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Determination of buprenorphine in plasma by liquid chromatography: application to heroin-dependent subjects

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Abstract

A rapid, sensitive, precise and accurate HPLC assay with UV detection was developed for the determination of buprenorphine (BN) in human plasma. This method involved a two-step extraction in the presence of clothiapine as internal standard. The compounds were chromatographied on a reversed-phase Spherisorb® C8 column with a mobile phase consisting of 0.06 M KH_2PO_4/Na_2HPO_4 pH 6.4–acetonitrile–triethylamine–Pic B5® (520:480:0.5:15, v/v) and detected at 214 nm. The recovery of BN was greater than 94% with an intra-day relative standard deviation $\leq 4.8\%$ and an inter-day relative standard deviation $\leq 14.6\%$ at any studied level. Studies of drug stability during sample storage at $-20°C$ and at $+4°C$ did not show any significative degradation of BN. This method was successfully applied to explore the overdose state of heroin-dependent subjects treated by high-dose BN.

Keywords: Buprenorphine; Reversed-phase chromatography; Heroin dependence; Toxicology

1. Introduction

Buprenorphine (Fig. 1), 21-cyclopropyl-7α-[(*S*)-1-hydroxy-1,2,2-trimethylpropyl]-6,14-endo-ethano-6,7,8,14-tetrahydroororipavine, a semi-synthetic, highly lipophilic opioid is 25–50 times more potent than morphine [1]. This compound, primarily used as an analgesic agent, now appears

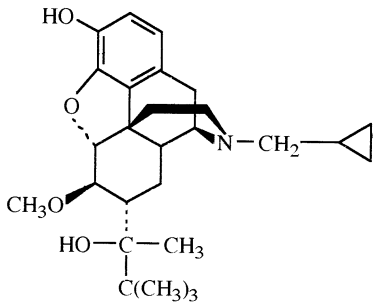

Fig. 1. Chemical structure of buprenorphine.

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to be useful as a maintenance drug for heroin dependent subjects (Subutex®: high-dose buprenorphine). In the first month of maintenance treatment, some overdose cases were reported. Accordingly, it seemed important to provide a specific analytical method to explore the etiology of these overdoses.

Several methods have been described to determine buprenorphine concentrations in plasma, such as radioimmuno-assays [2–4], gas chromatographic [5–7] and high performance liquid chromatographic (HPLC) [8,9] procedures.

Among the previously reported HPLC assays, different detections were used such as UV spectrophotometry [10,11], fluorometry [8,10], electrochemical [12–14] or mass spectrometry [9]. Both HPLC methods with UV detection are too time-consuming due to a three-step liquid extraction [10] or two-step solid extraction (C18 bonded silica column and thin-layer chromatography purification) [11].

The method described below is rapid, specific and adapted to analytical toxicology in overdose cases of heroin addicts.

2. Experimental

2.1. Chemicals

Buprenorphine (BN) was supplied by Sigma (Saint Quentin Fallavier, France) and the internal standard (clothiapine) by Sandoz (Rueil Malmaison, France). All chemicals were of analytical reagent grade; all solvents were of HPLC grade. Triethylamine was supplied by Info-Labo (Sainte Foy la Grande, France) and Pic B5® reagent by Waters Assoc. (Milford, MA). Water was deionized and doubly glass distilled. Human heparinized plasma of healthy subjects was purchased from the Aquitaine Establishment of Blood Transfusion (E.T.S.A. Bordeaux, France).

2.2. Chromatographic conditions

The HPLC system consisted of a Waters apparatus, including a Model 45 constant-flow pump, an U6K manual injector and a lambda Max Model 480 ultraviolet detector operated at 214 nm. The separation of compounds was carried on a 150×4.6 mm column packed with a Spherisorb C8 (Waters). Chromatograms were recorded on a 10-mV recorder Omniscribe (Houston Instruments) at a chart speed of 0.5 cm min^{-1}.

