d4 was used for the assay of NBUP.

3.2. Validation

3.2.1. Linearity and limit of quantitation

The assay was found to be linear for all the analytes under investigation, but there was a specific linearity range for each of them. The useful range was between 0.7 and 200 ng/ml plasma for BUP, 1.0 and 200 ng/ml for NBUP, 2.0 and 200 ng/ml for NDD and between 5.0 and 200 ng/ml for OXA. The calibration slopes and coefficients of determination (r^2) of the calibration curves are given in Table 1. The calibration intercepts were not significantly different from zero ($p > 0.05$ for each analyte). The average coefficients of variation (CV%) for specific concentrations on the calibration curves of BUP and NBUP were 5.64 and 5.00%, respectively, with values ranging from 0.91 to 9.10% and from 2.06 to 8.25%. They were 6.45% for NDD (range: 5.18–9.03%) and 5.54% for OXA (range: 2.39–7.34%). The errors from theoretical values went from 3.20 to 14.03% for BUP (average error: 6.92%), from 3.16 to 10.32% for NBUP (average error: 6.71%), from 5.43 to 9.63% for NDD (average error: 7.38%) and from 6.00 to 12.09% for OXA (average error: 9.03%).

The LLOQs of BUP and NBUP were similar and slightly lower than the LLOQ of NDD; they were five-times and 2.5-times lower than the LLOQ of OXA, respectively (Table 1). The associated coefficients of variation ranged from 10.03% (NDD) to 16.38% (NBUP), and accuracies from 12.35% (BUP) to 17.28% (NBUP). The estimated limits of detection were 0.35 ng/ml plasma for BUP, 0.50 ng/ml for NBUP, 0.80 ng/ml for NDD and 1.60 ng/ml for OXA.

3.2.2. Intra-assay and inter-assay variations

The validation data concerning intra- and inter-assay variations are shown in Table 2. All the analytes have three QC samples in common, but an additional QC sample was used for

Table 1
Calibration curves and lower limits of quantitation (LLOQs) of BUP, NBUP, NDD and OXA in rat plasma

Analyte	Standard curve			Lower limit of quantitation (LLOQ)		
	Useful linearity range (ng drug/ml plasma)	Calibration slope (±S.D.)	Determination coefficient (r^2)	ng drug/ml plasma	CV (%)	Error (%)
BUP	0.7–200	1.3999 ± 0.0167	0.9915	0.7	10.93	12.35
NBUP	1.0–200	0.6182 ± 0.0044	0.9974	1.0	16.38	17.28
NDD	2.0–200	1.5297 ± 0.0201	0.9902	2.0	10.03	15.99
OXA	5.0–200	1.4474 ± 0.0183	0.9924	5.0	15.92	16.97

Standard curves were analyzed in blank plasma samples (100 μl) spiked with different concentrations of the analytes (0.7, 1.0, 2.0, 5.0, 10, 20, 50, 100 and 200 ng drug/ml plasma for all analytes). The linear regression model used for the calculation of calibration curves was $y = a \times x$, because the calibration intercepts were not significantly different from zero ($p > 0.05$ for each analyte).The useful linearity range of each specific curve is presented along with its calibration slope and determination coefficient (r^2); the calibration slopes are given with their standard deviations. The data resulted from five replicates at each concentration level analyzed on 5 different working days. The LLOQs of BUP, NBUP, NDD and OXA are shown with associated coefficients of variation (CV%) and accuracies (error%) ($n = 10$ for each analyte).

BUP, NBUP and NDD, because their standard curves fell outside of the lower limit relative to OXA. The intra-assay precision was within 6% CV for BUP, roughly 11% for NBUP, and was 7% for both NDD and OXA. Intra-assay accuracy was within 7% of the nominal concentrations for NDD, roughly 9 and 12% for BUP and NBUP, respectively, and was within 14% for OXA. The inter-assay precision did not exceed 5 and 6% CV for BUP and OXA, respectively, and was roughly 11% for both NBUP and NDD. Inter-assay accuracy was within ±15% of the nominal values for all the analytes.

