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Development and validation of a gas chromatography–mass spectrometry method for the simultaneous determination of buprenorphine, flunitrazepam and their metabolites in rat plasma: application to the pharmacokinetic study

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Abstract

Buprenorphine (BUP), a synthetic opioid analgesic, is frequently abused alone, and in association with benzodiazepines. Fatalities involving buprenorphine alone seem very unusual while its association with benzodiazepines, such as flunitrazepam (FNZ), has been reported to result in severe respiratory depression and death. The quantitative relationship between these drugs remain, however, uncertain. Our objective was to develop an analytical method that could be used as a means to study and explore, in animals, the toxicity and pharmacological interaction mechanisms between buprenorphine, flunitrazepam and their active metabolites. A procedure based on gas chromatography–mass spectrometry (GC–MS) is described for the simultaneous analysis of buprenorphine, norbuprenorphine (NBUP), flunitrazepam, *N*-desmethylflunitrazepam (*N*-DMFNZ) and 7-aminoflunitrazepam (7-AFNZ) in rat plasma. The method was set up and adapted for the analysis of small plasma samples taken from rats. Plasma samples were extracted by liquid–liquid extraction using Toxi-tubes A. Extracted compounds were derivatized with *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), using trimethylchlorosilane (TMCS) as a catalyst. They were then separated by GC on a crosslinked 5% phenyl-methylpolysiloxane analytical column and determined by a quadrupole mass spectrometer detector operated under selected ion monitoring mode. Excellent linearity was found between 0.125 and 25 ng/μl plasma for BUP, 0.125 and 12.5 ng/μl for NBUP and *N*-DMFNZ, 0.125 and 5 ng/μl for FNZ, and between 0.025 and 50 ng/μl for 7-AFNZ. The limit of quantification was 0.025 ng/μl plasma for 7-AFNZ and 0.125 ng/μl for the four other compounds. A good reproducibility (intra-assay CV = 0.32–11.69%; inter-assay CV = 0.63–9.55%) and accuracy (intra-assay error = 2.58–12.73%; inter-assay error = 0.83–11.07%) were attained. Recoveries were 71, 67 and 81%, for BUP, FNZ and *N*-DMFNZ, respectively, and 51% for NBUP and 7-AFNZ, with CV ranging from 5.4 to 13.9%, and were concentration-independent. The GC–MS method was successfully applied to the pharmacokinetic study of BUP, NBUP, FNZ, DMFNZ and 7-AFNZ in rats, after administration of BUP and FNZ.

Keywords: Derivatization, GC; Pharmacokinetics; Buprenorphine; Flunitrazepam

1. Introduction

Buprenorphine (BUP), a semi-synthetic opioid derivative, is a powerful analgesic, 25–40 times more potent than morphine. Buprenorphine at low dosages (typically 0.3–0.6 mg intravenous or intramuscular) is widely prescribed for the treatment of moderate to severe pain, and also for premedication in anaesthesiology [1]. Additionally, buprenorphine has been recognized as an effective medication for the substitutive maintenance in opioid dependent-patients [2–4]. A high dosage formulation of buprenorphine (0.4, 2 and 8 mg tablets for sublingual use) is available in this specific

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medication in France since 1996. High-dose buprenorphine has been reported to substantially decrease heroin self-administration [2,5]. The limited respiratory effects of high-dose buprenorphine is of utmost importance regarding the safety of this drug for use in substitution treatment. However, numerous buprenorphine-related deaths have been reported by forensic toxicologists and other sources of information (e.g. intensive care units) since 1996. These fatalities may result from misuse (intravenous injection of crushed tablets) or overdose with substitution treatment [6,7], or a concomitant intake of psychotropics, mostly benzodiazepines [8,9]. Benzodiazepines are extensively prescribed to patients with insomnia in many countries, and are considered as relatively safe drugs since deaths involving benzodiazepines solely, in the absence of other pathologies, are very uncommon [10,11]. Some benzodiazepines, such as flunitrazepam (FNZ), nordiazepam and diazepam, have become popular among heroin addicts. The association of these drugs with substitution products (buprenorphine or methadone) has been found in many fatal intoxications [9,10,12]. Experimental studies further suggest that the combination of opioids and benzodiazepines is a major risk factor for lethal outcomes. Severe respiratory depression has been observed in rats when flunitrazepam is administered concurrently and/or acutely with opioids, as assessed by arterial blood gas measurements [13]. Furthermore, studying the acute toxicity of various combinations of opioids and flunitrazepam in rats, some of us observed that lethality was significantly increased in buprenorphine-treated rats relative to rats treated with methadone or morphine: there was both a large decrease in the median lethal dose of buprenorphine and time to death [14].

