Simultaneous Determination of Buprenorphine and Norbuprenorphine in Serum by High-Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry

Mariusz Scisłowski¹, Wojciech Piekoszewski¹,²*, Aleksandra Kamenczak³, and Ewa Florek⁴

¹Department of Clinical and Industrial Toxicology, Jagiellonian University, Złotej Jesieni 1, PL 31-826 Krakow, Poland; ²Institute of Forensic Research, Westerplatte 9, PL 31-033 Krakow, Poland; ³Toxicology Clinic, Jagiellonian University, Złotej Jesieni 1, PL 31-826 Krakow, Poland; and ⁴Laboratory of Environmental Research, Department of Toxicology University of Medical Sciences, Dojazd 30, PL 60-631 Poznan, Poland

Abstract

Buprenorphine is a strong narcotic analgesic. It is also used in the substitution therapy for opium alkaloid addicts. The aim of this paper was to develop and validate a highly sensitive high-performance liquid chromatography–electrospray ionization-mass spectrometry method for simultaneous determination of buprenorphine and norbuprenorphine in human serum. The developed methodology was then applied to real clinical cases in a clinical toxicology setting. Extraction of analytes has been done using solid-phase extraction. Chromatographic separation was achieved on a LiChroCART column with a Purospher RP-18e cartridge, and for detection an LCQ mass spectrometer with an ion trap analyzer was used. Quantitation of buprenorphine and norbuprenorphine was performed in a single ion monitoring mode (m/z 468 buprenorphine, m/z 414 for norbuprenorphine) in order to increase the sensitivity of the method. The standard curves for both compounds were linear over the range of 0.2-10 ng/mL ($r^2 > 0.995$). The quantitation limit was 0.2 ng/mL for both analytes. The method was used for quantitation of both buprenorphine and norbuprenorphine in the serum of 15 patients undergoing the buprenorphine substitution therapy. Serum concentrations ranged between 0.36 and 4.60 ng/mL for buprenorphine and 0.21 and 2.50 ng/mL for norbuprenorphine, with buprenorphine single dosages from 0.8 to 6.0 mg.

Introduction

Buprenorphine is a semi-synthetic alkaloid and a thebaine derivative. It is currently used as an analgesic and in the replacement therapy for opium alkaloid addicts. Buprenorphine is administered sublingually, intravenously, and intramuscularly. Buprenorphine is partial mu-receptor agonist and kappa-receptor antagonist (1–3). Due to a significant first pass effect, it shows only slight activity after oral administration. Bioavailability of buprenorphine for intramuscular administration varies from 30 to 55% (4,5). Buprenorphine is demethylated primarily by CYP3A4 in the liver and intestinal wall, which leads to the formation of N-demethyl metabolite norbuprenorphine. Then buprenorphine and norbuprenorphine conjugated with glucuronic acid (6).

The quantitation of buprenorphine and norbuprenorphine in biological material requires highly sensitive analytical techniques due to a low therapeutic concentration of the drug in body fluids. Bullingham et al. (7) have demonstrated that after administration of a single dose of 0.4 mg of buprenorphine sublingually, the concentrations in serum ranged from 0.4 to 0.8 ng/mL. After 1980, when the method for buprenorphine quantitation in serum was published for the first time (8), a number of articles became available describing the analysis of the drug and its metabolite norbuprenorphine in biological specimens. These include thin-layer chromatography; high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) (9–11), electrochemical (ECD) (12), fluorimetric, and UV detection (13); and gas chromatography (GC) with nitrogen-phosphorus detection, ECD, and MS (14,15).

In the method described in 1980 (8), a liquid–liquid extraction of analyte was performed followed by a GC–MS with mass selective detector operating in a selected ion monitoring mode. In this method, the limit of quantification was 20 ng/mL. In 1984, Tebbett et al. (13) described analytical methodology for quantitation of buprenorphine and norbuprenorphine in serum using HPLC with UV detection after extraction with diethyl ether from alkaline medium. High extraction recovery
(98–100%) and good sensitivity of the method makes it useful for monitoring the buprenorphine therapy after administration of drug in high dose (13). In 1985, Bloom et al. (9) published for the first time the GC–MS analytical methodology that allowed for buprenorphine quantitation 24 h after its administration [limit of detection (LOD) = 0.15 ng/mL]. The LC–ECD-based procedure developed by Schleyer et al. (12) made buprenorphine quantitation possible in a concentration range from 0.25 to 2.9 ng/mL. In general, LC–MS methods for detection of buprenorphine and its major metabolite in biological fluids are more sensitive and specific (10,11).

The aim of the study was to develop and validate a highly sensitive and specific LC–electrospray ionization (ESI)-MS method for buprenorphine and norbuprenorphine quantitation in serum and its subsequent application in therapeutic drug monitoring of buprenorphine.

Materials and Methods

Analytical standards

Buprenorphine, norbuprenorphine, buprenorphine-d4, and norbuprenorphine-d3, were purchased from Cerilliant (Austin, TX). Lyophilized human serum with certified concentration of buprenorphine and norbuprenorphine (BPM 1/01-A) were obtained from Medicem (Steinenbronn, Germany).