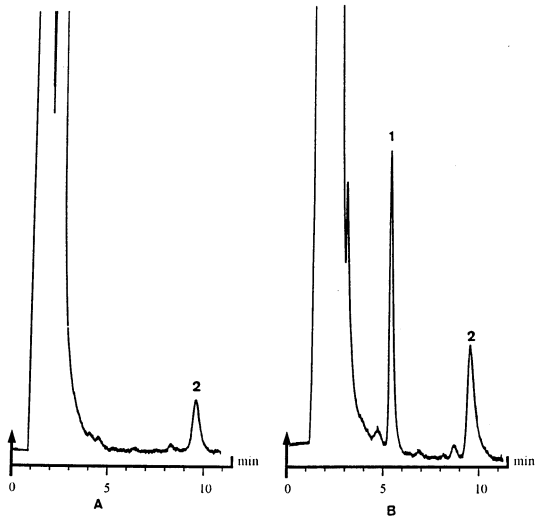

Fig. 2. Chromatograms of (A) human drug-free plasma; (B) blank human plasma with 50 ng ml^{-1} of buprenorphine. Peak 1: BN; peak 2: i.s.

The mobile phase consisted of phosphate buffer (0.06 M, pH 6.4)–acetonitrile–triethylamine–Pic B5® (520:480:0.5:15, v/v), the pH of the mixture was adjusted to 6.4 with orthophosphoric acid. Before use, the eluent was filtered through a 0.22-μm filter (Sartorius, Göttingen, Germany). The flow-rate was maintained at 1.6 ml min^{-1}.

2.3. Standard solutions

Stock solutions of BN and clothiapine (1 mg ml^{-1}) were prepared in methanol and stored at − 20°C without degradation for 3 months. Appropriate dilutions of BN were made in drug-free human heparinized plasma to provide final concentrations of 2, 5, 10, 20 and 50 ng ml^{-1}.

2.4. Sample preparation

To 2-ml human plasma placed in a 10-ml glass extraction tube (fitted with PTFE-lined screw cap)

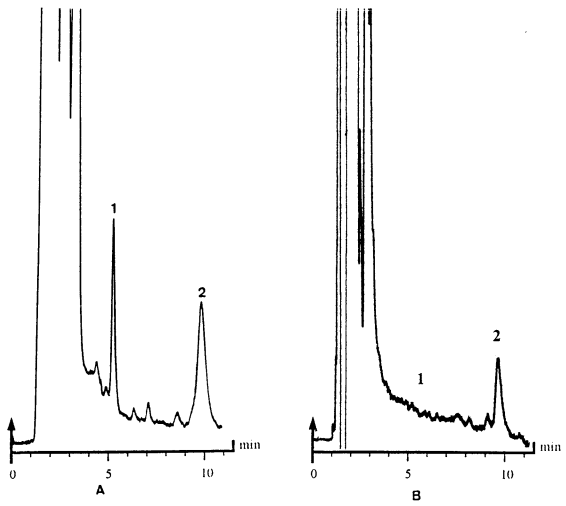

Fig. 3. Chromatograms of patient X sample: (A) containing 32 ng ml^{-1} of buprenorphine; (B) at the end of the hospitalisation without co-eluting endogenous compounds. Peak 1: BN; peak 2: i.s.

100 μl of internal standard (i.s) solution (0.5 μg ml^{-1}) and 100 μl of 1 N sodium hydroxide were added. The samples were extracted with 6 ml of a mixture of hexane–isopropyl alcohol (99:1, v/v) by rotary mixing for 10 min. After centrifugation at $2000 \times g$ for 5 min, the organic layer was transferred into another 10-ml glass tube containing 300 μl of 0.2 N hydrochloric acid. After 10 min on a rotary shaker and centrifugation at $2000 \times g$ for 5 min, the upper organic layer was discarded. Then, an aliquot of the acid phase (120 μl) was injected into the HPLC system.

3. Results and discussion

Under these chromatographic conditions, BN and the internal standard were well resolved from endogenous plasma compounds (Fig. 2AFig. 3B). The retention times of BN and clothiapine were 5.2 and 9.6 min, respectively. The ability of 29 compounds to interfere with BN or clothiapine was investigated. Their retention times and capacity factors are shown in Table 1.

Both the intra-day and the inter-day precision of this method were determined by replicates analysis of three different quality control (QC) plasma containing 3, 15 and 30 ng ml^{-1} of buprenorphine. Nine replicates were assayed to determine the intra-day precision. Three replicates were carried out per day during 6 days to access the inter-day precision. The results presented in Table 2 are expressed as the relative standard deviation (R.S.D.) of the replicate measurements.