The toxicity mechanisms of the buprenorphine–flunitrazepam association as well as the pharmacological and metabolic interactions between these drugs are poorly understood, calling for further investigations. The availability of diagnostic means of study and exploration in the animal is, however, a prerequisite for such work. Various analytical methods, including gas chromatography–mass spectrometry (GC–MS) assay methods, have been described for the analysis of buprenorphine [15–28], of flunitrazepam [29–32] and their major metabolites. However and until now, there had been no reported method for the simultaneous determination of these drugs.

Here, we describe a GC–MS analytical method which allows the simultaneous determination of buprenorphine and its major metabolite, norbuprenorphine (NBUP), as well as flunitrazepam and its active metabolites, *N*-desmethylflunitrazepam (*N*-DMFNZ) and 7-aminoflunitrazepam (7-AFNZ), in rat plasma. The assay method validation is also presented. We used previous studies from our laboratory [33,34] as a starting point for the extraction, derivatization and quadrupole MS analysis of the compounds under study. The method was set up and adapted for the analysis of the small plasma samples taken from rats. Its development then allowed us to explore the pharmacokinetics of these five drugs after administration of buprenorphine and flunitrazepam.

2. Experimental

2.1. Chemicals and reagents

Stock solutions of buprenorphine, norbuprenorphine, flunitrazepam, N-desmethylflunitrazepam and 7-aminoflunitrazepam, and of buprenorphine-d4 (BUP-d4) and *N*-desmethylflunitrazepam-d4 (*N*-DMFNZ-d4) were supplied by Cerilliant (Austin, TX, USA). Toxi-tubes A extraction cartridges were obtained from Toxi-Lab Ansys Diagnostic (Lake Forest, CA, USA). A mixture of 99% *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), acetonitrile HPLC-grade, as well as all other reagent grade chemicals, were purchased from Merck (Darmstadt, Germany). Water was doubly deionized to >18.2 MΩ with a Milli-Q ultrapure water system (Millipore Corp., Woburn, MA, USA).

2.2. Standards and solutions

Of all the aforementioned stock solutions, only FNZ was 1 mg/ml in acetonitrile while BUP, NBUP, *N*-DMFNZ, 7-AFNZ and internal standards were each 100 μg/ml in acetonitrile. The stock solutions were all stored at −20 °C. A combined stock solution of the drugs was then prepared from each of the BUP, NBUP, FNZ, *N*-DMFNZ and 7-AFNZ solutions, in order to give final concentrations of 10 μg/ml of acetonitrile for each drug. Two dilutions of the combined stock solution were prepared in acetonitrile at concentrations of 1 and 0.01 μg/ml for each drug. The combined stock solution and its dilutions were used as needed to prepare the calibration curves and quality control (QC) samples. These solutions were stored at −20 °C between experiments. The stock solutions of internal standards (ISTDs) (BUP-d4 and *N*-DMFNZ-d4) were diluted with acetonitrile to give working solutions at concentrations of 1 μg/ml and were stored at –20 °C.

2.3. Animals and plasma sample collection

Male Sprague–Dawley rats (OFA strain; 250–300 g; 8–10 weeks) were obtained from Iffa-Credo (L'Arbresle, France). Animals were maintained under standard conditions of temperature and lighting for 8 days with ad libitum access to food and water. There were two sample sets of animals. The first set was used to prepare blank plasma samples, and the second for the pharmacokinetic studies. Rats were anaesthetized with an intraperitoneal injection of the combination of ketamine hydrochloride (Panpharma, Fougères, France; 70 mg/kg) and xylazine (Bayer, Puteaux, France; 10 mg/kg). The ethical rules of the French Ministry of Agriculture for experimentation with laboratory animals (law no. 87–848) were followed.

Drug-free rat plasma samples were obtained as follows: blood was taken from anaesthetized animals by carotid bleeding and collected into (heparinized) borosilicate tubes. After centrifugation for 10 min at 2000 × *g* and at 5 °C, the harvested plasma samples were mixed to obtain a homogeneous pool of blank plasma which was stored at −20 °C until use.

On the day before the treatment with BUP and FNZ, the second sample set of animals was anaesthetized 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 o.d.; Dow Corning, Midland, MI, USA). The catheters were then tunneled subcutaneously and fixed at the back of the neck [35]. The rats were given at least a 24 h recovery period to allow for washout of anaesthesia. On the study day, rats were placed in a restraining chamber. They received a 30 mg/kg dose of BUP (buprenorphine hydrochloride, Schering-Plough, Levallois-Perret, France), in a volume of 1.3 ml, by intravenous injection via femoral vein cannulation. The injection was performed over 3 min and at a constant rate of 433 μl/min, using a perfusion pump (PHD 2000; Harvard Instruments, Holliston, MA, USA). Immediately after, the rats were given a dose of 40 mg/kg FNZ (Hoffmann-LaRoche, Neuilly-sur-Seine, France), also in a volume of 1.3 ml, by intravenous perfusion over 30 min and at a rate of 43.3 μl/min. Drug solutions were freshly prepared: BUP, 18.2 mg/ml, was prepared in a mixture of sterile water and ethanol (8.5% (v/v)) adjusted to pH 5.2 with HCl 0.1 M [35]; FNZ, 10.0 mg/ml, was prepared in a mixture of sterile water and Tween-80 (20% (v/v)) [14]. Prior to injection, the solutions were diluted with sterile water to adjust the doses of BUP and FNZ to the weight of each rat. The pharmacokinetic study involved serial arterial blood sampling (∼100 μl) with 11 samples obtained from each animal at the following time points: after BUP perfusion (−30 min), during FNZ perfusion (−25, −20, −15 and −10 min) and after FNZ perfusion (0, 5, 15, 60, 120 and 180 min). The blood samples were transferred to microtubes containing 5 μl heparin and then centrifuged at 2000 × *g* for 10 min and at 5 °C. The plasma (∼50 μl) was separated and frozen immediately at −20 °C until further analysis. No major problems were encountered during catheterization, drug administration or collection of arterial blood samples.

