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Journal of Pharmaceutical and Biomedical Analysis
32 (2003) 619–631
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Sensitive determination of buprenorphine and its *N*-dealkylated metabolite norbuprenorphine in human plasma by liquid chromatography coupled to tandem mass spectrometry

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Received 26 June 2002; received in revised form 13 July 2002; accepted 22 July 2002

Abstract

A highly sensitive method based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been developed for the quantitative determination of buprenorphine and its active metabolite norbuprenorphine in human plasma. Automated solid phase extraction (SPE) on disposable extraction cartridges (DEC) is used to isolate the compounds from the biological matrix and to prepare a cleaner sample before injection and analysis in the LC–MS/MS system. After conditioning, the plasma sample (1.0 ml) is loaded on the DEC filled with octyl silica (C8) and washed with water. The analytes are, therefore, eluted by dispensing methanol containing 0.1% of acetic acid. The eluate is collected and evaporated to dryness. The residue is dissolved in mobile phase and an aliquot is injected in the LC–MS/MS system. On-line LC–MS/MS system using atmospheric pressure chemical ionization (APCI) has been developed for the determination of buprenorphine and norbuprenorphine. The separation is obtained on a RP-18 stationary phase using a mobile phase consisting in a mixture of methanol and 50 mM ammonium acetate solution (50:50, v/v). Clonazepam is used as internal standard (IS). The MS/MS ion transitions monitored are m/z 468 → 468, 414 → 414 and 316 → 270 for buprenorphine, norbuprenorphine and clonazepam, respectively. The method was validated regarding recovery, linearity, precision and accuracy. The limits of quantification (LOQs) were around 10 pg/ml for buprenorphine and 50 pg/ml for norbuprenorphine.

Keywords: Buprenorphine; Norbuprenorphine; Pharmacokinetics; Solid phase extraction; LC–MS/MS

1. Introduction

Buprenorphine (B), (2S)-2-[17-(cyclopropylmethyl)-4,5α-epoxy-3-hydroxy-6-methoxy-6α,14-ethano-14α-morphinan-7α-yl]-3,3-dimethylbutan-

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doi:10.1016/S0731-7085(03)00169-9

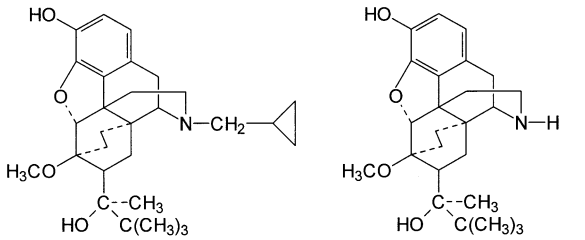


Fig. 1. Structures of Buprenorphine and its *N*-dealkylated metabolite, norbuprenorphine.

2-ol (cfr. Fig. 1), is a semi-synthetic opiate analgesic with a long duration of action [1]. As an analgesic, buprenorphine is 25–40 times more potent than morphine and has been used successfully by intramuscular, intravenous or sublingual routes for the treatment of moderate to severe pain. Analgesis is achieved by using a typical dose range from 0.3 to 0.6 mg [2]. At higher doses, from 2 to 32 mg, it acts as a potent opioid antagonist and is, therefore, used as an attractive alternative to methadone for the treatment of opiate dependence [3–6].

The metabolization of buprenorphine occurs in the liver by *N*-dealkylation to form the active metabolite norbuprenorphine (NB). Both B and NB are then conjugated with glucuronic acid. Due to the low dosage of buprenorphine administered in human and the metabolic pathway leading to glucuronides metabolites, the plasma concentrations of buprenorphine and norbuprenorphine are situated lower than the ng/ml range. This very low concentration level constitutes a challenging task for analysts.

The analysis of buprenorphine in biological samples is abundantly described in the literature. Different methods based on liquid chromatography with UV [7–9], fluorescence [10,11], electrochemical [12–19] and mass spectrometric [2,20–23] detection have been applied to the analysis of B and metabolites in plasma or serum, whole blood, urine, feces, cadaveric tissues or hair. Gas chromatography (GC) associated with mass [2,24–28], nitrogen–phosphorus [29], electron capture detectors [29–31] was also used to determine low concentrations of B or NB in biological samples.

The sample handling step prior a chromatographic analysis allows to remove proteins and to increase the selectivity and sensitivity of the method. The extraction of buprenorphine and metabolites from plasma samples has generally been carried out by liquid–liquid extraction [2,7,9,10,13,14,19,20,24,27,31] using organic solvents after alkalinization of the sample. An interesting alternative to this tedious and time-consuming sample preparation approach consists in the isolation of drugs by solid phase extraction (SPE) [8,16,18,21,23,25,28,29]. The SPE procedure can be easily automated by using column-switching systems [16] or by using sample processors such as the Automated Sample Preparation with Extraction Cartridges (ASPEC) system (Gilson) allowing, therefore, the treatment of a great number of samples to be analyzed. Automated SPE procedure can be coupled on-line [32–34] or at-line [35,36] to liquid chromatographic determinations.

Since the introduction of atmospheric pressure ionization interfaces, LC–MS has been increasingly used in the last few years to perform bioanalytical determinations with maximum sensitivity and selectivity. Moreover, this technique is generally characterized by relatively short analysis times and is, therefore, well indicated for pharmacokinetic studies where the number of samples is frequently high.