3.2.3. Recoveries

The recoveries of BUP, NBUP, NDD and OXA (Table 3) were studied at the same concentrations as those used to study the intra- and inter-assay variations. Recovery was apparently not concentration dependent. The recoveries of BUP, NDD and OXA were similar and almost quantitative, with average values of 88.7, 87.9 and 82.9%, respectively, and average coefficients of variation of 5.3, 7.1 and 6.9%. In comparison, the recovery of NBUP was approximately two-fold lower, with an average value of 39.0% and an average coefficient of variation of 7.2%. Nevertheless, the validation data was acceptable for this analyte.

As already seen, BUP, NBUP, NDD and OXA were isolated from rat plasma by using a liquid–liquid extraction procedure with Toxitubes A. Clean-up procedures using Toxitubes A had already been used by the authors for the GC–MS analysis of BUP, flunitrazepam and their metabolites in rat plasma [19], and a variety of substances including benzodiazepines, in human blood and urine [37,38]. In the former study as well as in the present one, the extraction recoveries of NBUP were less than 50%, and were approximately from 1.5 to 2-fold lower than the recoveries of the other analytes. Several attempts were made to improve the recovery of NBUP. The recovery was basically the same when the rather long liquid–liquid extraction procedure of Molinaro et al. [40] was used. The use of solid-phase extraction procedures with Oasis, Bond-Elut certify

Table 2
Intra-assay and inter-assay precisions and accuracies for the LC–ESI-MS assay of BUP, NBUP, NDD and OXA in rat plasma

Analyte	Concentration (ng drug/ml plasma)	Intra-assay		Inter-assay	
		Precision (CV%)	Accuracy (Error%)	Precision (CV%)	Accuracy (Error%)
BUP	5	4.14	9.15	3.84	12.95
	20	5.21	7.52	4.97	11.13
	100	3.26	6.44	4.13	7.42
	200	1.80	1.72	1.61	1.48
NBUP	5	11.26	12.12	9.50	14.71
	20	4.99	4.75	11.26	10.60
	100	6.10	7.39	4.51	4.30
	200	5.39	8.96	4.73	6.47
NDD	5	7.27	6.93	11.09	10.99
	20	4.39	4.21	8.72	11.93
	100	5.86	6.86	7.28	14.84
	200	3.28	3.32	9.41	9.27
OXA	20	7.16	13.64	3.72	13.18
	100	3.88	12.28	3.02	2.89
	200	7.01	10.00	5.60	6.89

Accuracy and precision were analyzed at three different concentrations for all the analytes (20, 100 and 200 ng drug/ml plasma). An additional concentration was analyzed for BUP, NBUP and NDD (5 ng drug/ml plasma), because the specific standard curves of these compounds fell outside of the lower limit relative to the standard curve of OXA. The number of replicates for each evaluated concentration of the analytes was 10.

Table 3
Analytical recoveries of BUP, NBUP, NDD and OXA from rat plasma

Analyte	Theoretical concentrations (ng drug/ml plasma)	Recovered concentrations (ng drug/ml plasma) (mean ± S.D.)	CV (%)	Recovery (%)
BUP	5	3.9 ± 0.5	12.40	77.5
	20	19.1 ± 0.3	1.77	95.5
	100	86.3 ± 2.5	2.89	86.3
	200	191.1 ± 7.8	4.08	95.5
NBUP	5	2.0 ± 0.2	11.42	40.7
	20	9.3 ± 0.5	5.62	46.6
	100	33.1 ± 1.0	3.11	33.1
	200	70.7 ± 6.0	8.54	35.4
NDD	5	4.2 ± 0.3	7.10	83.6
	20	17.5 ± 1.6	9.17	87.4
	100	87.3 ± 6.1	7.03	87.3
	200	186.6 ± 9.4	5.01	93.3
OXA	20	16.5 ± 2.4	14.29	82.7
	100	74.7 ± 3.0	4.05	74.7
	200	176.8 ± 6.0	3.41	88.4