2.4. Plasma sample preparation and extraction

This involved a clean-up procedure using Toxi-tubes A, followed by derivatization with *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide, according to previously reported methods [33,34]. The Toxi-tubes A contain a mixture of dichloromethane, 1,2-dichloroethane, heptane and isopropranol, and are used for the liquid–liquid extraction of neutral and basic drugs and unconjugated metabolites. Extraction was performed from 40 μl blank plasma samples spiked with analytes under investigation, or from 40 μl plasma samples from BUP/FNZ-treated rats. The volumes of combined stock solutions of the analytes which were added to do the spiking were between 5 and 100 μl The samples were also spiked with 80 μl of 1 μg/ml BUP-d4 and *N*-DMFNZ-d4, as internal standards. The volume of samples was adjusted to 1.0 ml with deionized water. After mixing, the samples were transferred into Toxi-tubes A which had been previously added with 2 ml of deionized water, in order to keep the sample volume in the tube between 2 and 5 ml, as recommended by the manufacturer. The loaded Toxi-tubes were automatically agitated for 5 min before being centrifuged at 100 × *g* for 5 min. These various steps were performed at ambient temperature. Following centrifugation, the organic phase was transferred to a clean tube and evaporated to dryness under a N_2 stream at 25 °C. Before being derivatized, the residue was heated to 80 °C for 5 min, in order to remove any traces of water.

Trimethylsilylated derivatives were formed by reaction of the dry residues with 40 μl of the mixture of BSTFA–TMCS (99:1) at 80 °C for 20 min. TMCS is a catalyst that increases the silylating power of BSTFA used as derivatization agent [36]. The derivatized samples were then allowed to cool down to ambient temperature prior to GC–MS analysis. FNZ cannot be silylated since it does not contain an exchangeable hydrogen [33].

2.5. GC–MS conditions and instrumentation

The GC–MS system consisted of a Hewlett-Packard 6890 series auto sampler injector and gas chromatograph which was coupled to an HP 5973 quadrupole mass spectrometer detector (Agilent, Massy, France).

Samples (1 μl) were automatically injected in splitless mode at a rate of 5 μl/s into a 30 m × 0.25 mm i.d., 0.25 μm film thickness, crosslinked 5% phenyl-methylpolysiloxane column (Hewlett-Packard-5 MS). The split vent was opened 1.5 min following the injection. The oven temperature was initially maintained at 220 °C for 1 min and programmed to 300 °C at 15 °C/min, where it was held constant for 9 min. The injector and the transfer line were operated at 300 and 290 °C, respectively. Helium was used as carrier gas at a flow rate of 1.0 ml/min.

The source and electrodes of the quadrupole mass filter were both set to 250 °C. Ionization was carried out in electron impact ionization (EI) mode at 70 eV. Detection was operated under selected ion monitoring (SIM) mode. Three qualifying ions were selected for analytes under investigation, which were: *m*/*z* 285.1, 286.1, 312.1 for FNZ; *m*/*z* 326.1, 327.1, 355.2 for 7-AFNZ; *m*/*z* 352.1, 370.1, 371.1 for *N*-DMFNZ; *m*/*z* 450.2, 451.2, 482.3 for BUP; *m*/*z* 468.2, 500.3, 510.2 for NBUP; *m*/*z* 356.1, 373.1, 375.1 for *N*-DMFNZ-d4; *m*/*z* 454.3, 455.3, 486.3 for BUP-d4. The ions: *m*/*z* 285.1 for FNZ; *m*/*z* 355.2 for 7-AFNZ; *m*/*z* 370.1 for *N*-DMFNZ; *m*/*z* 450.2 for BUP; *m*/*z* 468.2 for NBUP; *m*/*z* 375.1 for *N*-DMFNZ-d4; *m*/*z* 454.3 for BUP-d4, were the most abundant and used for quantification.

2.6. Method validation

Calibration standards and QC samples were prepared from working solutions of stock sources on each validation day.

