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Research paper

Development and validation of a sensitive analytical method for the simultaneous determination of buprenorphine and norbuprenorphine in human plasma

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Abstract

A sensitive, specific, and robust capillary gas chromatography–mass spectrometry method has been developed and validated for simultaneous determination of buprenorphine and its active metabolite, norbuprenorphine, in human plasma. Sample preparation involved a clean-up procedure using a Bond Elut Certify cartridge followed by derivatization with pentafluoropropionic anhydride. Separation was carried out on a HP-1 fused silica capillary column using helium as the carrier gas. Selected ion monitoring was used in the electron impact mode. Excellent linearity was found between 0.10 and 20.0 ng/ml with a limit of quantitation of 0.05 and 0.10 ng/ml for buprenorphine and norbuprenorphine, respectively. Interday and intraday assay precisions (%CV) and accuracies were within 15.0% for buprenorphine and norbuprenorphine, respectively. Recoveries were quantitative and concentration-independent. This method will be applied to pharmacokinetic/pharmacodynamic/bioequivalence studies of buprenorphine in humans.

Keywords: Bioanalysis; Buprenorphine; Norbuprenorphine; Human plasma; Gas chromatography–mass spectrometry

1. Introduction

Buprenorphine, a synthetic opioid analgesic (Fig. 1), is about 30 times more potent than morphine [1] and is well known to be effective in the treatment of post-operative pain in patients. It is currently marketed in the parenteral form as Buprenex®. Buprenorphine has been of great interest to drug abuse researchers since 1978 when Jasinski and co-workers, in a clinical study, first recognized the drug's capacity to block the euphoria produced by opiates [2]. After Mello and associates reported that buprenorphine decreased heroin self-administration [3,4], extensive clinical trials have demonstrated that buprenorphine is effective for maintenance in opioid-dependent patients.

The pharmacokinetics and disposition of buprenorphine, and the metabolite norbuprenorphine (Fig. 1), in humans or animals have not been well characterized due to the lack of sensitive analytical methods to determine the very low plasma concentrations (less than 1.0 ng/ml) [5,6]. Limited information suggests that buprenorphine is metabolized in humans to norbuprenorphine and to conjugated buprenorphine and conjugated norbuprenorphine [7,8]. These glucuronides are then elimiated via biliary excretion. First-pass inactivation by gut wall and entero-hepatic cycling has also been demonstrated [8,9].

Various methods have been developed for the determination of buprenorphine in biological specimens, using either gas chromatography with electron capture detector [10–12], gas chromatography-mass spectrometry (GC-MS) [7,13–17], gas chromatography-tandem mass spectrometry (GC-MS-MS) [18,19], or high-performance liquid chromatography (HPLC) with electro-chemical detector [20] and fluorescence detector [21]. One of the major problems with traditional HPLC methods was their lack of sensitivity.

Most of the methods either did not report any assay validation or reported incomplete assay validation [7,10,13,18–21,23], could not perform simultaneous determination of buprenorphine and norbuprenorphine in human plasma [7,10,13,17,20,21], lacked sensitivity [7,13,18] or had difficulties in being adapted for routine analytical use. Very little work in characterizing the pharmacokinetics/disposition of

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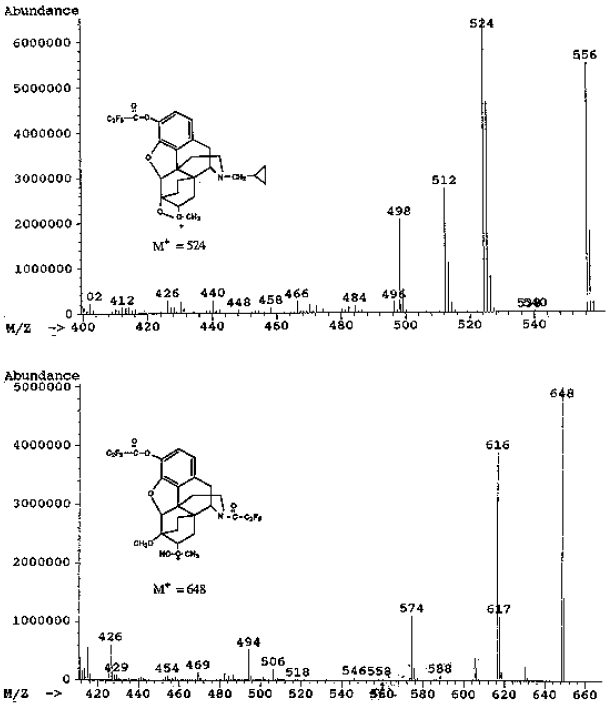

