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Journal of Chromatography B, 754 (2001) 447–459

JOURNAL OF CHROMATOGRAPHY B

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Simultaneous determination of buprenorphine, norbuprenorphine, and buprenorphine–glucuronide in plasma by liquid chromatography–tandem mass spectrometry

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Received 15 August 2000; received in revised form 24 November 2000; accepted 4 January 2001

Abstract

For the first time, an LC–MS–MS method has been developed for the simultaneous analysis of buprenorphine (BUP), norbuprenorphine (NBUP), and buprenorphine–glucuronide (BUPG) in plasma. Analytes were isolated from plasma by C_{18} SPE and separated by gradient RP-LC. Electrospray ionization and MS–MS analyses were carried out using a PE-Sciex API-3000 tandem mass spectrometer. The m/z 644→m/z 468 transition was monitored for BUPG, whereas for BUP, BUP-d_4, NBUP, and NBUP-d_3 it was necessary to monitor the surviving parent ions in order to achieve the required sensitivity. The method exhibited good linearity from 0.1 to 50 ng/ml ($r^2 \geq 0.998$). Extraction recovery was higher than 77% for BUPG and higher than 88% for both BUP and NBUP. The LOQ was established at 0.1 ng/ml for the three analytes. The method was validated on plasma samples collected in a controlled intravenous and sublingual buprenorphine administration study. Norbuprenorphine–glucuronide was also tentatively detected in plasma by monitoring the m/z 590→m/z 414 transition. Published by Elsevier Science B.V.

Keywords: Buprenophine; Nobuprenophine

1. Introduction

Buprenorphine (BUP) (Fig. 1) is a semi-synthetic opiate derived from the alkaloid thebaine (oripavine) that exhibits partial agonist activity at the μ-opiate receptor and antagonist activity at the κ-opiate receptor. The main pharmacological properties of BUP are a high analgesic potency (25–40 times higher than morphine), a prolonged duration of action [1], a limited respiratory depressant activity [2], and a less rapid and less intense withdrawal syndrome compared to pure mu receptor agonists, e.g. morphine [3]. Chronically administered, BUP has been shown to attenuate or block the effects of opioid agonists likely due to its high affinity and low efficacy for mu receptors and/or to the development of cross-tolerance [4–7]. After administration of sublingual BUP doses from 0 to 32 mg, the maximal physiological and subjective effects were observed at 4–8 mg, with no increase at higher doses [8]. This ''ceiling effect'' may reduce BUP abuse liability. This pharmacological profile makes BUP an attractive alternative to methadone for the treatment of opioid dependence. The use of BUP as an opioid

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0378-4347/01/$ – see front matter Published by Elsevier Science B.V.
PII: S0378-4347(01)00029-9

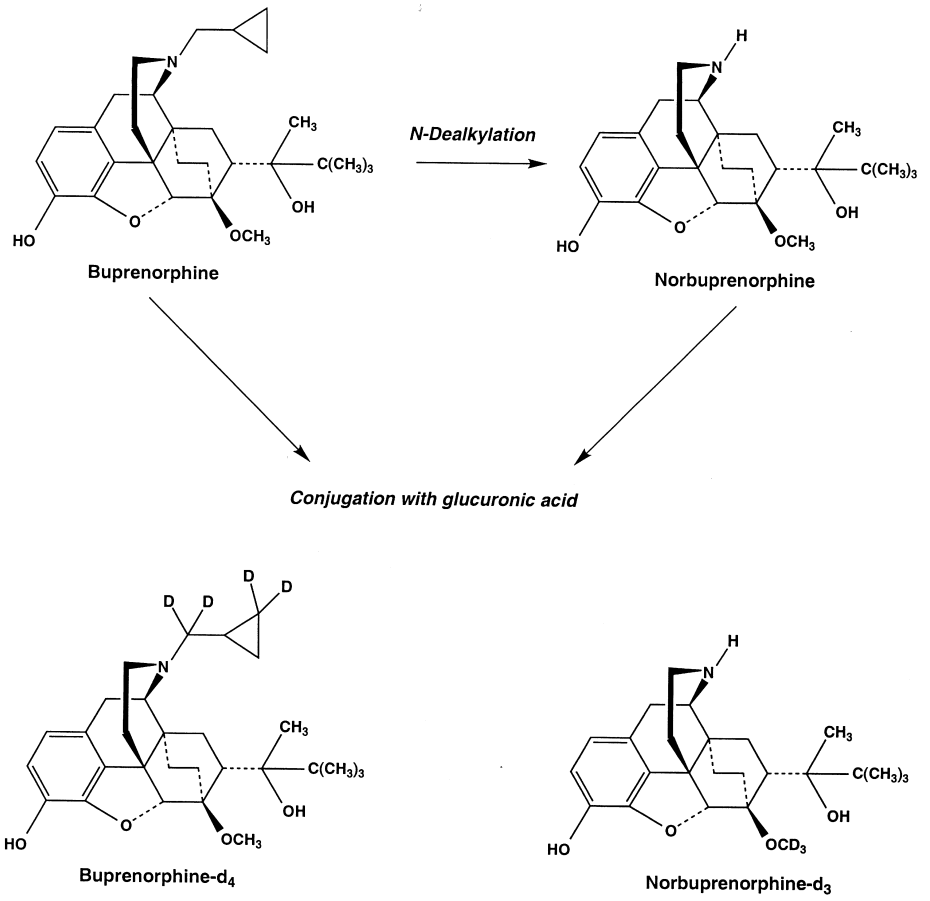

Fig. 1. Structures of buprenorphine, metabolites, and deuterated analogs used in the present study.

pharmacotherapeutic has been authorized in France in 1996 in doses up to 8 mg per tablet [9], and it is in the process of approval in the United States for dosages up to 24 mg. However, a number of recently reported BUP and benzodiazepine-related deaths have raised concern about the possible abuse and misuse of this drug (intravenous injection of crushed tablets, concomitant intake of other neurodepressant drugs) [9–11].