Linearity was studied by analyzing blank plasma samples (40 μl) spiked with different concentrations of the drugs: 0.025, 0.125, 0.25, 0.50, 1.25, 2.5, 5, 12.5 and 25 ng/μl plasma for all compounds. BUP-d4 (2 ng/μl of plasma) was used as internal standard for BUP and NBUP and *N*-DMFNZ-d4 (2 ng/μl of plasma) for FNZ, *N*-DMFNZ and 7-AFNZ. Samples were extracted and analyzed as described in Sections 2.4 and 2.5. Response (peak area) ratios between compounds and internal standards were used for calculations. Regression analysis for a linear model was used for the calculation of all calibration curves. Linearity was determined by checking five calibration curves on five different working days. An analysis of variance was carried out on the factors day and peak area ratio as recorded from the mass spectrometer.

The QC samples for intra-assay variation, inter-assay variation and quantification limit (LOQ) were prepared by spiking 40 μl aliquots of blank plasma with 80 ng of ISTDs and the tested concentrations of the analytes, followed by extraction and analysis. Two groups of 10 replicates of blank samples spiked with 0.025 and 0.125 ng/μl of the analytes were used to determine the LOQ. Three replicates at five different concentrations of the drugs (0.125, 0.5, 2.5, 5 and 25 ng/μl plasma) spiked in blank plasma were used for the determination of intra-assay precision and accuracy. Inter-assay precision and accuracy were determined on five different experimental days. Precision is expressed as coefficient of variation (CV (%)) for specific added target concentrations, and accuracy as percentage error (error%) of concentration found as compared with target added concentrations.

Extraction recoveries were analyzed at two different concentrations of the drugs, 0.5 and 2.5 ng/μl plasma, using six replicates for each evaluated concentration. The QC samples for recovery were prepared and extracted as described above, except that the internal standards (80 ng of BUP-d4 and *N*-DMFNZ-d4) were added to the collected extract from Toxi-tubes A cartridge before evaporation of the organic phase. In parallel, a set of samples were prepared by adding the same amounts of reference substances and ISTDs to acetonitrile (1.0 ml). After evaporation to dryness, a 40 μl volume of the mixture of BSTFA–TMCS (99:1) was added to the residue, and derivatization was proceeded as described under Section 2.4. Following analysis, recoveries were calculated by comparison between the concentrations in the spiked plasma samples and those determined in the samples prepared in acetonitrile.

2.7. Quantification of the analytes in plasma specimens from rats treated with BUP and FNZ

The plasma levels of BUP, NBUP, FNZ, *N*-DMFNZ and 7-AFNZ in treated rats 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 the calibration curves prepared as described above. QC samples (0.5 and 2.5 ng drug/μl plasma) were included in each analytical batch to check calibration, accuracy and precision.

3. Results and discussion

3.1. Analytical method

Fig. 1 displays the GC–MS-SIM fragmentograms from an extract of a blank plasma (40 μl) (Fig. 1A) and an extract of a blank plasma (40 μl) fortified with 0.25 ng of each analyte per μl plasma (Fig. 1B). As shown in Figs. 1A and 2A, the blank plasma extract was clean with no significant interfering peaks. All the analytes showed sharp and/or well defined peaks (Fig. 1B) at the retention times of 5.90, 6.72 and 7.15 min for *N*-DMFNZ, FNZ and 7-AFNZ, respectively, and 11.20 and 14.00 min for NBUP and BUP. The relatively short retention times of the two latter drugs enabled a chromatographic run time of 15.3 min, making it possible to analyze up to 60 samples per day including those used for the standard curves and quality controls. The internal standards *N*-DMFNZ-d4 and BUP-d4 had retention times of 5.87 and 13.95 min, respectively and were co-eluted with their non deuterated analogues. Resolution of *N*-DMFNZ and *N*-DMFNZ-d4, and of BUP and BUP-d4 was possible under selected ion monitoring mode: the respective *m/z* ions of the deuterated and non deuterated compounds were recorded on separate channels so that they could be separately visualized and integrated. Other internal standards than *N*-DMFNZ-d4 and BUP-d4 were tested in preliminary experiments: flunitrazepam-d7 for the FNZ/*N*-DMFNZ/7-AFNZ assay, and norbuprenorphine-d3 for the BUP/NBUP assay. We finally chose *N*-DMFNZ-d4 and BUP-d4 because of their higher sensitivity.

3.2. Method validation

Data on method validation are reported in Tables 1–3. Table 1 shows that there is a specific linearity range for each analyte. Of the nine concentrations tested to establish the calibrations curves, the useful range is between 0.125 and 25 ng/μl plasma for BUP, 0.125 and 12.5 ng/μl for NBUP and *N*-DMFNZ, 0.125 and 5 ng/μl for FNZ and between 0.025 and 5 ng/μl for 7-AFNZ. The coefficients of correlation (r^2) of the standard curves stem from 0.985 to 0.999 (Table 1). The regression equations for the analytes are given in Table 1. Using an analysis of variance, we found that the day had no significant effect on MS response ratio between analyte and ISTD. The average coefficients of variation for specific concentrations on the standard curves of BUP and NBUP were 0.91 and 2.17%, respectively, with values ranging from 0.63 to 1.32% and from 0.45 to 3.83%.