This study reports a validated method combining automated SPE and liquid chromatography coupled to tandem mass spectrometry to determine simultaneously buprenorphine and its active *n*-dealkylated metabolite, norbuprenorphine in human plasma. The SPE procedure has been optimized in order to obtain sufficiently high recoveries for both analytes, regarding particularly the selection of the extraction sorbent. The MS/MS conditions were also investigated in order to achieve very low concentrations for these substances. The method has been validated by considering different parameters such as selectivity, linearity, precision and accuracy. The limit of quantitation of the method was found to be 0.01 ng/ml for buprenorphine and 0.05 ng/ml for norbuprenorphine. Finally the method reported was successfully used to perform the determination

of B and NB in real human plasma samples and was found to be applicable for the quantification of these compounds in pharmacokinetics studies which requires high sensitivity and selectivity.

2. Experimental

2.1. Chemicals

Buprenorphine was obtained from Diosynth (Apeldoorn, The Netherlands) and the internal standard (IS) (clonazepam) was supplied by Sigma (Saint-Louis, MO, USA). Ammonium acetate and glacial acetic acid were of analytical grade from Merck (Darmstadt, Germany). Methanol and water were of HPLC grade from Merck. Nitrogen was produced by an on-site nitrogen generator from Air Liquide (Milmort, Belgium).

Isolute DECs (1 ml capacity) filled with 50 mg octylsilica (C8) were obtained from International Sorbent Technology (IST, Mid-Glamorgan, UK). Other Isolute DECS filled with 50 mg of other sorbents such as diol, cyanopropyl (CN), endcapped ethyl ($C2^{EC}$), endcapped octyl ($C8^{EC}$), phenyl (Ph), octadecyl (C18) and endcapped octadecyl ($C18^{EC}$) were also tested.

The analytical column was prepacked with Purospher Star RP-18e (particle size 3 μm) from Merck.

2.2. Apparatus

The ASPEC system from Gilson (Villiers-le-Bel, France) consisted of an automatic sampling injector module equipped with four needles, four model 401 dilutor pipettors and a set of racks and accessories for handling DECs, plasma samples and solvents.

The LC system consisted in a Model 1100 Series liquid chromatograph equipped with a binary pump, a vacuum degasser, a thermostatted column compartment and an autosampler, all from Agilent Technologies (Palo-Alto, CA, USA).

Mass spectrometric detection was carried out using a Applied Biosystems API 3000 Triple Quadrupole instrument (Thornhill, Toronto, Canada) equipped with an APCI interface. A PC Dell Optiplex GX1 (Round Rock, TX, USA) equipped with a ANALYST 1.1 version software from Applied Biosystems was used to control the LC–MS/MS system and to collect and treat the data. The INTRV@L® software (Arlenda, Belgium) was used to determine the accuracy profiles.

2.3. Chromatographic technique

All chromatographic experiments were carried out in the isocratic mode. A Manu-Cart cartridge system which consisted of a Purospher STAR RP-18e analytical column (55×4 mm i.d.) from Merck was thermostatted at 30 °C. The mobile phase consisted of a mixture of methanol and 50 mM ammonium acetate adjusted to pH 4.5 (50:50, V/V). Before use, the mobile phase was degassed for 15 min in an ultrasonic bath. The flow-rate was 0.8 ml/min.

2.4. Mass spectrometric detection

Mass spectrometric detection was achieved by means of an Applied Biosystems API 3000 apparatus operating in the positive ion mode using an APCI interface. The following conditions were applied: the auxiliary gas (N_2) flow-rate was 2 l/min, the curtain gas flow-rate was 1.2 l/min at 60 psi, the heated nebulizer temperature was 250 °C and the corona discharge was 3 μA. The mass spectrometer was set to generate and select the pseudomolecular ion [MH^+] at *m/z* 468 for buprenorphine, 414 for norbuprenorphine and 316 for clonazepam (IS) via the first quadrupole mass filter (Q1). The pseudomolecular ions were then introduced into the collision cell (Q2) with a collision energy of 47 eV. Signals from the third quadrupole (Q3) were monitored at *m/z* 468, 414 and 270 for buprenorphine, norbuprenorphine and IS, respectively.

2.5. Standard solutions

2.5.1. Solutions used for method development

Stock solutions of B and NB were prepared independently by dissolving the appropriate amount of each compound in methanol in order to obtain a final concentration of 10 μg/ml. These