Table 1
Retention times (t_r) and capacity factors (k') of potentially interfering compounds

Compounds	t_r (min)	k'	Compounds	t_r (min)	k'
Buprenorphine	5.20	2.58	Doxepine*	4.60	2.17
Internal standard	9.60	5.62	Nortriptyline*	5.17	2.56
Norbuprenorphine	1.50	0.03	Oxaflozane*	5.20	2.58
Viloxazine	1.65	0.14	Maprotiline*	5.20	2.58
Naloxone	2.20	0.52	Dosulepine*	5.67	2.91
Tianeptine	2.50	0.72	Imipramine	6.17	3.25
Trazodone	2.67	0.84	Quinupramine	6.50	3.48
Opipramol	3.00	1.07	Mianserine	6.50	3.48
Diazepam	3.00	1.07	Amitryptyline	7.00	3.83
Metapramine	3.17	1.19	Methadone	7.10	3.90
Demexiptilline	3.66	1.52	Carpipramine	8.66	4.97
Amoxapine	3.67	1.53	Clomipramine*	9.16	5.27
Normaprotiline	4.00	1.76	Codeine		No response
Paroxetine	4.17	1.88	Pholcodine		No response
Medifoxamine	4.50	2.10	Codethyline		No response
Desipramine	4.50	2.10	Morphine		No response

* Interfering compounds.

Table 2
Accuracy and precision of results for plasma spiked with buprenorphine

Quality control sample concentration (ng ml^{-1})	Replicates (n)	Mean measured concentration (ng ml^{-1})	RSD[a] (%)	Accuracy[b] (% Bias)
Intra-day				
3	9	3.3	3.5	8.51
15	9	14.5	2.9	−3.55
30	9	29.9	4.8	−0.51
Inter-day				
3	3	2.8	12	−4.88
15	3	14.6	14.6	−2.66
30	3	28.7	10.4	−4.69

[a] Relative standard deviation = (S.D./mean) × 100.
[b] Accuracy is expressed as % Bias:
% Bias = [(measured concentration − true concentration)/true concentration] × 100.

The variance homogeneity was controlled by a Cochran test which was non-significant at each concentration of the QC samples ($C_{obs} < C_{(0.05; 6; 2)}$ and $< C_{(0.01; 6; 2)}$). There was no day effect according to the results of one-way ANOVA at each concentration (NS; $p > 0.05$; $F_{(5, 12)} = 2.544$, 1.212 and 1.307 for 3, 15 and 30 ng ml^{-1}, respectively)

The accuracy is reported in Table 2 as percentage bias. It is calculated from the following expression: % Bias = [(measured value − true value)/true value] × 100, where the measured concentrations were those obtained during the estimation of precision [15].

The limit of detection for buprenorphine was 1 ng ml^{-1} (signal to noise ratio = 3).

The lower and upper limits of quantification (LLOQ and ULOQ), using LOQ quality control samples, were 2 and 50 ng ml^{-1}, respectively. The precisions and accuracies were 14.64 and 4.08% at the LLOQ, and 3.20 and −3.35% at the ULOQ, respectively. The linearity of dilution were not accessed since the samples are diluted with plasma which is the matrix of validation [15].

From 18 calibration curves each constructed with five unique calibration points ranging from 2–50 ng ml^{-1}, a high correlation coefficient was found ($r^2 = 0.976$; $p < 0.001$). The relationship between the peak-height ratio (y) and the concentration (x) was: $y = 0.062x + 0.005$ with standard deviations being equal to 0.006 for the slope and 0.067 for the intercept.

To determine the influence of temperature on the stability of buprenorphine, QC human plasma samples spiked with 3, 15 and 50 ng ml^{-1} of buprenorphine were stored at −20°C for 28 days and at +4°C for 8 days. The mean concentrations values of stability samples were compared against freshly prepared 100% controls analysed in the same analytical run, in replicates of six. Statistical analysis were performed by the Fisher–Snedecor test for the variance and the Student's t-test for the means (Table 3). No deterioration was observed in the quick frozen samples during four weeks. Storage for as long as 1 week at +4°C produced no significant decrease of buprenorphine concentrations ($p > 0.05$). This agrees with the results of Ho et al. [8].