Table 1
Calibration curves and limits of quantification of BUP, NBUP, FNZ, *N*-DMFNZ and 7-AFNZ in rat plasma

Analyte	Standard curve			Limit of quantification (LOQ)		
	Useful linearity range (ng drug/μl plasma)	Regression equation	r^2	ng drug/μl plasma	CV (%)	Error (%)
BUP	0.125–25	$y = 1.516x - 0.751$	0.9998	0.125	3.85	10.09
NBUP	0.125–12.5	$y = 0.542x - 0.893$	0.9996	0.125	3.63	15.85
FNZ	0.125–5	$y = 0.571x + 0.721$	0.9996	0.125	13.45	14.37
N-DMFNZ	0.125–12.5	$y = 1.498x - 0.505$	0.9974	0.125	11.76	11.22
7-AFNZ	0.025–5	$y = 2.589x - 0.860$	0.9847	0.025	10.27	14.54

Standard curves were analyzed in blank plasma samples (40 μl) spiked with different concentrations of the analytes (0.025, 0.125, 0.25, 0.50, 1.25, 2.5, 5, 12.5 and 25 ng drug/μl plasma for all analytes). The useful linearity range of each specific curve is presented along with its regression equation and correlation coefficient (r^2). The data result from five replicates analyzed on five different working days. The limits of quantification of BUP, NBUP, FNZ, *N*-DMFNZ and 7-AFNZ are shown with associated accuracies (error%) and coefficients of variation (CV (%)) ($n = 10$ for each analyte).

Table 2
Intra-assay ($n = 3$) and inter-assay ($n = 5$) precision and accuracy calculated for the determination of BUP, NBUP, FNZ, *N*-DMFNZ and 7-AFNZ in rat plasma

Analyte	Concentration (ng drug/μl plasma)	Intra-assay		Inter-assay	
		Precision (CV (%))	Accuracy (error%)	Precision (CV (%))	Accuracy (error%)
BUP	0.5	1.00	6.16	0.64	1.58
	2.5	6.79	10.29	0.63	0.83
	5.0	3.11	3.04	7.55	7.38
	25.0	3.55	5.67	1.24	1.15
NBUP	0.5	5.21	7.59	3.80	8.54
	2.5	10.00	10.43	8.51	8.41
	5.0	6.95	6.21	1.14	1.83
FNZ	0.5	4.69	12.34	6.45	5.96
	2.5	3.99	5.63	6.67	6.18
	5.0	4.45	5.96	9.55	9.06
N-DMFNZ	0.5	7.70	6.95	2.81	2.74
	2.5	0.32	2.58	4.29	3.84
	5.0	4.06	3.79	9.38	9.42
7-AFNZ	0.125	10.43	12.73	8.65	10.59
	0.5	5.33	11.93	3.75	5.35
	2.5	8.65	9.61	3.55	11.07
	5.0	11.69	12.16	5.91	6.80

Accuracy and precision were analyzed at three different concentrations for all the analytes (0.5, 2.5 and 5 ng drug/μl plasma). An additional concentration was analyzed for 7-AFNZ (0.125 ng drug/μl plasma) and BUP (25 ng drug/μl plasma), because the specific standard curves of these compounds fell outside of the upper (BUP) or lower (7-AFNZ) limit relative to the standard curves of the other analytes.

Table 3
Analytical recoveries at two different concentrations (0.5 and 2.5 ng/μl plasma) of BUP, NBUP FNZ, *N*-DMFNZ and 7-AFNZ from rat plasma ($n = 6$ for each evaluated concentration)

Analyte	Theoretical amounts (ng drug in 40 μl plasma)	Recovered amounts (ng drug in 40 μl plasma) (mean ± S.D.)	CV (%)	Recovery (%)
BUP	20	13.9 ± 0.9	6.3	69.5
	100	71.8 ± 9.9	13.9	71.8
NBUP	20	9.8 ± 1.0	10.4	49.1
	100	52.3 ± 5.0	9.6	52.3
FNZ	20	13.6 ± 0.9	6.3	68.2
	100	64.9 ± 3.6	5.5	64.9
N-DMFNZ	20	15.8 ± 2.1	13.2	79.0
	100	82.3 ± 13.8	9.5	82.3
7-AFNZ	20	10.5 ± 0.6	5.4	52.6
	100	48.3 ± 4.4	9.2	48.3