The bioavailability of BUP (4 mg) by the sublingual and buccal route has been estimated as 51.4% and 27.8%, respectively [12]. The drug is highly bound to plasma proteins (96%) and is metabolized in the liver by *N*-dealkylation to norbuprenorphine (NBUP), mainly by cytochrome P450 3A4 [13]. Both BUP and NBUP undergo conjugation with glucuronic acid (Fig. 1). BUP is largely excreted through the bile, whereas less than 30% of the dose is found in urine, mainly as phase II metabolites [14,15]. Both BUP and NBUP are subject to enterohepatic circulation [14,16,17].

Different methods based on combined chromatographic and mass spectrometric techniques have been applied to the analysis of BUP and metabolites in biological samples, such as plasma and serum [18–23], whole blood [21,24], urine [16,21,25,26], feces [16], cadaveric tissues [9,10,21], hair [21,26,27], and sweat patches [28]. Gas chromatography–mass spectrometry (GC–MS) either in the electron impact [25,26] or positive-ion chemical ionization [16,19,22] modes has been widely applied in the past. Methods based on this technique obviously suffer the limitations of GC separation, i.e. required derivatization prior to instrumental analysis, direct

determination of free metabolites only (BUP and NBUP), and degradation of unstable analytes. Derivatization has been performed using acylating [16,19,20,22] and silylating [26] agents or by extractive methylation [25]. However, reproducible derivatization may be difficult to achieve. Vincent et al. [26] encountered problems in obtaining the quantitative derivatization of the secondary amino group of NBUP with silylating agents, whereas other authors reported decomposition of BUP and NBUP with heating during derivatization with acylating agents [29]. A two-step analysis, with and without hydrolysis, has to be carried out in order to determine indirectly the concentration of conjugated metabolites in the sample. Enzymatic hydrolysis [16,24–26] has been applied to plasma [24] and urine [16,25,26] samples. Alternatively, total BUP and NBUP can be measured following chemical hydrolysis in strongly acid and heated medium. In this case, BUP, NBUP and other 6,14-*endo*-ethanotetrahydrooripavine derivatives undergo an acid-catalyzed rearrangement by elimination of a molecule of methanol and formation of a tetrahydrofuran ring [30]. The resulting degradation product of BUP, demethoxybuprenorphine, can be extracted and analysed by GC–MS after acylation providing that the reaction is brought to completion [18]. Thermal degradation of BUP has been observed in the heated particle beam liquid chromatography–mass spectrometry (LC–MS) interface [31].

Extraction of BUP and metabolites from biological fluids has generally been carried out by liquid–liquid partition [16,22,25]. Isolation of BUP and NBUP from hair has been carried out by overnight incubation in diluted hydrochloric acid and subsequent extraction using mixed mode solid-phase cartridges [26]. An interesting report on the application of GC coupled with tandem mass spectrometry (MS–MS) to the determination of BUP and NBUP in plasma utilized solid-phase extraction (SPE) with mixed mode cartridges, heptafluorobutyryl derivatization and negative chemical ionization mode-detection [20].

After the introduction of atmospheric pressure ionization LC–MS interfaces, a considerable number of LC–MS procedures for the determination of free and conjugated heroin and morphine metabolites have been published [32–42]. On the other hand, only a few reports on the analysis of buprenorphine metabolites by LC–MS [21,14] and LC–MS–MS [15,16] are available in the literature, and all of them measure only the free metabolites (BUP and NBUP).

An LC–MS–MS method for the simultaneous determination of BUP, NBUP, and buprenorphine-*O3*–glucuronide (BUPG) in plasma is described. The method was validated with plasma samples collected in a clinical study of controlled sublingual and intravenous buprenorphine administration. Norbuprenorphine–glucuronide was tentatively identified in plasma extracts on the basis of its expected chromatographic, mass spectrometric and pharmacokinetic properties.

2. Experimental

2.1. Reagents

Buprenorphine (BUP, for the preparation of calibrators), buprenorphine-d_4, norbuprenorphine (NBUP), and norbuprenorphine-d_3 free bases in methanol solution (100 μg/ml) were purchased from Radian International (Austin, Texas). Buprenorphine-*O3*–glucuronide (BUPG) was obtained from Research Triangle Institute (Raleigh, NC). Buprenorphine hydrochloride (for the preparation of validation and quality control samples) was obtained from Reckitt & Colman (Hull, UK).

Ammonium carbonate, ammonium formate and formic acid were purchased from Sigma (St. Louis, MO). All solvents used were HPLC grade. Water was prepared by filtering distilled water on a Milli-Q A10 filtration system from Millipore (Bedford, MA).

Cellulose and nylon 0.45 μm-pore size filters for mobile phase filtration were obtained from Hewlett-Packard (Palo Alto, CA). C_{18} solid-phase extraction cartridges, 200 mg, 40 μm particle size, (Cat. No. 1211-3024) were purchased from Varian (Walnut Creek, CA).

The following procedure was followed for the preparation of calibrators. A methanolic solution of BUPG (0.28 mg/ml) was prepared and stored in the dark at −20°C. Combined solutions of BUP, NBUP, and BUPG at different concentrations (from 1 to 1000 ng/ml), and of BUP-d_4 and NBUP-d_3 (50 ng/ml each), were prepared in water, divided into

1- and 3.5-ml aliquots, respectively, and stored in polypropylene tubes in the dark at −20°C. Aliquots from each solution were thawed, briefly vortexed before use, and added to blank plasma samples. The same procedure, using a fresh methanol solution of buprenorphine hydrochloride (Reckitt & Colman) (100 μg/ml as free base), a separate methanol standard of NBUP, and a separate weighing for BUPG, was used for the preparation of validation and quality control samples.

Ammonium carbonate buffer (0.01 *M*, pH 9.3) was prepared by adjusting 900 ml of a 0.01 *M* solution of ammonium carbonate to pH 9.3 with ammonium hydroxide. The final volume was adjusted to 1000 ml with the ammonium carbonate solution. The mobile phase consisted of a mixture of water (A) and acetonitrile/water (99.5:0.5 v/v, B) both containing 2 m*M* ammonium formate and 0.1% (v/v) formic acid. Component B of the mobile phase was prepared by dissolving 126 mg of ammonium formate in 5 ml water. After the addition of 1 ml of formic acid, the solution was made up to 1000 ml with acetonitrile. Both components of the mobile phase were filtered on 0.45 μm cellulose (A) and nylon (B) filters and degassed with helium before and during use.