Chemicals and reagents

All reagents were analytical or HPLC grade: buffer TRIS (pH 9) (Sevra, Heidelberg, Germany), methanol, acetonitrile, SPE columns C18 (Merck, Darmstadt, Germany), formic acid (Riedel de-Haën, Seelze, Germany), acetic acid (POCH, Gliwice, Poland).

Buffer TRIS (pH 9) was prepared by mixing 60 mL of 0.2M 2-amino/2-hydroxymethyl/1,3-propandiol with 250 mL of 0.1M HCl and 690 mL of distilled water. Mobile phase A was prepared by adding 1 mL of formic acid to 999 mL of water, and B was prepared by mixing 50 mL of phase A and 950 mL of acetonitrile.

Samples preparation and extraction

Two milliliters of Tris-buffered solution was added to 1 mL of serum, and then 10 μL (100 ng/mL) standard solutions of internal standards, buprenorphine-d4, and norbuprenorphine-d3, were added to reach a final solution of 1.0 ng/mL. Samples were mixed and allowed for equilibration for approximately 5 min. Next, the samples were centrifuged at 2000 rpm and extracted using solid-phase extraction (C18 columns). Prior to the extraction, all columns were conditioned with 2 mL methanol, 2 mL water, and 2 mL Tris buffering solution. After the serum was added, the columns were rinsed with 0.2 mL of methanol/Tris buffering mixture solution (50:50, v/v) and vacuum-dried for approximately 10 min. For elution of analytes, a 2-mL volume of methanol/acetonitrile (50:50, v/v) with 5% of acetic acid was used. Eluates were evaporated in the stream of air at 56°C and the dry residue was dissolved in 100 μL of methanol and centrifuged at 10,000 rpm. A 20-μL volume was then injected on column.

Instrumental analysis

The analysis of buprenorphine and norbuprenorphine was performed using Finnigan-MAT HPLC coupled with an LCQ ion trap detector (Finnigan-MAT, San Jose, CA) equipped with an ESI interface. The separation of sample components was achieved on LiChroCART Purospher 60 RP-18e column (125-mm length × 3-mm i.d., 5-μm particle size, Merck). For the analysis, a gradient elution was applied using mixture of phase A and B. The gradient elution as follows: from 0 to 2 min 95% of A, linear decrease to 5% A in 10 min, 1-min hold at 5% A, linear increase to 95% A in 2 min with a final hold of 5 min at a constant flow rate of 0.4 mL/min. Data acquisition time was 20 min.

Ionization of analytes was carried out using the following settings: sheath gas flow rate (nitrogen): 80 arb, auxiliary gas flow rate (helium): 10 arb, spray voltage: 4.2 kV, capillary temperature: 200°C, capillary voltage: 17.0 V, the first octapole offset: -3.0 V, the second octapole offset: -7.0 V, lens voltage: -16.0 V.

Molecular ions were monitored for both analytes and their deuterated analogues in routine analysis (m/z 468 for buprenorphine, m/z 414 for norbuprenorphine, m/z 472 for buprenorphine-d4, and m/z 417 for norbuprenorphine-d3).

Tandem MS was performed only for the examination of fragmentation of analytes. Nitrogen was used as collision gas. For examination of the fragmentation of analytes in MS–MS full scan mode, the m/z 468 transition was monitored for buprenorphine with relative collision energy of 40%, and the m/z 414 transition was monitored for norbuprenorphine with relative collision energy of 34%. The m/z 472 and m/z 417 transitions were monitored for buprenorphine-d4 and norbuprenorphine-d3, respectively, with relative collision energies of 40% for buprenorphine-d4 and 34% for norbuprenorphine-d3 (Figures 1 and 2).

Validation

For validation of the method, the following parameters were determined: range of linearity, precision between groups and inside groups, accuracy, LOD, and limit of quantification (LOQ).

Linearity

A standard curve was prepared by spiking negative serum with known quantities buprenorphine and norbuprenorphine and their deuterated analogues. Six-point standard curves for buprenorphine and norbuprenorphine were prepared by spiking aliquots (1 mL) of negative serum. The final concentrations of buprenorphine and norbuprenorphine were 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/mL.

Precision and accuracy

The intraday precision expressed as a relative standard deviation was determined by extraction of the serum spiked with buprenorphine and norbuprenorphine using the following concentrations: 0.5, 1.0, and 10.0 ng/mL in four attempts made every two days.

The interday precision also expressed as a relative standard deviation was determined in the same way as precision between groups, but the four-element series of samples was tested on one day.
The accuracy of the method was estimated on the basis of the analysis of the certified reference material, freeze-dried serum with known buprenorphine and norbuprenorphine contents quantified by independent forensic medicine laboratories.

**LOD and LOQ**

LOQ was determined on the basis of the signal-to-noise ratio (S/N) 3:1. In the case of the detection limit the ratio was 2. S/N values were determined by a computer program for LC–MS handling, on the basis of the algorithms provided by the manufacturer.