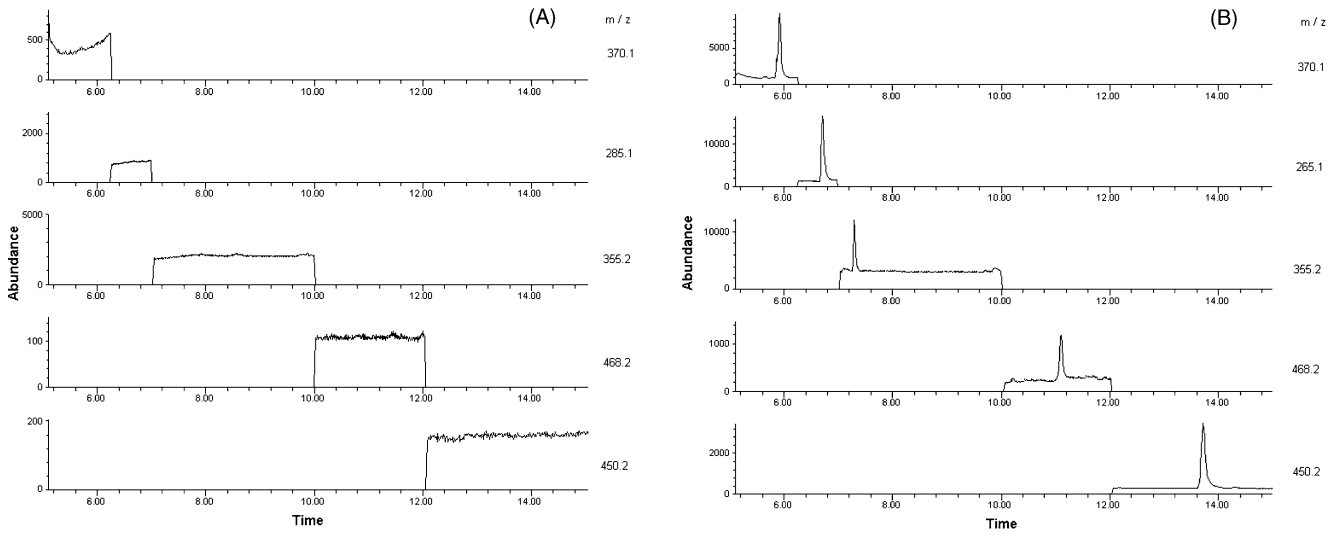

Fig. 1. GC–MS-SIM fragmentograms of BSTFA-derivatized extracts from (A) rat blank plasma (40 μl) and (B) rat blank plasma (40 μl) spiked with 0.25 ng/μl BUP, NBUP, FNZ, *N*-DMFNZ and 7-AFNZ, respectively, and 2 ng/μl BUP-d4 and *N*-DMFNZ-d4. The analytes that were silylated are those including a protic functional group (BUP, NBUP, *N*-DMFNZ and 7-AFNZ). (1) *N*-DMFNZ, *m*/*z* 370.1; (2) FNZ, *m*/*z* 285.1; (3) 7-AFNZ, *m*/*z* 355.2; (4) NBUP, *m*/*z* 468.2; (5) BUP, *m*/*z* 450.2. The retention times of the analytes and ISTDs are given in the text. *N*-DMFNZ-d4 (*m*/*z* 375.1) and BUP-d4 (*m*/*z* 454.3) were co-eluted with the corresponding non-deuterated analytes.

They were 7.06% for FNZ (range: 4.04–9.62%), 3.54% for *N*-DMFNZ (range: 0.93–9.17%) and 5.48% for 7-AFNZ (range: 2.42–8.80%). The errors from theoretical value go from 0.95 to 10.92% for BUP (average error: 3.62%), from 0.63 to 9.86% for NBUP (average error: 4.58%), from 6.02 to 15.27% for FNZ (average error: 8.93%), from 2.76 to 13.52% for *N*-DMFNZ (average error: 7.36%), and from 3.77 to 11.54% for 7-AFNZ (average error: 8.11%).

The limit of quantification of each analyte is presented in Table 1 with associated accuracy and coefficient of variation ($n = 10$). The limit of quantification of 7-AFNZ was five-fold lower than that of BUP, NBUP, FNZ and *N*-DMFNZ, respectively. The coefficients of variation were between 3.63% (NBUP) and 13.45% (FNZ), and accuracies between 10.09% (BUP) and 15.85% (NBUP) (Table 1). Using a peak-to-noise ratio of 3 as a criterion, the estimated limits of detection were 0.0125 ng drug/μl plasma for 7-AFNZ, 0.025 ng/μl for FNZ, 0.050 ng/μl for BUP and *N*-DMFNZ, and 0.0625 ng/μl for NBUP.