Fig. 1. Proposed structures and mass spectra of the PFPA derivatives of (a) buprenorphine and (b) norbuprenorphine.

buprenorphine in humans has been conducted until recently with the development of HPLC-MS-MS methods [17,22,23]. However, the high cost and relative inaccessibility of HPLC-MS-MS facilities remain major obstacles in pharmacokinetic/pharmacodynamic studies of buprenorphine in humans and animals.

The GC-MS method described here presents the advantages of being sensitive, comprehensive, reproducible, specific and robust for simultaneous measurement of buprenorphine and norbuprenorphine levels in human plasma.

2. Materials and methods

2.1. Reagents

Buprenorphine hydrochloride was purchased from Sigma (St. Louis, MO), norbuprenorphine hydrochloride from Ultrafine Chemicals (Manchester, UK) and buprenorphine-D4 (Internal Standard) from Radian International (Austin, TX). Pentafluoropropionic anhydride (PFPA) was of analytical grade and purchased from Sigma. Methanol, methylene chloride and 2-propanol were of HPLC grade and purchased from Fisher Chemical (Fair Lawn, NJ). All other chemicals (sodium acetate, sodium phosphate monobasic, sodium phosphate dibasic, glacial acetic acid, ammonium hydroxide) were of analytical grade and purchased from Fisher Chemical. Bond Elut Certify solid-phase extraction (SPE) cartridges were purchased from Varian International (Walnut Creek, CA).

2.2. Standards and solutions

Standard stock solutions of buprenorphine hydrochloride (10 mg/10 ml), norbuprenorphine hydrochloride (10 mg/10 ml) and buprenorphine-D4 (1 mg/10 ml) were prepared in methanol and refrigerated at 8°C. The working solutions were prepared as and when required by serial dilution of the stock solutions with methanol.

2.3. Instrumentation and chromatographic conditions

GC-MS determination under selected ion monitoring (SIM) mode was carried out on a HP 5890 gas chromatograph equipped with a 5971A mass selective detector. A HP-1 (cross-linked methyl silicone gum) fused silica capillary column (12 m long, 0.2 mm i.d. and 0.33 μm film) was used). The injector and detector temperatures were set at 280 and 325°C, respectively. The oven was programmed as follows: 120°C for 2.5 min, increased at the rate of 20°C/min until 280°C, and held constant for 4 min. Helium was used as carrier gas at a flow rate of 1.05 ml/min. The mass selective detector was operated in the selected ion monitoring mode and set at *m/z* 528 and 560 for the internal standard, *m/z* 524 and 556 for buprenorphine and *m/z* 648 and 616 for norbuprenorphine, respectively.

2.4. Extraction and derivatization procedures

All glassware used in this study was silanized with 5% solution of dimethylchlorosilane in toluene prior to use. To plasma aliquots (1 ml) were added 2 ng of internal standard, 4 ml deionized water and 2 ml of phosphate buffer (pH 6.0, 100 mM). The samples were then vortexed, centrifuged and loaded on to the preconditioned SPE columns. The SPE columns were preconditioned with 1×3 ml methanol, 1×3 ml water and 1×1 ml phosphate buffer. The loaded columns were then washed with 1×2 ml water, 1×2 ml acetate buffer (pH 4.5) and 1×3 ml methanol. The columns were dried for 5 min at >10 mmHg. The sample was then eluted with 1×4 ml of methylene chloride-isopropanol-ammonium hydroxide (78:20:2). The extracts were evaporated to dryness under nitrogen. One hundred and fifty microliters of PFPA were added to the tubes and reacted for 60 min at room temperature. The excess PFPA was removed by evaporating under nitrogen. The residues were reconstituted with 15 μl of ethyl acetate and 4 μl was injected for GC-MS analysis.