2.2. Sample preparation

One-millilitre volumes of centrifuged (4500 *g*/5 min) plasma samples and calibrators (blank plasma fortified with BUP, NBUP, and BUPG at concentrations of 0, 0.1, 0.5, 1.0, 5, 10, and 50 ng/ml) were spiked with 100 μl of the internal standards solution, briefly vortexed, and allowed to equilibrate for 10 min. Ammonium carbonate buffer (3 ml) was added and the samples were gently vortexed, centrifuged (1600 *g*/5 min), and submitted to the following SPE procedure. Samples were poured into SPE cartridges previously conditioned with 3 ml of methanol and 3 ml of ammonium carbonate buffer and filtered at 1–2 ml/min. SPE cartridges were washed with 3 ml of water and dried for 5 min under vacuum. Elution of analytes was carried out with two consecutive 1-ml aliquots of methanol. Extracts were evaporated to dryness with air at 55°C, reconstituted with 70 μl of component A of the mobile phase (vortex, 30 s), centrifuged (1600 *g*/5 min), and transferred with Pasteur pipettes into 200-μl autosampler microvials. After further centrifugation (5800 *g*/2 min), 20-μl volumes of the extracts were submitted for instrumental analysis.

2.3. Instrumental analysis

LC–MS–MS instrumentation consisted of the following components: (a) a Perkin-Elmer (Norwalk, CT, USA) Series 200 Autosampler (injector needle and valve were flushed in between sample injections with a water/methanol, 75:25 v/v, solution); (b) two Perkin-Elmer Series 200 pumps connected together through a 10-μl tee mixer (Lee Company, Westbrok, CT); (c) a PE-Sciex (Foster City, Canada) API 3000 tandem mass spectrometer equipped with a TurboIonSpray® interface.

Chromatographic separation was carried out using a Chrompack Inertsil ODS-3 column 100×3 mm I.D., 3 μm particle size (Varian, Walnut Creek, CA) equipped with Chrompack 10×2 mm RP-guard column. Gradient elution at a constant flow-rate of 0.7 ml/min was performed as follows: 84% A for 0.1 min, linear decrease to 70% A in 5.1 min, 2.5 min hold at 70% A, step increase to 84% A with 5-min equilibration before the following injection (total run time: 12.7 min).

Ionization of analytes was carried out using the following settings of the electrospray interface: source temperature, 475°C; ion source voltage, 5100 V; orifice and ring voltages, 60 and 350 V, respectively; nebulizer and curtain gases (nitrogen) settings, 10 and 12, respectively; heater gas (nitrogen), 8 l/min.

Tandem mass spectrometric analysis was performed using nitrogen as collision gas (setting, 12). The m/z 644→m/z 468 transition (collision energy, −58 eV) was monitored for BUPG, whereas for BUP (collision energy, −38 eV) and NBUP (collision energy, −28 eV) and their deuterated analogues it was necessary to monitor the surviving parent ions (BUP, m/z 468→m/z 468; BUP-d_4, m/z 472→m/z 472; NBUP, m/z 414→m/z 414; NBUP-d_3, m/z 417→m/z 417) in order to achieve the required sensitivity.

2.4. Plasma samples

Plasma samples were collected prior to and up to 72 h after administration of 12 mg sublingual