For intra- and inter-assay variations, all the analytes have three QC samples in common (Table 2). However, for BUP and 7-AFNZ, an additional QC sample was used because their standard curves fell outside of both the upper and lower limits, respectively, relative to the other analytes. The intra-assay coefficients of variation were within 7% for BUP, 10% for NBUP, 5% for FNZ, 8% for *N*-DMFNZ and within 12% for 7-AFNZ. Intra-assay accuracy was reasonably good with errors from nominal concentrations within 10% for BUP and NBUP, 7% for *N*-DMFNZ and roughly 12 and 13% for FNZ and 7-AFNZ, respectively. The inter-assay coefficients of variation were relatively low with values not exceeding 10% for all the analytes (Table 2). Inter-assay accuracy was good with errors from nominal concentrations less than 8% for BUP, 9% for NBUP and 10% for FNZ and *N*-DMFNZ, and roughly 11% for 7-AFNZ.

Table 3 shows the recoveries of BUP, NBUP, FNZ, *N*-DMFNZ and 7-AFNZ analyzed at two different concentrations, 0.5 and 2.5 ng drug/μl plasma. Recovery was apparently not concentration dependent. The recoveries of BUP, FNZ and *N*-DMFNZ were similar and almost quantitative, with average values of 70.7, 66.7 and 80.7%, respectively, and average coefficients of variation of 10.1, 5.9 and 11.4%. These recoveries were greater than those of NBUP and 7-AFNZ which were 50.7 and 50.5%, respectively, with average coefficients of variation of 10.0 and 7.3%. The relatively low coefficients of variations illustrate the good reproducibility of the recoveries (Table 3).

Previous authors have already reported low extraction recoveries for 7-AFNZ and also *N*-DMFNZ by using liquid–liquid extraction [37] and solid-phase extraction (SPE) [38] procedures. In the latter case, an improvement of the recoveries has been obtained at the price of careful optimization of the extraction conditions [38]. However, our method showed a lower extraction recovery of NBUP than other methods [28,39]. Gopal et al. [39] have reported quantitative recoveries for NBUP (90%) and BUP (80%) from human plasma, using a SPE with Bond-Elut Certification followed by sample analysis by GC–MS. These authors have emphasized that silanization of all glassware, including glass liner, was essential to maintain the sensitivity of both NBUP and BUP. The fact that we did not use silanized glassware in our method cannot explain the relatively low recovery of NBUP since the recovery determined for BUP was significantly higher (70%). In preliminary experiments, we tested SPE procedures for the extraction of BUP, NBUP, FNZ, *N*-DMFNZ and 7-AFNZ from rat plasma. Higher

analytical recoveries relative to those reported here were obtained for NBUP and 7-AFNZ, but the extracts resulted in dirtier extracts following SPE, giving rise to problems during GC injection and thereby, to chromatographic interferences in sample analysis by GC–MS. Other liquid–liquid extraction procedures than that using Toxi-tubes A were also tested and compared during method development. For example, testing the procedure by Molinaro et al. [40], we observed that the analyte recoveries resulted sensibly the same as those determined here using Toxi-tubes A. The Molinaro's method also proved to be rather long and tedious. In view of these results, we have considered that the extraction procedure using Toxi-tubes A was a good compromise between recovery of analytes, clean up of extracts and absence of chromatographic interferences. Furthermore, due to its simplicity and rapidity, this procedure is suitable for studies including multiple sample analysis, such as kinetic studies.

Finally, the intra- and inter-assay variation results satisfactorily met current acceptance criteria for bioanalytical method validation [41]. The limits of quantification and analytical recoveries were considered adequate for the purpose of this study.

3.3. Pharmacokinetic study of BUP, FNZ and their metabolites in rats

Fig. 2 shows some plasma concentration-time profiles for BUP, FNZ and their metabolites obtained in rats following intravenous perfusion of BUP (30 mg/kg) over 3 min and then of FNZ (40 mg/kg) over 30 min. The concentration-time data presented were determined in a single animal. Sample analysis by GC–MS was performed in duplicates.

As shown in Fig. 2A, BUP had a maximum plasma concentration at the first sampling time (5.774 ± 0.197 ng/μl; −30 min). Between times –25 and −15 min post-perfusion, its concentration declined rapidly and then, more slowly. The BUP level was 0.365 ± 0.003 ng/μl at 180 min. NBUP was formed very rapidly after BUP administration, but its plasma level was very low over all the duration of the kinetics. The peak concentration of NBUP was observed at −30 min (0.130 ± 0.006 ng/μl). Thereafter, its concentration decreased only slightly: it represented more than 50% of its peak concentration at the end of the kinetic study. It is noticeable that the concentrations of NBUP were nearly parallel to those of BUP during the major part of the kinetic study.