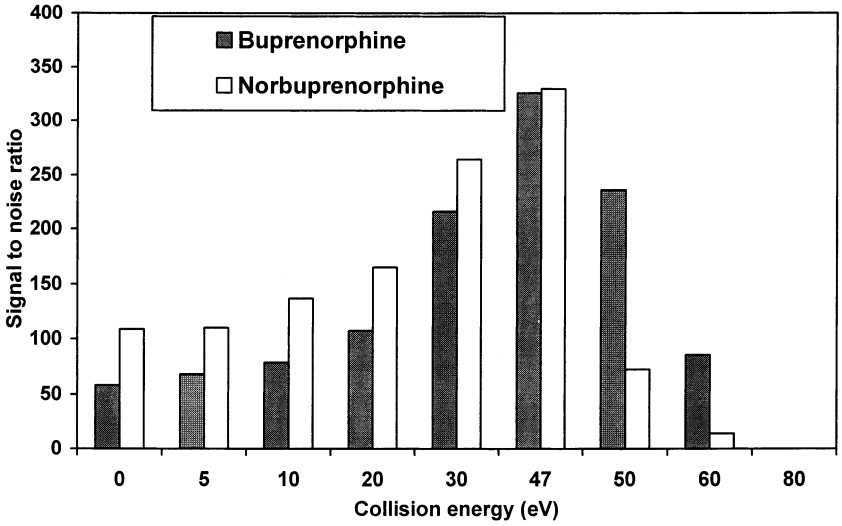


Fig. 2. Influence of collision energy on signal-to-noise ratio.

solutions were then diluted with a methanol/water mixture (50:50, v/v) in order to achieve a final concentration of 100 ng/ml for each compound.

2.5.2. Solutions used for method validation

Six mixed solutions of B and NB were prepared by diluting stock solutions with the methanol/water mixture to reach concentrations ranging from 0.2 to 20 ng/ml for both compounds. These aqueous solutions were then used to spike plasma samples either for calibration curves (0.01–5.0 ng/ml for B and 0.05–5.0 ng/ml for NB) or for quality control during the pharmacokinetic study.

A stock solution of clonazepam (IS) was prepared in methanol. This solution was then diluted with the methanol/water mixture to obtain a final concentration of 0.1 ng/ml.

2.6. Sample preparation

Plasma samples were centrifuged at 3000 × *g* for 10 min and a 1.0-ml volume was transferred manually to a sample vial on the appropriate rack of the ASPEC system. A 200-μl volume of IS solution (0.1 ng/ml) was then automatically added and mixed. The DEC sorbent was first treated with 2.0 ml of methanol and then with 1.0 ml of water. A 1.0-ml volume of sample was then aspirated by the autosampler needle from the corresponding vial and applied onto the DEC. 1.0 ml of water was, therefore, dispensed twice on the DEC to perform the washing step. The elution of analytes was obtained by dispensing twice 0.75 ml of methanol containing 0.1% of acetic acid. Both eluates were collected in the same vial and homogenized. All these operations were performed automatically by the ASPEC system in the batch mode. The resulting extract was then evaporated to dryness for 120 min in a rotational-vacuum concentrator at 60 °C, reconstituted in a 250-μl volume of mobile phase and transferred manually to the LC autosampler rack for analysis. The injection volume was 100 μl.

2.7. Pharmacokinetioc study

The developed LC–MS/MS procedure was used to investigate the plasma profiles of buprenorphine and norbuprenorphine after a single oral dose of an immediate release formulation of buprenorphine. A clinical study on healthy volunteers was conducted. The subjects received a single dose of 400 μg. 25 blood samples were withdrawn at different times until 24 h after the administration of the medication.

Table 1
Recovery of analytes on different sorbents

Type of sorbent	Analyte recovery (%, $n = 3$)	
	Buprenorphine	Norbuprenorphine
Diol	10	47
CN	42	73
C2 endcapped	101	99
C8 endcapped	66	77
C8	83	92
Phenyl endcapped	84	76
C18 endcapped	73	77
C18	59	62

3. Results and discussion

3.1. Optimization of MS conditions

The LC–MS/MS method for the determination of buprenorphine and norbuprenorphine was investigated. For the optimization of MS conditions, each compound was directly introduced in the MS detector using APCI ionization and parameters such as corona discharge, orifice voltage, ring voltage, flow of nebulizer and auxiliary gas (N_2) and temperature of auxiliary gas (N_2) were investigated in order to obtain the protonated pseudomolecular ions of buprenorphine, norbuprenorphine and clonazepam (IS).

The pseudomolecular ions [MH^+] observed on the full scan mass spectra of buprenorphine and norbuprenorphine were m/z 468 and m/z 414 for B and NB, respectively. Moreover, it can be observed that the collision energy in Q2 did not produce significant fragment ions from both compounds of interest. The MS/MS transitions 468/468 and 414/414 for B and NB, respectively, were then selected to perform the quantitation of these compounds.

The most suitable collision energy was determined by observing the signal-to-noise ratio. The collision energy was ranged from 0 to 60 eV. The 0 eV collision energy corresponds to single MS conditions. Fig. 2 illustrates clearly that the best signal-to-noise ratio is situated at 47 eV. This also demonstrates that the sensitivity of the method can be increased by using MS/MS rather single MS conditions. Indeed, increasing the collision energy induces fragmentation of the background ions and allows a significant increase of the signal-to-noise ratio [2]. When the collision energy is increased higher than 47 eV, B and NB are shattered and many low intensity products ions are formed, leading to a significant decrease of the signal-to-noise ratio and, therefore, of sensitivity. The selective reaction monitoring (SRM) mode was then used since the determination of B and NB can be performed with great sensitivity and selectivity.