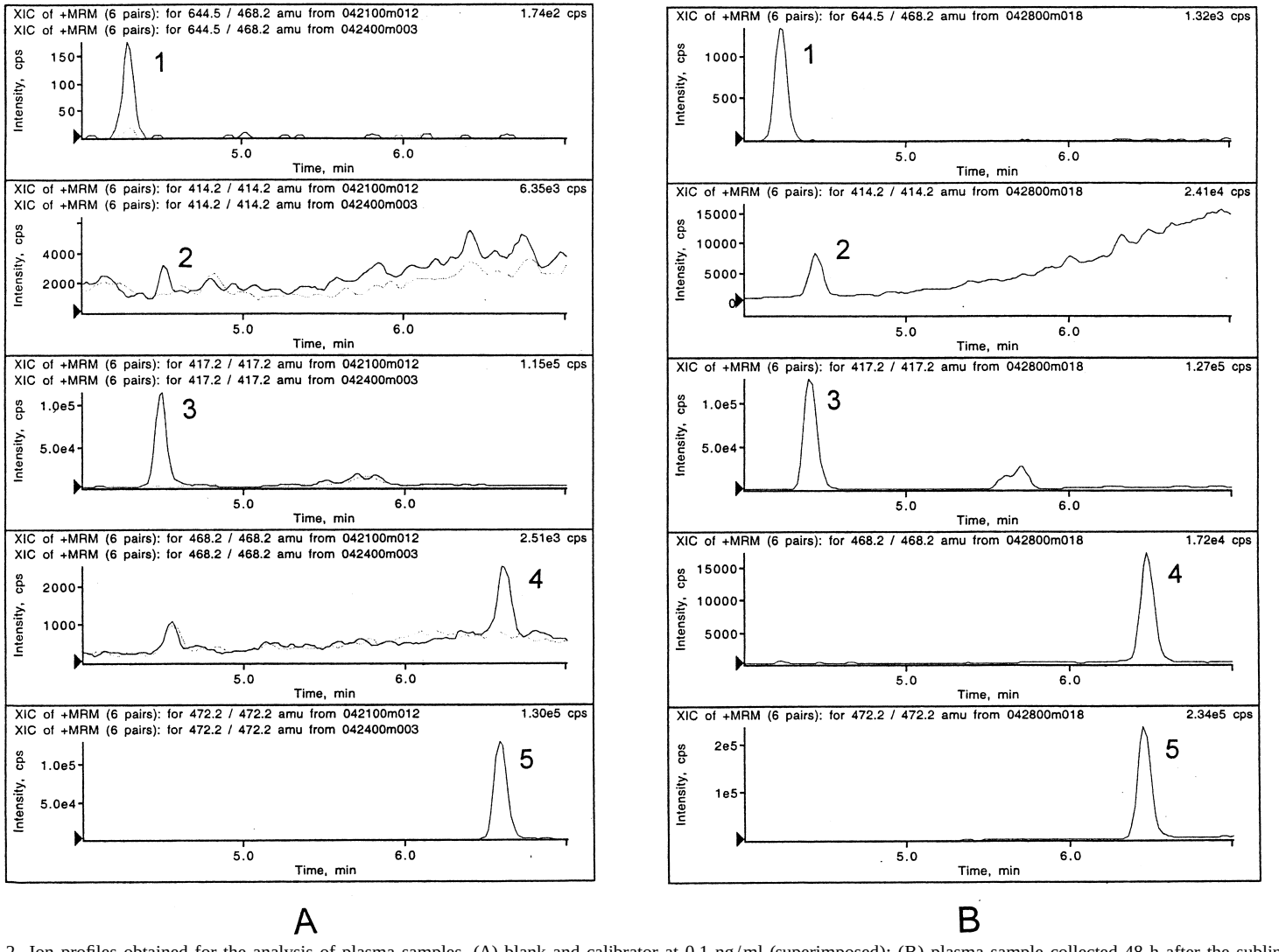

Fig. 2. Ion profiles obtained for the analysis of plasma samples. (A) blank and calibrator at 0.1 ng/ml (superimposed); (B) plasma sample collected 48 h after the sublingual administration of 12 mg BUP to a human volunteer. The following concentrations were measured: buprenorphine (BUP)=0.36 ng/ml; norbuprenorphine (NBUP)=0.33 ng/ml; buprenorphine–glucuronide (BUPG), 0.41 ng/ml. Peaks: 1, BUPG; 2, NBUP; 3, NBUP-d_3; 4, BUP; 5, BUP-d_4.

buprenorphine to participants in an IRB-approved study. Volunteers provided informed consent and were paid for their participation. Plasma samples were stored at −20°C until analysis.

3. Results and discussion

3.1. Chromatography

Good chromatographic separation was achieved for BUP and NBUP. Gradient elution was necessary in order to reduce retention time and avoid excessive broadening of the BUP peak. Initial gradient conditions were adjusted in order to make BUPG elute as close as possible to NBUP (Fig. 2). This was necessary in order to use NBUP-d_3 as internal standard for BUPG, since a deuterated analog of this compound is not yet commercially available. Deuterated internal standards are highly recommended in LC–MS analysis. In fact, differences in chromatographic behavior between analyte and internal standard, particularly under gradient elution, are known to be a major source of imprecision in LC–MS and LC–MS–MS analysis. Compounds may variably respond to possible fluctuations of the parameters involved in ionization and collisionally activated dissociation. However, if a deuterated internal standard is not available, as in this case, the problem can be reduced by minimizing the retention time difference between analyte and internal standard, providing, of course, that they can be separated by MS and/or MS–MS (Figs. 3 and 4).

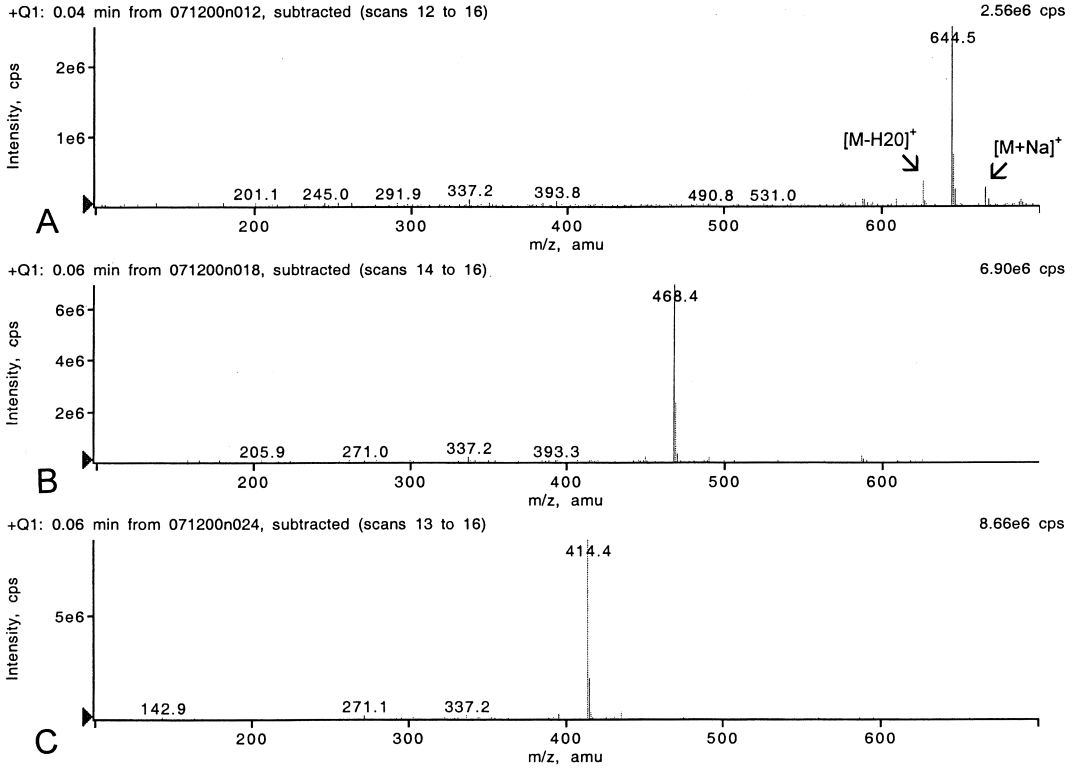

Fig. 3. Full scan mass spectra of buprenorphine–glucuronide (A), buprenorphine (B), and norbuprenorphine (C) obtained by flow injection of the respective aqueous solutions at 1 ng/μl. Mobile phase composition: 75% A, 25%B (see text for details on instrumental conditions).