Recoveries were determined at three different concentrations for all the analytes (20, 100 and 200 ng drug/ml plasma). An additional concentration was analyzed for BUP, NBUP and NDD (5 ng drug/ml plasma), because the specific standard curves of these compounds fell outside of the lower limit relative to the standard curve of OXA. The number of replicates for each evaluated concentration was five.

or Extrelut-3 extraction columns gave rise to higher analytical recoveries for NBUP, but also to an overall loss of reproducibility for all analytes (data not shown). Finally it was considered that the extraction of BUP, NBUP, OXA and NDD using Toxitubes A was a good compromise between recovery of analytes and reproducibility of extraction. Furthermore, the rapidity and simplicity of this extraction procedure make it suitable for applications involving multiple sample analysis, such as kinetic studies.

3.3. Determination of BUP, NDD and their metabolites in rat plasma samples

In this study, the doses of BUP and NDD given to the rats were chosen in order to mimic situations frequently observed among addicts where poisonings or deaths appeared to be related to the concomitant use of a high dose of BUP and a low dose of benzodiazepine [4,8]. The high dose of BUP given to the rats was similar to that used by Gopal et al. [41] to characterize the pharmacokinetics of BUP and NBUP after i.v. bolus administration of BUP.

The plasma concentration–time data of BUP, NDD and their metabolites obtained in rats ($n = 5$) following intravenous perfusion of a 30 mg/kg dose of BUP over 3 min and then, a dose of 10 mg/kg NDD over 30 min are given in Table 4. Examples of the chromatograms obtained for the LC–ESI-MS analysis of plasma samples collected at 20 min post-perfusion of NDD are illustrated in Fig. 4. While the concentrations of NBUP and OXA could be directly measured in the tested specimen, the concentrations of the parent drugs, BUP and NDD, were all above the upper limits of quantitation. Therefore, the levels of BUP and NDD were determined in a second set of analyses, using 1:10 and 1:50 dilutions of the plasma specimen. The dilution factors were chosen according to the LC–MS analysis of non-diluted samples and preliminary kinetic studies. The dilutions were prepared with blank plasma in order to avoid matrix effects, and 100 μl-aliquot of the dilutions were extracted and analyzed as described above. The concentrations of BUP and NDD in the dilutions all fell within the lower and upper limits of quantitation on the corresponding standard curves.

BUP had a mean maximum plasma concentration at the first sampling time (−30 min, ∼11.6 μg/ml). Its concentration then declined rapidly: it represented only 13% of the maximum concentration 30 min after the perfusion of BUP (i.e. time 0 in Table 4), and then remained nearly constant at the other times (Table 4). NBUP was formed very rapidly after BUP administration. The peak concentration of NBUP was also observed at −30 min (103 ng/ml). Thereafter, its concentration decreased slowly and was approximately half of its peak concentration at the other sampling times. It is noticeable that, while the concentrations of NBUP were much lower than those of BUP, the

Table 4
Plasma concentration-time data of BUP, NBUP, NDD and OXA in normal adult rats after intravenous perfusion of BUP and NDD

Sampling time (min)	Plasma drug concentration (ng/ml)			
	BUP	NBUP	NDD	OXA
−30	11623 ± 5607	103 ± 34	–	–
0	1497 ± 492	45 ± 27	1 561 ± 464	29 ± 18
5	1519 ± 231	50 ± 15	1 370 ± 397	42 ± 16
20	1628 ± 444	61 ± 27	1 002 ± 350	30 ± 12
60	976 ± 251	51 ± 21	558 ± 293	32 ± 21

The intravenous perfusion of BUP (30 mg/kg) was performed over 3 min and was followed by intravenous perfusion of NDD (10 mg/kg) over 30 min. The drug concentrations at different sampling times after BUP perfusion (−30 min) and after NDD perfusion (0, 5, 20 and 60 min) were all determined from analysis of 100 μl plasma samples. For the determination of BUP and NDD, the samples were diluted 1:10 or 1:50 with pooled blank plasma, because the plasma concentrations of these drugs fell outside of the upper limits of quantitation of the standard curves. Each drug concentration represents the mean of the five values determined in the rats, and is given with its standard deviation.