3.2. Selection of SPE sorbent

Different kinds of DECs containing bonded silicas with various polarities were tested. Spiked plasma solutions were used as samples and the corresponding recoveries of B and NB were determined (Table 1). The recoveries were calculated by comparing the peak areas obtained from freshly prepared samples extracts with those found by direct injection of aqueous solutions at the same concentration into the LC–MS/MS system, using the same autosampler. As can be seen in Table 1, very low recoveries for B and NB were observed with the Diol phase. This can be explained by analytes losses during the loading and washing steps. The best recoveries for the two compounds of interest were observed when DECs filled with ethyl endcapped (C2) sorbent was used. However, some interferences were observed in the chromatograms obtained from plasma samples prepared with C2 DECs. DECs filled with octyl silica (C8) were finally selected regarding the high recoveries obtained both for buprenorphine and norbuprenorphine.

3.3. Validation

3.3.1. Stability

The stability of the whole procedure was studied by considering the different steps of the method. The stability of stock solutions (30 days at 8 °C), autosampler eluate (48 h at 20 °C), plasma sample (24 h at 20 °C), plasma storage (3 months at −80 °C) and after three freeze and thaw cycles was investigated. The determination of B, NB and IS were performed at the beginning and at the end of

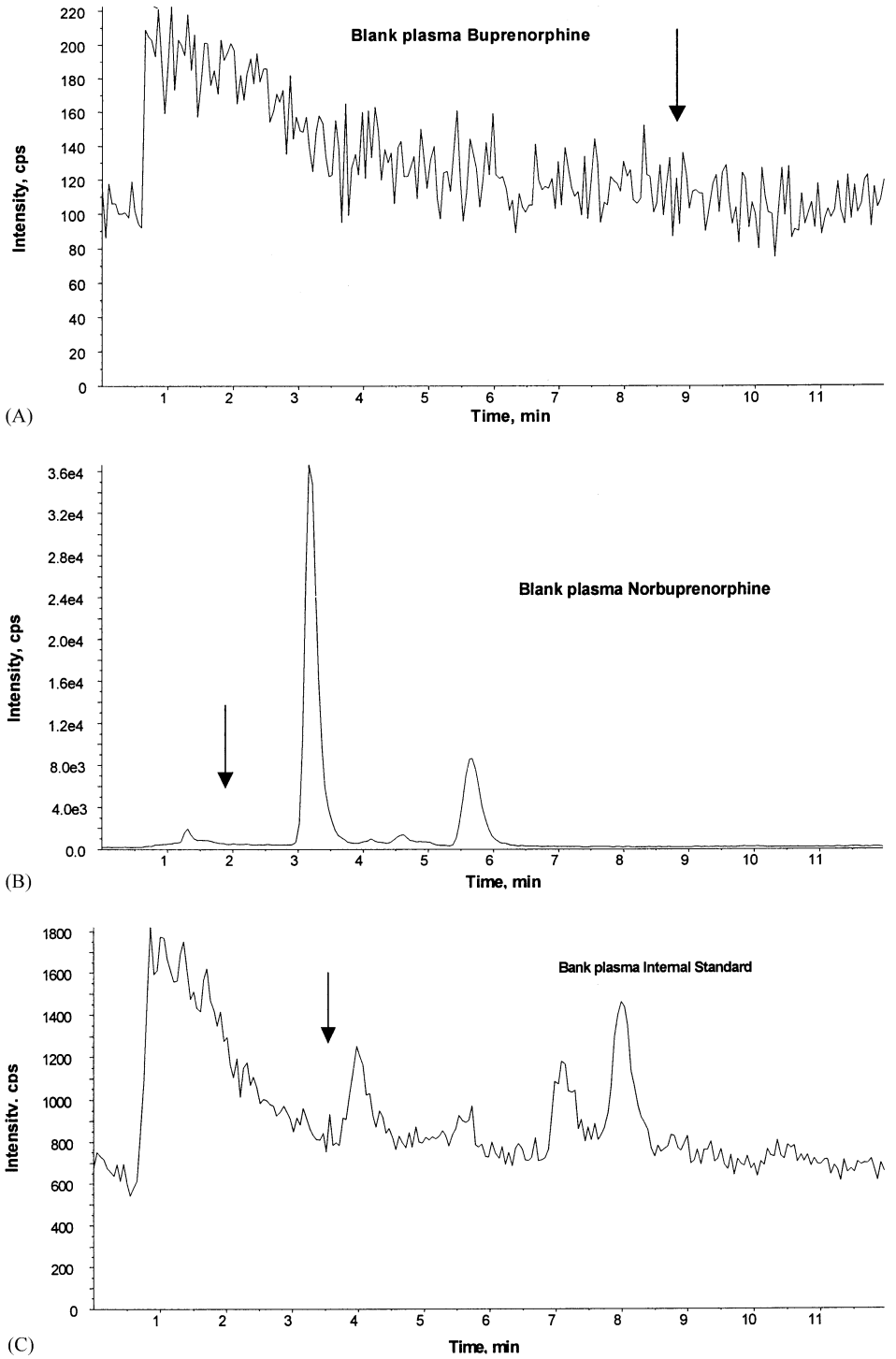
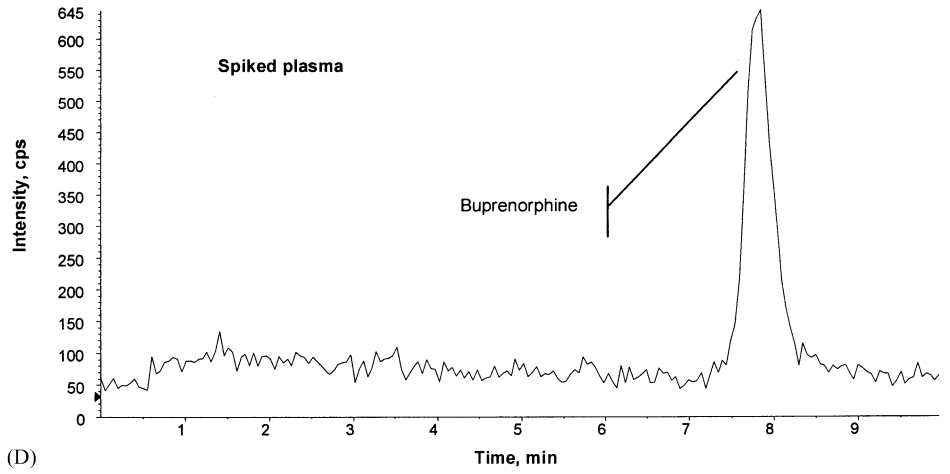
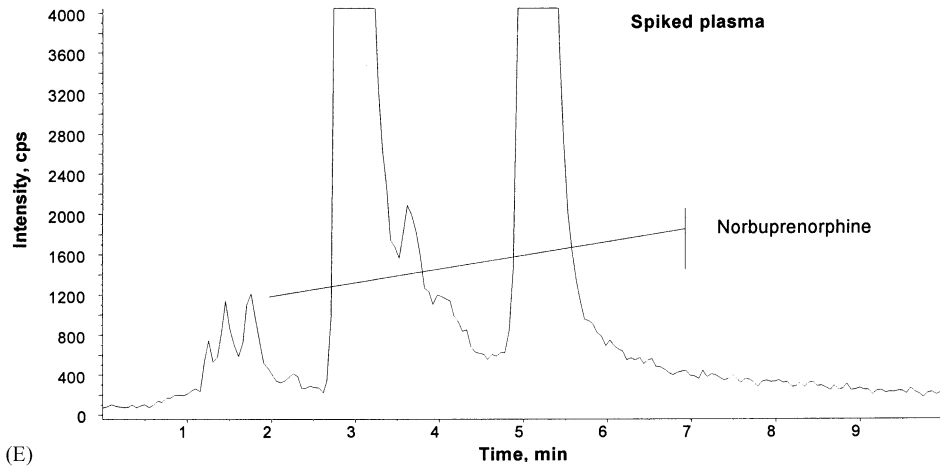