3.2. Mass spectrometry

Fig. 3 shows the full scan mass spectra of the analytes at an orifice voltage of 60 V. Preliminary experiments carried out by flow injection at different orifice voltages indicated that the intensity of the $[M+H]^+$ ions of BUP, NBUP, and BUPG maximized at 60 V. As expected, all analytes exhibited low fragmentation. Besides the pseudomolecular ion, $[M-H_2O]^+$ and $[M+Na]^+$ ions were present in the full scan spectrum of BUPG and, with lower relative abundance, in the mass spectra of BUP and NBUP. No evidence of the mass peak corresponding to the aglycone (m/z 468) was found in the mass spectrum of BUPG, indicating that this analyte was stable at the adopted ionization conditions.

Fig. 4 shows the product ions mass spectra of the respective $[M+H]^+$ ions of the three analytes. BUPG produced a very intense and almost unique deconjugation fragment at m/z 468 (minor fragments were the *N*-dealkylation ion at m/z 590 and the respective aglycone at m/z 414). This feature makes BUPG an optimal candidate for highly sensitive MS–MS analysis as almost all the signal of the precursor ion is transferred to one product ion. On the contrary, as previously observed also by Moody et al. [22], several attempts to obtain similar results for BUP and NBUP by varying both the collision gas pressure and the collision energy failed. At collision energies below 30–40 eV no significant fragmentation of the precursor ion was observed, whereas higher collision energies produced extensive fragmentation, with an overall significant loss of sensitivity. Therefore, it was decided to monitor the

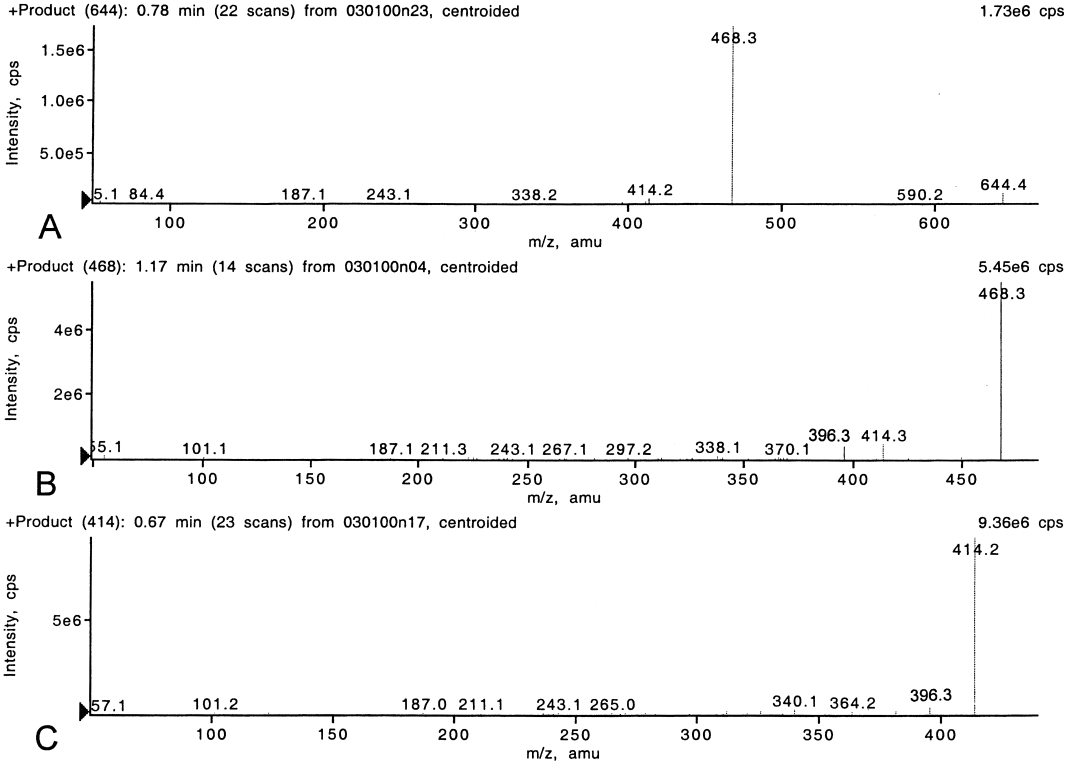

Fig. 4. Product ion mass spectra of the $[M+H]^+$ ions of buprenorphine–glucuronide (A), buprenorphine (B), and norbuprenorphine (C) obtained by flow injection of the respective aqueous solution at 1 ng/μl. Mobile phase composition: 75% A, 25%B (see text for details on instrumental conditions).

surviving precursor ion of BUP and NBUP and of their respective deuterated analogs in the second mass analyzer. This analytical strategy exploits the different behavior of substances at the conditions present in the collision cell (collision energy and gas pressure). Highly stable analytes, in our case BUP and NBUP, are transferred as intact molecules to the second mass analyzer, whereas less chemically stable interferences fragment in the collision cell. The selectivity of this approach is certainly lower than that of classical MS–MS analysis (precursor ion→product ion). In addition, the simultaneous monitoring of the specific product ion of BUPG (*m*/*z* 644→*m*/*z* 468) helps to provide further evidence of the correct identification of the analytes in the sample.