The plasma concentration-time profiles of FNZ and its metabolites (Fig. 2B) were different from those of BUP and NBUP. The FNZ plasma level was seen to increase during the first 15 min of the perfusion of this drug, reaching a maximum at time −10 min (5.119 ± 0.017 ng/μl). From time 0 post perfusion until the end of the kinetic study, FNZ declined rapidly: its plasma level did not exceed 0.10 ng/μl at 180 min. Both *N*-DMFNZ and 7-AFNZ were formed rapidly after intravenous administration of FNZ and their kinetic profiles mimicked that of the parent drug. Like

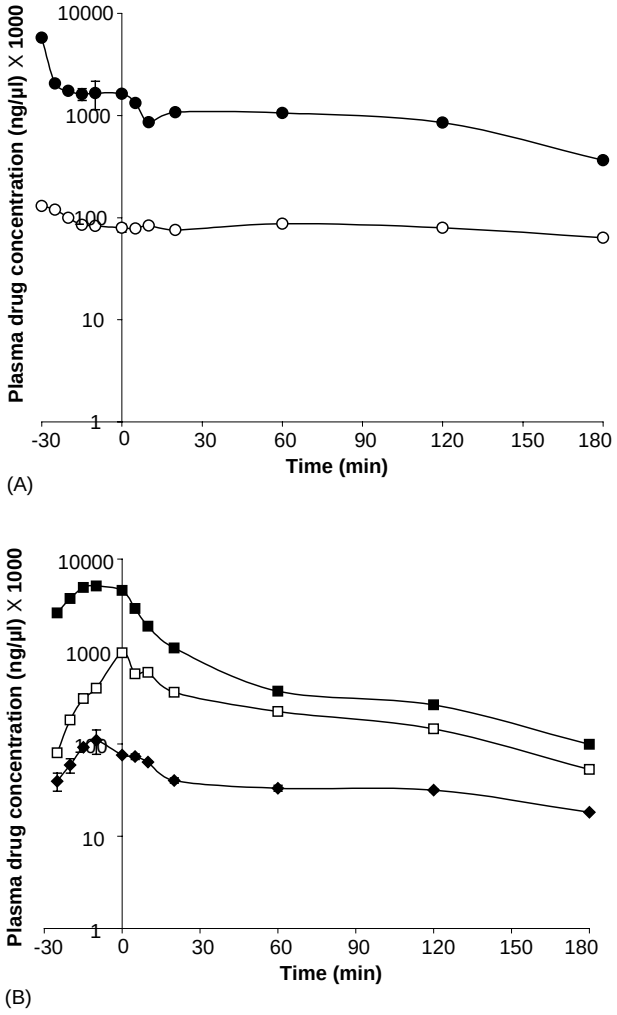

Fig. 2. Plasma concentration-time profiles of: (*A*) BUP (●) and NBUP (○), and of (*B*) FNZ (■), *N*-DMFNZ (□) and 7-AFNZ (◆) in normal adult rats, after intravenous perfusion of BUP (30 mg/kg) over 3 min, followed by intravenous perfusion of FNZ (40 mg/kg) over 30 min. Serial blood sampling was performed after BUP perfusion (−30 min), during FNZ perfusion (−25, −20, −15 and −10 min) and after FNZ perfusion (0, 5, 15, 60, 120 and 180 min). The kinetic profiles were all determined from analysis of plasma samples (40 μl) taken from one animal. Sample analysis by GC–MS was performed in duplicates. The plasma drug concentrations are shown as means ± S.D.

FNZ, 7-AFNZ reached its peak concentration at −10 min (0.109 ± 0.004 ng/μl), while the peak of *N*-DMFNZ was slightly shifted and observed at time 0 post perfusion (0.967 ± 0.010 ng/μl). At further sampling times, the plasma levels of *N*-DMFNZ and 7-FNZ were almost parallel to each other and also to those of their parent drug. The levels of *N*-DMFNZ and 7-AFNZ were two- and five-times lower, respectively, than the level of FNZ at the end of the kinetic study.

4. Conclusion

The GC–MS method reported in this paper to simultaneously analyze BUP, FNZ and their respective metabolites,

NBUP, *N*-DMFNZ and 7-AFNZ, in rat plasma was validated according to internationally accepted criteria [41]. The method consists of sample liquid–liquid extraction, chromatographic separation on 5% phenyl-methylpolysiloxane column and detection in SIM mode by GC–MS. Due to the relative simplicity and rapidity of the extraction and derivatization procedures used, the method is suitable for analysis of a large number of samples. The method showed adequate range of linearity, intra- and inter-assay accuracy and precision for its application in plasma analysis of these drugs for assessment of their pharmacokinetics following treatment with toxic doses of BUP and FNZ. However, assessment of the drug plasma kinetics for the use of lower doses of BUP and FNZ in the animals requires to improve the sensitivity to determine the metabolites of these drugs, NBUP, 7-AFNZ and *N*-DMFNZ. An improvement of the sensitivity can be obtained by using larger volumes of samples for the extraction, but the number of blood sampling in the animals will be consequently fewer. Anyway, because this method is able to simultaneously determine BUP, FNZ and their metabolites, its development should make it possible to explore the toxicity mechanisms of the BUP–FNZ association.

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