Fig. 3. SRM ion chromatograms of blank plasma (A, B, C) and spiked plasma samples (D, E, F) with 0.01 ng/ml for B, 0.05 ng/ml for NB and 0.1 ng/ml for IS. See text for operating conditions.



(D)



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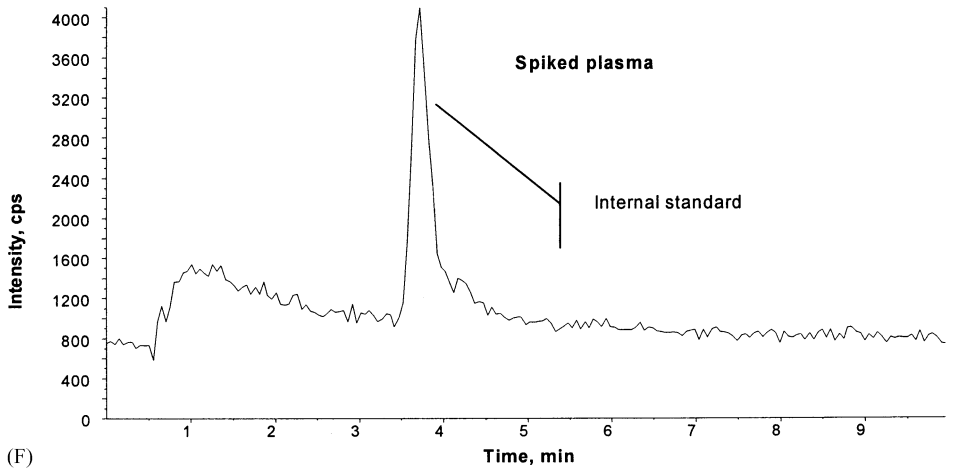

(F)

Fig. 3 (*Continued*)