At optimal collision energies for the transmission of the $[M+H]^+$ ion, the product ion spectrum of BUP was characterized by two low-intensity fragments at *m*/*z* 414 and *m*/*z* 396. While *m*/*z* 414 likely originates from *N*-dealkylation of BUP, the structure of the second fragment is more difficult to establish. One possibility is that *m*/*z* 396 derives from *m*/*z* 414 following dehydration. However, in this case, a fragment at *m*/*z* 396 should be present also in the product ion spectrum of BUP-d_4, owing to the position of the four deuterium atoms in the methylcyclopropyl moiety (Fig. 1). Instead, the corresponding fragment in the BUP-d_4 spectrum (Fig. 5) keeps the 4-mass units difference (*m*/*z* 400), indicating that the methylcyclopropyl moiety is still present in the structure. It is likely, therefore, that *m*/*z* 396 primarily originates from another fragmentation pathway, e.g. from the combined loss of a methyl and the *tert.*-butyl groups. The fragment at *m*/*z* 396 was present also in the product ion spectrum of NBUP. However, this fragment likely originates from dehydration and should, therefore, have a different structure from the fragment at *m*/*z* 396 of BUP. This hypothesis was verified by examining the

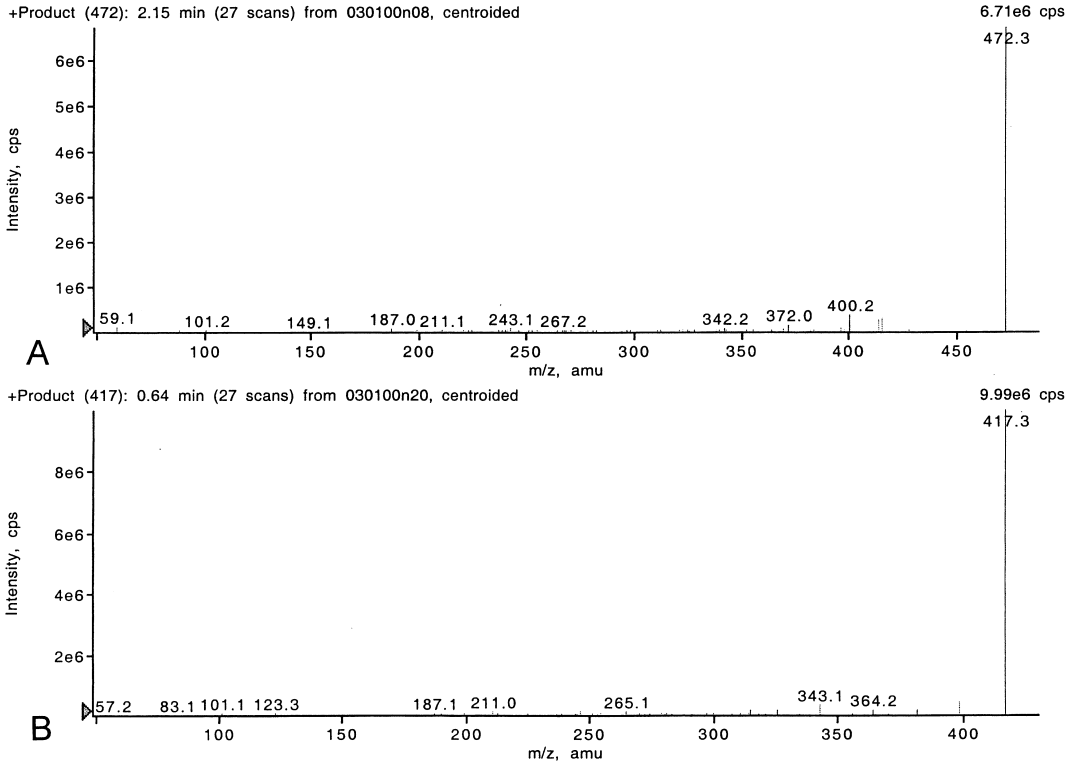

Fig. 5. Product ion mass spectra of the $[M+H]^+$ ions of buprenorphine-d_4 (A), and norbuprenorphine-d_3 (B) obtained by flow injection of the respective aqueous solution at 1 ng/μl. Mobile phase composition: 75% A, 25% B (see text for details on instrumental conditions).

product ion spectra of the two fragments (Fig. 6): m/z 396 from BUP exhibits a prominent ion at m/z 342, likely due to *N*-dealkylation, while this ion is virtually absent in the product ion spectrum of m/z 396 from NBUP.

3.3. Recovery, precision, linearity, limit of quantitation

Absolute recovery of the analytes was measured by spiking blank plasma samples with BUP, NBUP, and BUPG at different concentrations: 0.1, 5.0 and 50.0 ng/ml (four replicates each). An additional set of blank samples was also extracted with analytes added just before injection into the liquid chromatograph. In all cases, the internal standard solution was added just before injection. The absolute recovery was obtained by comparing the ratio of the peak areas analyte/internal standard measured in the two sets of samples: recoveries were higher than 77% for BUPG and higher than 88% for both BUP and NBUP (Table 1). Recoveries of BUP in the range 70–83% were previously obtained for the liquid–liquid extraction of plasma samples adjusted to pH 10.5 with a mixture of *n*-butyl chloride/acetonitrile (4:1 v/v) [22]. Lower recoveries (57–59% and 56–59% for BUP and NBUP, respectively) using a sample preparation procedure based on enzymatic hydrolysis and Extrelut-3 extraction columns [24] have been reported from whole blood.

Intra-day and inter-day precision and accuracy data are shown in Table 2. Intra-day precision was measured at 0.1, 1.0, 10.0 and 50.0 (five replicates each), whereas the inter-day precision was determined for the same concentrations within a 2-week (five separate analytical sessions) period ($n=13$).

Typical equation curves ($1/x$ weighted regression analysis) were: $y = 0.197x - 0.009$ (BUP); $y =$