2.5. Standard calibration curve

For the standard curve, 1 ml blank plasma samples were spiked with 2 ng of internal standard and different concentrations of buprenorphine and norbuprenorphine solutions followed by extraction and analysis using the method described earlier in Section 2.4. The concentrations used

for the calibration curve were 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 7.5, 10.0, 15.0 and 20.0 ng/ml plasma and that of internal standard was 2 ng/ml. The standard curve was constructed with the amount of buprenorphine/norbuprenorphine added to the blank plasma specimens and the response (peak area) ratio of buprenorphine/norbuprenorphine to the internal standard as recorded from the mass spectrometer.

2.6. Quantitation of buprenorphine and norbuprenorphine

Buprenorphine/norbuprenorphine levels in the plasma specimens were quantified by the ratio of the responses (peak area) of buprenorphine/norbuprenorphine and internal standard. With this ratio, buprenorphine/norbuprenorphine concentration in the plasma specimen was computed on the basis of the standard curve prepared as described in Section 2.5. Weighted linear regression was used for the calculation of all standard calibration curves.

2.7. Assay method validation

Assay method validation consisted of interday variation, intraday variation, recovery, and limit of quantitation (LOQ). Interday variation was tested on five different working days. Calibration standards, blank plasma samples and QC samples were prepared on each validation day. The QC samples for interday variation, intraday variation and recovery were 0.75, 3 and 18 ng/ml human plasma and those for LOQ were 0.05, 0.1 and 0.2 ng/ml human plasma. The QC samples were prepared in triplicate for interday variation and in six replicates for intraday variation, recovery and LOQ, respectively. The QC samples were prepared by spiking 1 ml blank plasma samples with 2 ng of internal standard and the different QC concentrations of buprenorphine and norbuprenorphine followed by extraction and analysis using the method described under Section 2.5.

2.8. Absolute recovery

The absolute extraction recovery of buprenorphine and norbuprenorphine from human plasma was estimated using spiked plasma samples at the above QC concentrations. These samples were extracted as described earlier (Section 2.7) except that the internal standard was added to the collected extract from solid-phase cartridge before evaporation of the eluate. In addition, a set of drug-free human plasma was also extracted (Section 2.4). Each drug-free plasma extract was then supplemented with buprenorphine and norbuprenorphine (0.10, 0.20, 0.50, 1.0, 2.0, 5.0, 7.5, 10.0, 15.0 and 20.0 ng/ml) along with the internal standard, and was analyzed to construct a calibration curve. The concentrations of the spiked plasma samples were calculated from the curve and compared to the theoretical values to derive the extraction recovery.

3. Results and discussion

3.1. Performance of chromatographic system and specificity

The mass spectra of the PFPA derivatives of buprenorphine and norbuprenorphine are shown in Fig. 1 where ions 524, 556, 616, and 648 exhibited the highest abundance for buprenorphine and norbuprenorphine, respectively, and hence selected as the major ions for monitoring. The chromatogram of blank plasma extract was relatively clean with no significant interfering peaks (Fig. 2). Fig. 3 displays a representative chromatogram of calibration plasma containing 1 ng/ml of buprenorphine and norbuprenorphine. Both buprenorphine and norbuprenorphine showed sharp, well-defined peaks at the retention times of 9.98 and 10.77 min, respectively, with baseline separation. The relatively short retention times of buprenorphine and norbuprenorphine enabled a chromatographic run time of 15 min. This made it possible to analyze about 60 samples per day, including those used for the standard curve and quality controls. Cleanliness and silanization of the glass liner were essential to maintain the sensitivity of buprenorphine and norbuprenorphine. This was achieved by changing the silanized glass liner every day.