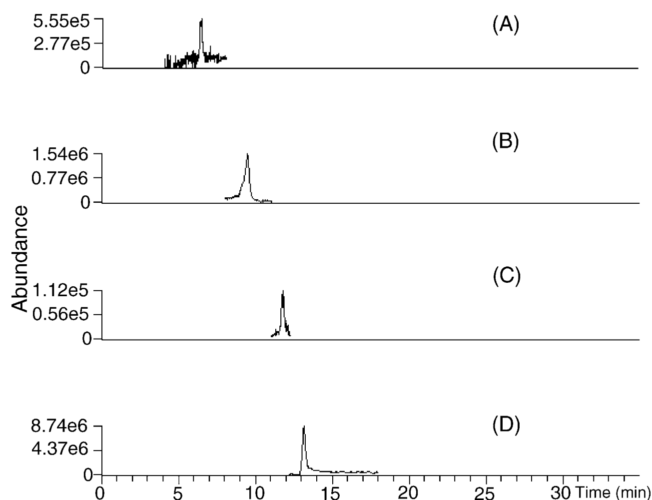

Fig. 4. LC–ESI-MS chromatograms of an extract of real plasma sample (100 μl) collected after intravenous perfusion of BUP and NDD to rats: (A) NBUP, (B) BUP, (C) OXA and (D) NDD. The chromatograms were all obtained from the analysis of a plasma sample taken from one animal at 20 min post-perfusion of NDD.

concentration–time profile of NBUP mirrored that of its parent drug (Table 4). Very different results were obtained for NDD and its metabolite, OXA. The NDD plasma level reached a maximum at time 0 post-perfusion (∼1.6 μg/ml plasma). From time 0 to 60 min, NDD declined slowly and, at 60 min, its concentration still represented more than 35% of its peak concentration. OXA was formed rapidly after intravenous administration of NDD, but its levels were very low in all samples (Table 4). In comparison to NDD, the peak concentration of OXA was slightly shifted and observed at 5 min post-perfusion. At further sampling times the concentration–time profile of OXA mimicked that of NDD, though the plasma levels of OXA represented less than 6% of those of NDD.

4. Conclusion

The described LC–ESI-MS method enables the simultaneous determination of BUP, NDD and their respective metabolites, NBUP and OXA, in rat plasma. Analytes were extracted by liquid–liquid extraction and separated by chromatography on a X-terra C18 column. Their quantitation was performed in the SIS mode, with deuterated analogs as internal standards. The method was shown to be linear and satisfactorily met current acceptance criteria for bioanalytical method validation: intra- and inter assay precisions within the required limits of ≤15% CV and accuracies within the acceptance interval of ±15% of the nominal values [39]. The LLOQs fulfilled the LLOQ requirements: precision ≤20% CV and accuracy within ±20% of the nominal values. Both the relatively simple and rapid sample preparation and acceptable HPLC run time make the method suitable for multiple sample analysis. The method was applied to plasma analysis of the tested drugs for assessment of their kinetics in rats following treatment with a high dose of BUP and a comparatively low dose of NDD.

Because of its sensitivity in determining the active metabolites of these drugs, this method should allow its application in kinetic studies using smaller volumes of plasma, and thus a larger number of blood sampling in the animals, and also the use of lower doses of BUP and NDD for treatment. The evaluation of the metabolic profile of a drug in biological samples, as well as the parent drug-to-metabolite ratios, are of great importance in interpretative toxicology. In this respect, this method should be useful to explore the toxicity mechanisms of the BUP–NDD association, as well as the pharmacological and metabolic interactions between these drugs.

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