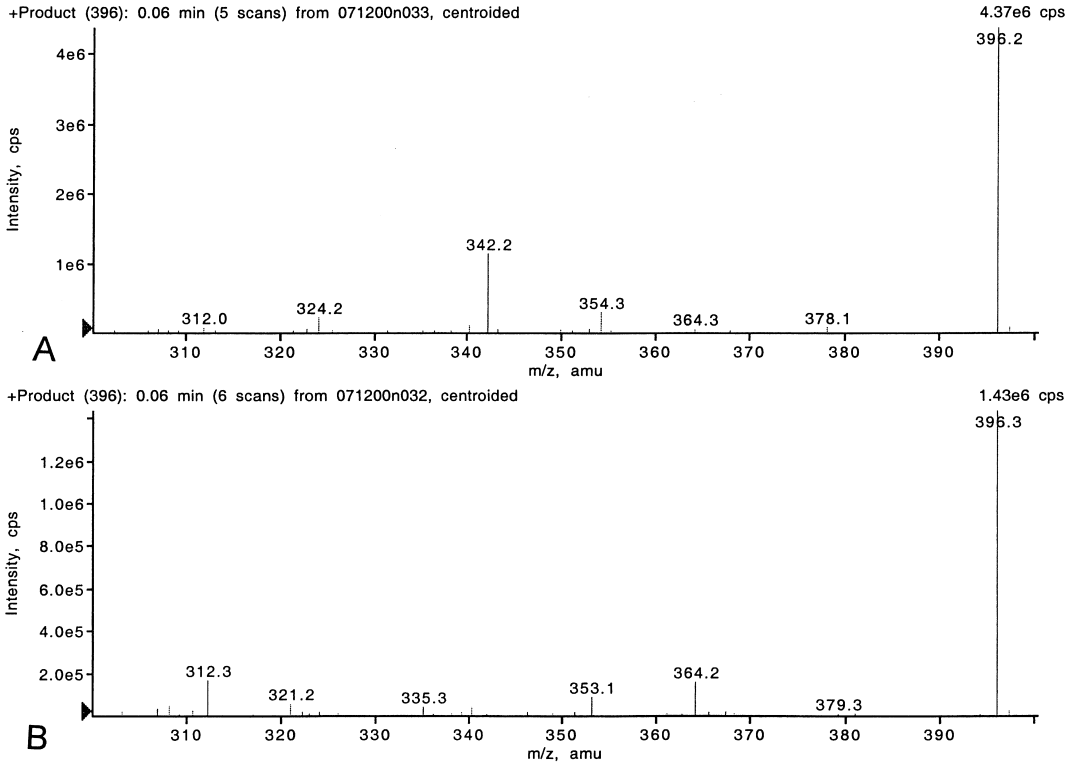

Fig. 6. Product ion mass spectra of m/z 396 of buprenorphine (A) and norbuprenorphine (B) obtained by flow injection of the respective aqueous solutions at 1 ng/μl. Mobile phase composition: 75% A, 25%B; orifice voltage, 120 V; collision energy, −48 eV (see text for details on instrumental conditions).

Table 1
Absolute recovery of BUP, NBUP, and BUPG (% mean±SD, $n=4$)

Compound	0.1 ng/ml (% mean±SD)	5 ng/ml (% mean±SD)	50 ng/ml (% mean±SD)
Buprenorphine	88.3±6.3	82.2±3.5	93.5±4.4
Norbuprenorphine	89.0±7.5	91.6±5.4	99.9±6.8
Buprenorphine–glucuronide	77.3±6.9	86.9±9.1	88.1±3.0

$0.160x - 0.008$ (NBUP); and $y = 0.028x + 0.001$ (BUPG), where y is the peak-area ratio (analyte/internal standard) and x is the theoretical concentration. Correlation coefficients were higher than 0.998. Deviation from linearity was evaluated by comparing the slopes of the calibration curves from 0.1 to 1 ng/ml and from 1 to 50 ng/ml with the overall slope for the three analytes: differences between slopes were always less than or equal to 5%.

The limit of quantitation was established at 0.1 ng/ml for the three analytes on the basis of the accuracy of the determinations at this concentration (deviation from the nominal value within 20%). Quantitation limits of 0.1 ng/ml have been reported for BUP [22] in plasma, and for BUP and NBUP [24] in whole blood by other methods based on coupled liquid chromatographic–mass spectrometric techniques using a sample volume of 1 ml.

Fig. 2A shows the superimposed mass chromatograms obtained for the analyses of a calibrator at the quantitation limit (0.1 ng/ml) and of a blank. Fig. 2B shows the corresponding mass chromatograms obtained for a plasma sample collected 48 h after the sublingual administration of 12 mg of buprenorphine in which the following concentrations were measured: BUP, 0.36 ng/ml; NBUP, 0.33 ng/ml; BUPG, 0.41 ng/ml.

The method selectivity was demonstrated by the absence of interferences coeluting with the analytes in three different lots of blank human plasma. Furthermore, the addition of a mixture of opiates (6-acetylmorphine, morphine, codeine, normorphine, norcodeine) at 250 and 1000 ng/ml levels to validation plasma samples containing 1 ng/ml of BUP, NBUP, and BUPG did not affect the quantitation of the analytes.

Stability of BUP in frozen plasma samples has been previously demonstrated for up to 55 days [22]. In the present study, seven positive plasma samples containing variable concentrations of BUP, NBUP, and BUPG were reanalyzed after been stored for

Table 2
Intra-day and inter-day validation statistics for buprenorphine (BUP), norbuprenorphine (NBUP) and buprenorphine–glucuronide (BUPG) in plasma

Compound		Validation sample concentration (ng/ml)							
		Intra-day ($n=5$)				Inter-day ($n=13$)			
		0.1	1.0	10.0	50.0	0.1	1.0	10.0	50.0
BUP	Mean	0.10	1.02	10.33	51.71	0.10	1.00	9.91	51.96
	%RSD	3.9	4.4	2.6	3.6	8.8	7.4	7.7	7.7
	%R.E.	3.0	1.8	3.3	3.4	4.5	0.2	−0.9	3.9
NBUP	Mean	0.11	1.11	10.59	51.35	0.11	1.03	10.06	51.36
	%RSD	4.0	3.3	3.6	4.0	7.1	9.6	7.7	5.1
	%R.E.	5.8	11.0	5.9	2.7	7.2	2.8	0.6	2.7
BUPG	Mean	0.10	0.98	10.25	46.85	0.09	1.01	9.86	47.47
	%RSD	9.3	5.2	2.7	4.6	11.1	8.5	5.4	4.5
	%R.E.	4.0	−2.2	2.5	−6.3	−2.9	0.7	−1.4	−5.1

6 months at −20°C. The recoveries after storage were 101±11%, 99±12%, and 103±7% for BUP, NBUP, and BUPG, respectively.