3.2. Calibration curve and sensitivity

The calibration curves exhibited excellent linearity with a mean correlation coefficient of 0.993 for buprenorphine and 0.995 for norbuprenorphine ($n = 5$). The regression equations for buprenorphine and norbuprenorphine were: $y = 0.87x - 0.01$; $r^2 = 0.993$ and $y = 0.50x + 0.01$;

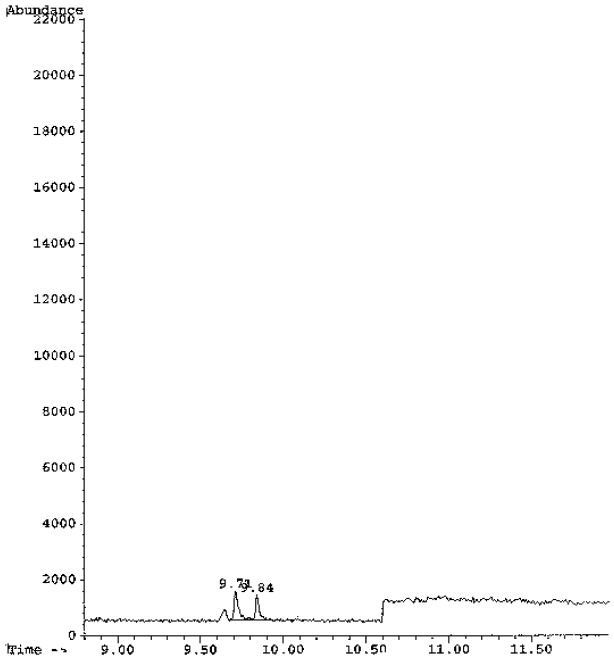

Fig. 2. Representative gas chromatogram of blank plasma.

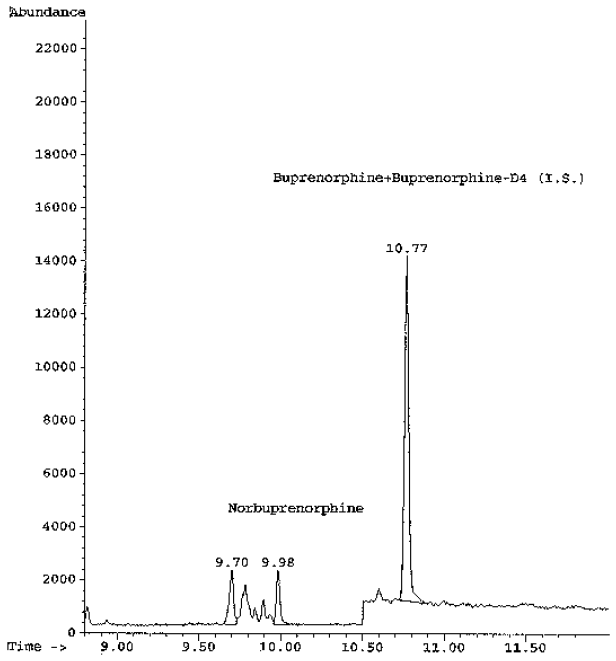

Fig. 3. Representative gas chromatogram of buprenorphine (1.0 ng/ml) and norbuprenorphine (1.0 ng/ml) spiked in blank plasma.

$r^2 = 0.995$, respectively. The error from theoretical value ranged from −16.40 to 6.60% for buprenorphine and from −0.81 to 6.41% for norbuprenorphine, respectively.

The LOQ of buprenorphine was 0.05 ng/ml with an associated accuracy of −3.34% and a CV of 14.8% and that of norbuprenorphine was 0.1 ng/ml with an associated accuracy of −8.70% and a CV of 15.4%. The average error from theoretical value for buprenorphine was 1.30% with an average CV of 11.5%. The average error from theoretical value for norbuprenorphine was 0.23% with an average CV of 18.8%. Using a peak to noise ratio of 3 as a criterion, the estimated limits of detection were 0.03 and 0.04 ng/ml for buprenorphine and norbuprenorphine, respectively. This method provided a better sensitivity when compared to other GC-MS methods where the reported LOQs were 0.2 ng/ml for BN and NBN [13], 0.2 and 0.03 ng/ml for BN and NBN [18] and 0.15 ng/ml for BN [7].