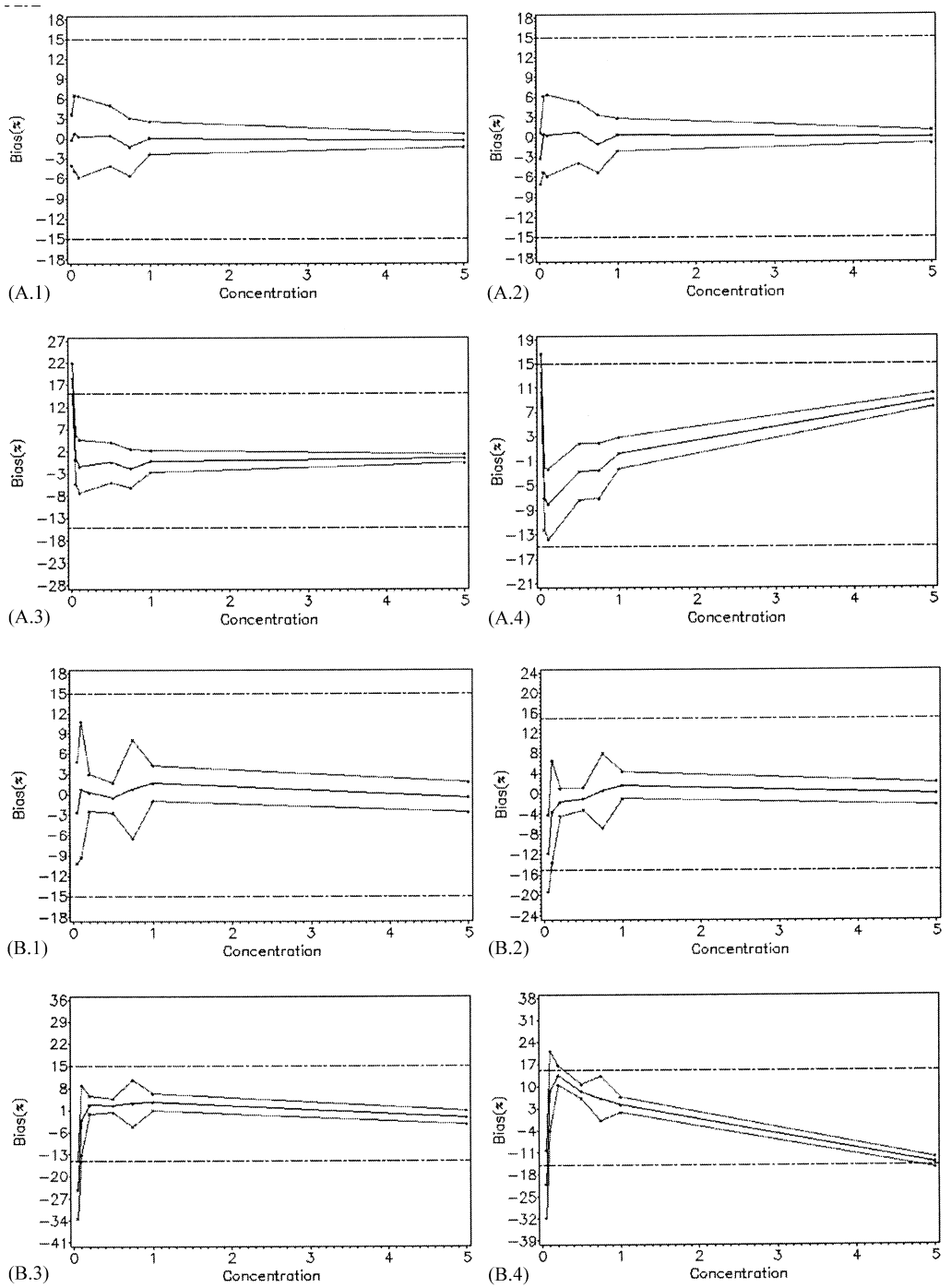

Fig. 4. Accuracy profiles of buprenorphine (A) and norbuprenorphine (B) (concentration in ng/ml) using a (1) weighted linear regression model with a weight equal to 1/X, (2) linear regression model, (3) linear regression model after square root transformation and (4) linear regression model after logarithm transformation.

each storage period. The results obtained were all comprised between 95 and 105% of the initial value. No significant degradation of buprenorphine, norbuprenorphine and IS was observed.

3.3.2. Selectivity

Potential interfering substances in a biological matrix include endogenous matrix components, related substances, metabolites and concomitant medication drugs such as OTC drugs (aspirin, acetaminophen, caffeine, ibuprofen). The selectivity was studied by injecting aqueous solutions of these compounds in the chromatographic system and by analyzing six different sources of plasma. No endogenous source of interference was observed at the retention times of the analytes. Typical chromatograms obtained with a blank plasma and a plasma containing 0.01 ng of B, 0.05 ng/ml of NB and 0.1 ng/ml of IS are presented in Fig. 3.

3.3.3. Response function

An important step of the validation phase must be the assessment of the relationship between the response and concentration in order to avoid serious difficulties in the estimation of other validation criteria. In order to select the most appropriate response function, the SFSTP approach [37] based on two-sided 90% confidence intervals for total measurement error—including both bias and precision—of validation samples has been used. Such an approach reflects more directly the performance of individual assays and will result in fewer rejected in-study runs than the current procedure that compares point estimates of observed bias and precision with the target acceptance criteria, i.e. 15% according to the Washington conference [38]. As illustrated in Fig. 4, once the validation experiments have been performed, the response function can be determined by applying different regression models and, from both analytical responses and regression line obtained, selecting the most suitable accuracy profile for the intended use of the analytical method. On the basis of the accuracy profile generated, regression analysis should be performed in the present study using a weighted least-squares, with weights equal to 1/X for the two compound,