The absolute recovery of the method was established by comparing the peak heights of extracted plasma samples at all levels of the calibration curve in replicates of nine with those of non-extracted standard prepared in mobile phase. The percentage recoveries were between 94 and 106% for buprenorphine and between 86 and 99% for the internal standard (Table 4). This extraction procedure requires non-hemolized plasma since the plasma/red blood cell ratio of buprenorphine is close to unity [2]. The N-dealkylated BN metabolite extracted under the procedure described here would not be dosed since it does not cross the hemo-encephalic membranes and has poor pharmacological activity on the μ receptors as compared with BN [16]. The BN and N-

Table 3
Buprenorphine stability in plasma using the Fisher–Snedecor test for the variances (F-value) and the Student's t-test for the means ($|t|$-value)

Quality control sample concentration (ng ml^{-1})	Replicates	Mean concentration found (ng ml^{-1})	S.D. (ng ml^{-1})	F-value[a]	$\|t\|$-value[a]
Stability at −20°C				(5.05)	(2.23)
3	6	3.23	0.10	2.16	1.11
15	6	14.45	0.35	1.47	0.17
30	6	29.78	0.95	1.98	1.69
Stability at +4°C				(5.05)	(2.23)
3	6	3.17	0.11	1.57	0.21
15	6	14.43	0.34	1.42	0.09
30	6	30.03	0.63	1.13	1.53

Values given between brackets are theoretical values ($p < 0.05$ for all tests).
[a] Reference for statistical comparison are the values of freshly prepared controls analysed in the same analytical run.

dealkylated BN glucuronides are not extracted. However, we showed (Fig. 4) that there is no possibility for the N-dealkylated BN to co-elute with either BN or i.s. With the chromatographic conditions used, this metabolite eluted strongly before BN (Table 1).

4. Patients results

We reported the results of three patients (X, Y, Z) who have recently begun a maintenance treatment by sublingual buprenorphine. The administered dose and route were unknown. Patients X (Fig. 3A) and Y, were admitted in the emergency department for a comatose episode, the measured plasma concentrations of BN were 32 and 45 ng ml^{-1}, respectively. The chromatogram of patient X obtained at the end of the hospitalisation, showed neither BN nor endogenous compounds at $t = 5.20$ min (Fig. 3B). Alternatively, patient Z presented a measured concentration of 4 ng ml^{-1}.

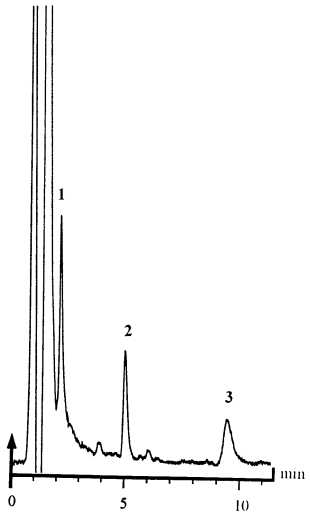

Fig. 4. Chromatograms of human drug-free plasma spiked with norbuprenorphine (peak 1), BN (peak 2) and i.s. (peak 3).

For patients X and Y, but not for Z the results were higher than usually reported after unique sublingual administration of BN 8 mg (mean $C_{max} = 3.1$ ng ml^{-1}) [17]. Since administered dose and route were unknown it appears difficult to explain the measured plasma concentrations. Nevertheless, this was not the aim of the study. Alternatively, these data provide strong evidence that buprenorphine can be easily dosed with the outlined method, this is of major interest since it is well recognized that respiratory depression after BN is resistant to naloxone [18,19]. However, respiratory depression following BN administration [20] has not been a major problem in addicts, which have developed pharmacological tolerance.

This analytical procedure is sensitive enough to explore the possible clinical overdose states at the beginning of a maintenance treatment or in forensic toxicology since buprenorphine has a potential for abuse [21]. Sublingual buprenorphine, that appears useful for the treatment of opiate dependence, still has potential for parenteral abuse since

Table 4
Absolute recoveries of buprenorphine and the internal standard from spiked plasma samples

	Buprenorphine concentration (ng ml^{-1})				
	2	5	10	20	50
Replicates	9	9	9	9	9
BN recovery[a]	104 ± 7.2	96 ± 5.2	102 ± 5.7	106 ± 12.5	94 ± 12.4
i.s. recovery[a]	95 ± 4.3	86 ± 5.9	99 ± 1.9	95 ± 5.7	97 ± 7.2

[a] Absolute recovery = [peak heights of analyte spiked into plasma (extracted)]/[peak heights of analyte prepared in mobile phase (unprocessed)].

her formulation is not a combination with an opiate antagonist of low sublingual absorption [22].

5. Conclusion

The HPLC method described for the determination of buprenorphine in human plasma was specific, reliable and rapid. The LLOQ (2 ng ml^{-1}) and the total time of this assay were especially adapted to emergency use. Thus, this method could be successfully used in pharmacokinetic studies for intoxicated people suspected or not of abuse. Therefore, this assay is now proposed for the determination of plasma levels of buprenorphine in addicts admitted in maintenance program for heroin-dependent subjects.

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