Table 2
Intraday assay variation for buprenorphine and norbuprenorphine

Theoretical concentrations (ng/ml)	Recovered concentrations (mean ± SD) (ng/ml)	CV (%)	DT (%)
Buprenorphine			
0.75	0.83 ± 0.13	10.2	− 3.08
3.00	2.93 ± 0.27	9.13	2.34
18.0	18.1 ± 0.84	4.63	0.38
Norbuprenorphine			
0.75	0.80 ± 0.10	14.5	− 6.67
3.00	2.83 ± 0.21	4.00	5.67
18.0	19.6 ± 0.44	2.05	8.89

3.3. Precision and accuracy

The results of interday and intraday assay variation for buprenorphine and norbuprenorphine are shown in Tables 1 and 2, respectively. The interday coefficients of variation were relatively low with values within 10% for buprenorphine and norbuprenorphine. Accuracy was very good with deviation of nominal concentrations within 0.4% for buprenorphine and within 3.0% for norbuprenorphine. The average deviations from theoretical value for buprenorphine and norbuprenorphine were −0.24% ranging from 0.13 to 0.34% and 1.87% ranging from 0.33 to 2.94%, respectively. The average coefficients of variation for buprenorphine and norbuprenorphine were 6.82%, ranging from 5.24 to 7.78% and 6.46%, ranging from 4.22 to 9.72%, respectively.

The intraday coefficient of variations was within 11% for buprenorphine and within 15% for norbuprenorphine. Accuracy was reasonably good with deviation of nominal concentrations within 5% for buprenorphine and within 10% for norbuprenorphine, respectively. The average deviation from theoretical value for buprenorphine was 1.93%, ranging from −3.08 to 2.34% and for norbuprenorphine was 7.08%, ranging from −6.67 to 8.89%, respectively. The

Table 1
Interday assay variation for buprenorphine and norbuprenorphine

Theoretical concentrations (ng/ml)	Recovered concentrations (mean ± SD) (ng/ml)	CV (%)	DT (%)
Buprenorphine			
0.75	0.75 ± 0.06	7.78	0.34
3.00	3.00 ± 0.22	7.44	0.13
18.0	18.0 ± 0.94	5.24	0.34
Norbuprenorphine			
0.75	0.73 ± 0.07	9.72	2.35
3.00	2.99 ± 0.13	4.22	0.33
18.0	17.5 ± 0.95	5.45	2.94

Table 3
Absolute recovery of buprenorphine and norbuprenorphine

Theoretical concentrations (ng/ml)	Recovered concentrations (mean ± SD) (ng/ml)	CV (%)	Recovery (%)
Buprenorphine			
0.75	0.59 ± 0.08	13.9	79.2
3.00	2.39 ± 0.21	8.92	79.6
18.0	14.8 ± 4.66	31.4	82.4
Norbuprenorphine			
0.75	0.75 ± 0.13	17.0	100
3.00	2.45 ± 0.33	13.3	81.7
18.0	17.4 ± 2.93	16.9	96.5

average coefficients of variation for buprenorphine and norbuprenorphine were 7.99%, ranging from 4.63 to 10.2 and 6.85%, ranging from 2.05 to 14.5%, respectively.

3.4. Absolute recovery

As shown in Table 3 the absolute recoveries of buprenorphine and norbuprenorphine were almost quantitative, being almost 80% for buprenorphine and greater than 92% for norbuprenorphine over a 200-fold concentration range. Recovery was apparently not concentration dependent.

In conclusion, this assay method compared to the others was completely validated and offers a one-step sample preparation and high recovery, resulting in high sensitivity and excellent reproducibility. This method was successfully used to explore the pharmacokinetic/pharmacodynamic relationship of buprenorphine and norbuprenorphine.

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