Table 2
Validation of the LC–MS/MS method

Validation criterion	Buprenorphine	Norbuprenorphine
Recovery (mean, $n=9$, %)	83.2	92.2
Linearity ($n=3$, k = 7)		
Range (ng/ml)	0.01–5.0	0.05–5.0
Slope ± S.D.	13.0 ± 0.03	6.13 ± 0.02
Intercept ± S.D.	0.06 ± 0.007	−0.15 ± 0.01
r^2	0.9999	0.9998
LOD (pg/ml)	2	12
LOQ (pg/ml)	7	40
Repeatability ($n=6$, %)		
0.01 ng/ml	3.4%	–
0.05 ng/ml	3.6%	4.1%
1.0 ng/ml	2.7%	2.8%
5.0 ng/ml	2.4%	1.9%
Intermediate precision ($N=6$; 3 days)		
0.01 ng/ml	3.7%	–
0.05 ng/ml	4.0%	4.4%
1.0 ng/ml	3.2%	2.8%
5.0 ng/ml	5.0%	2.4%
Accuracy (*recovery* ± *IC*, %; $n=6$)		
0.01 ng/ml	101.6 ± 2.1%	–
0.05 ng/ml	101.5 ± 2.1%	101.0 ± 3.8%
1.0 ng/ml	100.0 ± 2.0%	100.0 ± 2.8%
5.0 ng/ml	100.1 ± 2.0%	101.5 ± 1.6%

where X is the theoretical concentration. Indeed, better results were obtained using this latter regression model, especially at the lower concentration levels.

The following equations were obtained (concentration range 0.01–5.0 ng/ml for B and 0.05–5.0 ng/ml for NB):

Buprenorphine: $y = 13.0\ (\pm 0.03)x + 0.06\ (\pm 0.007)$

$r^2 = 0.9999$

Norbuprenorphine: $y = 6.13\ (\pm 0.02)x - 0.15\ (\pm 0.01)$

$r^2 = 0.9998$

The determination coefficient (r^2) obtained for the regression line of B and NB demonstrates the relationship between peak area ratio and concentration (Table 2). During routine analysis, the calibration equation was computed by weighted least-squares regression as mentioned, and the

concentration of each calibration sample was calculated. If the back-calculated concentration of a calibration sample did not fall within ±15% of nominal, that sample was discarded and the equation was recalculated. However, for the calibration and the run to be valid, no more than two calibration samples were discarded and at least five accepted calibration samples had to be kept.

3.3.4. Detectability

The limits of detection and quantification for B and NB were estimated as analyte concentrations giving rise to signal-to-noise ratios of 3 and 10, respectively. The LODs and LOQs were found to be 2 and 7 pg/ml, and 12 and 40 pg/ml for B and NB, respectively (Table 2). However, it is probably better to consider that LOQ is around 10 pg/ml for B and 50 pg/ml for NB since the validation has demonstrated that the method is precise and accurate at these concentrations.

3.3.5. Precision

The precision of the bioanalytical method was estimated by measuring repeatability and intermediate precision for both compounds at different concentration levels ranging from 0.01 to 5.0 ng/ml. The variance of repeatability and time dependent intermediate precision as well as the corresponding relative standard deviation (R.S.D.) were calculated from the estimated concentrations. The R.S.D. values presented in Table 2 were relatively low, less than 5% for the lowest concentration of the range and illustrated the good precision of the proposed method.

3.3.6. Accuracy

The accuracy of the procedure was assessed by calculating the ratio between the analyte amount found versus the amount spiked in the plasma at different concentrations levels ranging from 0.01 to 5.0 ng/ml. The accuracy, defined as the mean%± interval of confidence ($P > 0.05$) illustrates that the LC–MS/MS procedure developed can be considered as accurate within the concentration range investigated (Table 2). Mean values are very close to the theoretical concentrations and the joined intervals of confidence are relatively tightened, illustrating the good accuracy of the method.

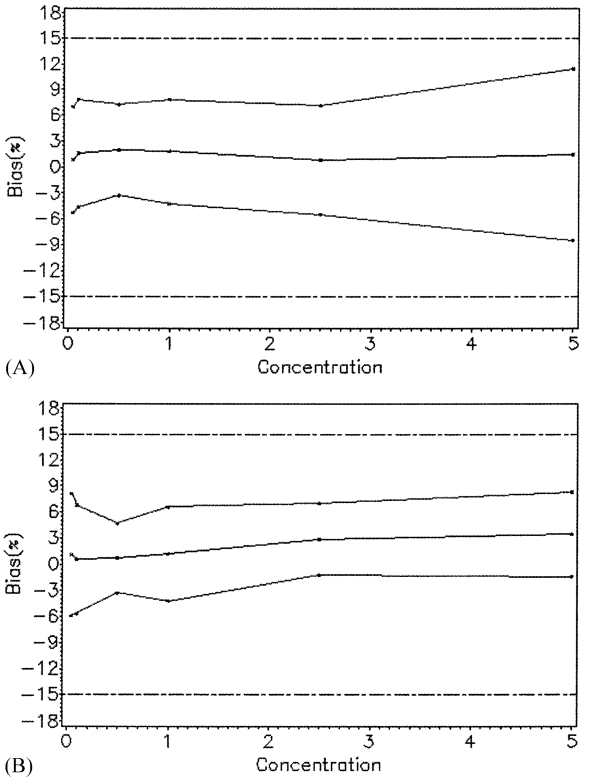

Fig. 5. Accuracy profiles of buprenorphine (A) and norbuprenorphine (B)—concentration in ng/ml.