3.4. Identification of norbuprenorphine–glucuronide

NBUP, as well as BUP, undergoes glucuronidation [14–17]. Therefore, attempts were made to detect and identify norbuprenorphine–glucuronide (NBUPG) in plasma samples collected in the controlled clinical study of buprenorphine administration. Although, in principle, both the *O3*- and the *N*-glucuronide may be formed from NBUP, conjugation in the *O3* position is more likely to occur, analogous to what has previously been described for normorphine [43,44]. Therefore, we decided to also include in the acquisition program of the tandem mass spectrometer the reaction m/z 590→m/z 414, assuming that NBUPG (MW=590) would behave similarly to BUPG under the same MS–MS conditions, producing an intense protonated aglycone (m/z 414). In fact, in all plasma samples containing detectable amounts of NBUP the ion profile of m/z 590→m/z 414 was characterized by the presence of a unique peak with a retention time relative to BUPG of 0.54 (Fig. 7). In addition to the mass spectrometric properties, the chromatographic features exhibited by this compound are compatible with the identification of NBUPG. In fact, NBUPG is expected to be less retained than BUPG in a reversed-phase-LC system owing to its higher polarity. A further confirmation of the identity of this peak was obtained by comparing the time course of the NBUP concentration in plasma with the time course of the absolute area of the suspected NBUPG peak: the two curves showed an almost identical trend (Fig. 8). Unfortunately, attempts to characterize the full scan

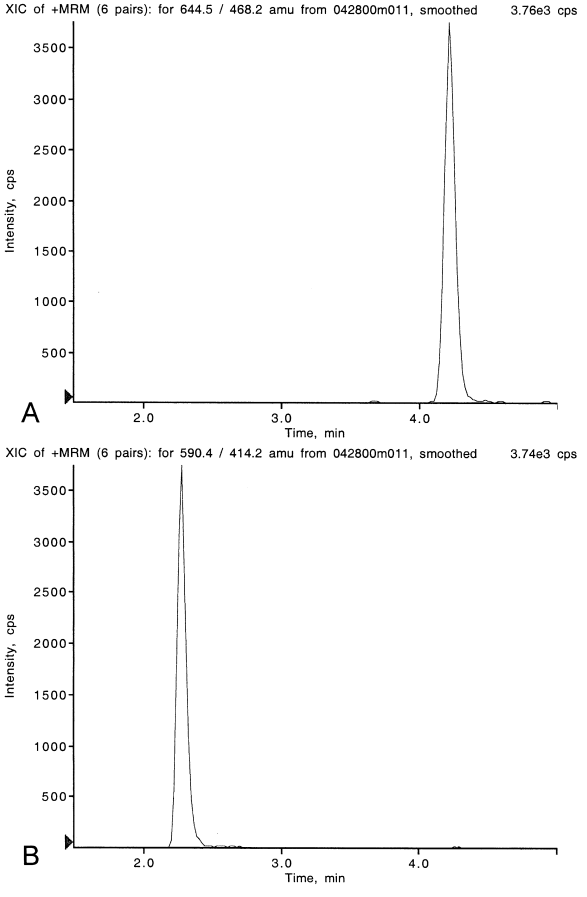

Fig. 7. Ion profiles of m/z 644→m/z 468 (buprenorphine–glucuronide (A) and of m/z 590→m/z 414 [suspected norbuprenorphine–glucuronide (B)] obtained for a plasma sample collected 1 h after the sublingual administration of 12 mg BUP to a human volunteer.

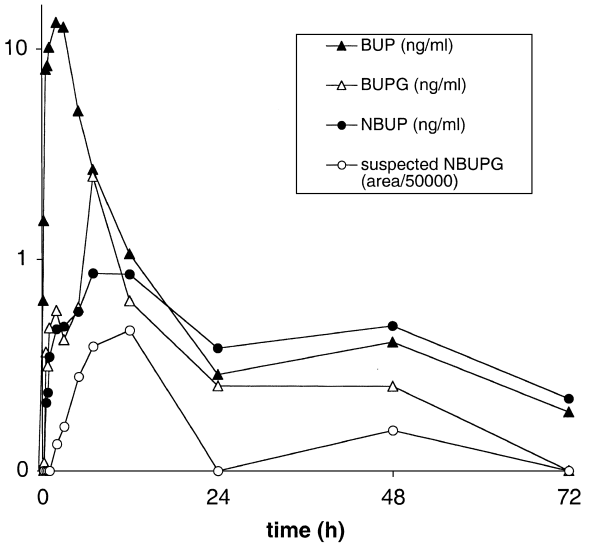

Fig. 8. Time course of BUP, NBUP, BUPG plasma concentrations and of the absolute area of the suspected norbuprenorphine–glucuronide peak after the sublingual administration of 12 mg buprenorphine to a human volunteer.

spectrum of the suspected NBUPG failed, most likely due to the low concentrations in plasma.

4. Conclusions

The described method enables the simultaneous determination of BUP, NBUP and BUPG in plasma samples at a quantitation limit of 0.1 ng/ml. This method appears also to be suitable for the direct determination of NBUPG providing that an analytical standard becomes available. The evaluation of the metabolic profile of a drug in biological samples, including glucuronide conjugates, is of great practical importance in interpretive forensic and clinical toxicology. Different factors, such as the development of metabolic tolerance, pharmacokinetic interactions with other substances, pharmacogenetic differences, and metabolic pathologies, may modify the metabolic profile of a drug and, as a consequence, the intensity and/or the type of biological response. Furthermore, parent drug-to-metabolite ratios may be useful in the assessment of the time elapsed after drug administration.

LC–MS–MS analysis of buprenorphine and metabolites simplified sample preparation and shortened analysis time, especially when compared to GC methods. Isolation of BUP and metabolites from plasma was achieved using a simple and fast SPE procedure. With this method glucuronide hydrolysis and derivatization are not required. Due to these features, the described method is applicable to the study of the pharmacokinetics of BUP and metabolites following different routes of drug administration in controlled clinical studies, as well as in the evaluation of the profile of BUP and metabolites in clinical and forensic intoxication cases.

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