3.3.7. Method follow-up during routine analysis

Once the assay method has been established for routine use, its performance should be regularly monitored to ensure that it continues to work properly. This is done by using quality control samples at different concentration levels in order to assess method performances during routine analysis. The procedure most widely used for the continuing evaluation of assay performance involves the construction of QC charts. Many schemes for constructing such charts have been suggested [39]. Since the objective of a method of determination consists in, for each sample analyzed, providing with results that are the nearest from their real values, a control, a control chart based on the accuracy profile [37,40,41] has been used in the present study (Fig. 5). The acceptance

Table 3
Control chart calculations: accuracy ± two-sided 90% confidence intervals

QC concentration levels (ng/ml)	Accuracy ($n = 6$)	Precision ($n = 6$)	
	Bias (%)	Lower confidence limit	Upper confidence limit
Buprenorphine			
0.05	0.88	−5.22	6.98
0.1	1.63	−4.59	7.84
0.5	2.00	−3.24	7.25
1.0	1.84	−4.17	7.85
2.5	0.86	−5.43	7.15
5.0	1.51	−8.46	11.48
Norbuprenorphine			
0.05	1.13	−5.85	8.12
0.1	0.57	−5.66	6.81
0.5	0.73	−3.26	4.72
1.0	1.22	−4.17	6.61
2.5	2.90	−1.21	7.01
5.0	3.46	−1.38	8.31

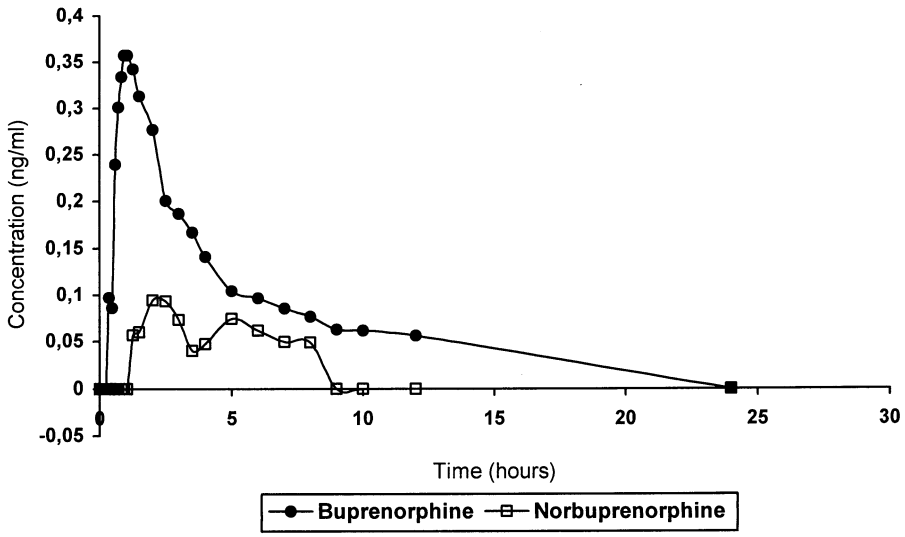

Fig. 6. Plasma concentration–time profiles of B and NB following a single oral dose of 400 μg buprenorphine.

limits have been fixed at ±15% of the bias according to the Washington conference [38]. In order to build the accuracy profiles of B and NB, six concentration levels (Table 3) for each compound ranging from to 0.05 (concentration near the lower limit of quantitation) to 5 ng/ml (upper concentration level of the standard curve) have been daily monitored. Accuracy data are presented in Table 3. As illustrated in Fig. 5, the accuracy profile of buprenorphine is very good since the bias at each concentration level is very close to zero and the joined confidence intervals are within the acceptance limits. The accuracy profile of norbuprenorphine indicates a slight trend in the

bias data (from 0.57 to 3.46%) but the two-sided 90% confidence limits are comprised between the acceptance limits of ±15%. The process is thus under control and analytical results can be used to investigate the plasma profiles of buprenorphine and norbuprenorphine.

3.4. Pharmacokinetics

The LC–MS/MS procedure developed was used to investigate the plasma profile of buprenorphine and its metabolite norbuprenorphine after multiple oral dose of an immediate release formulation of buprenorphine (tablet containing 400 μg of buprenorphine). The Pharmacokinetic profile of plasma concentrations of B and NB versus post-dose sampling time is presented in Fig. 6.

4. Conclusions

A sensitive, accurate and precise procedure based on the SPE coupled at-line to a LC–MS/MS determination has been developed for the simultaneous assay of buprenorphine and its metabolite norbuprenorphine. The extraction procedure and the MS/MS conditions were optimized in order to increase the sensitivity of the method. The procedure was validated to meet the requirements of the pharmacokinetic investigation of these two compounds. The procedure developed was successfully applied to the determination of B and NB plasma levels for investigating a pharmacokinetic study.

Acknowledgements

Many thanks are due to S. Bodart, C. Laine, R. Sibenaler and C. Zimmer for technical assistance.

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