**Patients**

Blood samples (7 mL) were taken 4 h after the sublingual administration of buprenorphine from 15 patients participating in the buprenorphine replacement therapy program. The drug single dosages varied from 0.8 to 6.0 mg. All patients gave informed consent, and the study was approved by the Ethical Committee of Jagiellonian University. The study was done in compliance with the principles enunciated in the declaration of Helsinki. All samples were stored at -20°C prior to analysis.

### Results and Discussion

Native serum spiked with buprenorphine and its demethylated metabolite norbuprenorphine were used for analytical method development and validation. A good separation of both compounds was achieved. Example of chromatographic separation of buprenorphine and norbuprenorphine extracted from serum collected from one patient is shown in Figure 3.

Before analysis, the mass detection parameters were optimized. For this, standard solutions of buprenorphine and norbuprenorphine as well as their deuterated analogues in the mixture of A and B phases at the ratio 50:50 (v/v) were placed directly in the MS (without chromatographic separation). The optimization of parameters was performed by selection of the value of apparent molecular ion [M+H]+ and selecting the option of automatic optimization in the program, thus optimizing capillary voltage, tube lens offset, the first octapole offset, the second octapole offset, and inter-octapole lens. Analyte fragmentation was conducted in the MS–MS full scan option.

In mass spectrum of buprenorphine, an ion with the mass-to-
charge ratio value of 414 and created by elimination of the methylocyclopropyl group was shown. The second ion with m/z 396 suggests dehydration of the 414 structure (Figure 1A).

In this situation, the m/z 396 ion also should be present in the mass spectrum of buprenorphine-d₄, although the mass spectrum of buprenorphine-d₄ contains a m/z 400 ion, that is, an ion larger by four mass-to-charge ratio units than the 396 ion on the spectrum of a non-labeled buprenorphine (Figure 1B). This can suggest the presence of deuterium atoms in the molecule, located in the methylocyclopropyl group. That means that the group remains in the molecule, and the structure with the mass of 396 in the spectrum of non-labeled buprenorphine originates from another manner of fragmentation (e.g., from elimination of tert-butyl group). The observations are consistent with suggestions of other authors (11). In the case of norbuprenorphine, the most intensive fragmentary ion is the one with the mass-to-charge ratio value of 396 (Figure 2A).

The structure represented by that ion may be similar to the structure with the same mass-to-charge ratio value on the spectrum of buprenorphine, stemming from the presence of similar mass-to-charge ratio values on the spectra of buprenorphine and norbuprenorphine.

Validation data

For evaluating precision and accuracy of the method, a calibration curve was constructed for each drug by measuring bank serum, which were fortified with analytes’ standards in the range 0–10 ng/mL and internal standards in concentration of 1 ng/mL. The spiked samples were extracted as described and analyzed by HPLC–ESI-MS. Calibration curves were linear in tested range. Regression and correlation coefficients of the calibration curves were for buprenorphine \( y = 0.3376x + 0.0002 \),

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Buprenorphine</th>
<th>Norbuprenorphine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity (ng/mL)</td>
<td>0.2–10.0</td>
<td>0.2–10.0</td>
</tr>
<tr>
<td>LOD (ng/mL)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>LOQ (ng/mL)</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Intraday precision (n = 4) RSD (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 ng/mL</td>
<td>11.9</td>
<td>10.4</td>
</tr>
<tr>
<td>1.0 ng/mL</td>
<td>10.3</td>
<td>8.8</td>
</tr>
<tr>
<td>10.0 ng/mL</td>
<td>9.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Interday precision (n = 4) RSD (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 ng/mL</td>
<td>14.7</td>
<td>12.6</td>
</tr>
<tr>
<td>1.0 ng/mL</td>
<td>12.2</td>
<td>13.8</td>
</tr>
<tr>
<td>10.0 ng/mL</td>
<td>13.5</td>
<td>12.3</td>
</tr>
<tr>
<td>Accuracy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measured concentration (ng/mL)</td>
<td>4.3</td>
<td>8.5</td>
</tr>
<tr>
<td>Target concentration (ng/mL)</td>
<td>3.7</td>
<td>7.3</td>
</tr>
<tr>
<td>Confidence range (ng/mL)</td>
<td>2.3–5.1</td>
<td>4.8–9.8</td>
</tr>
</tbody>
</table>

Figure 3. Ion chromatograms of buprenorphine and norbuprenorphine: spiked serum (concentration 5 ng/mL) (A) and patient serum (buprenorphine 0.88 ng/mL, norbuprenorphine 0.54 ng/mL) (B).

Figure 4. Correlation between buprenorphine dose and buprenorphine and norbuprenorphine concentration.
\[ r^2 = 0.9949, \text{ and norbuprenorphine } y = 0.0251x + 0.0007 \]
\[ r^2 = 0.9955. \]

Small values of the \( b \) coefficient in calibration equations indicate the small systematic error of the developed method, and high correlation coefficients confirm the suitability of the method for analysis of these drugs in the biological material.

The LOD was evaluated as the lowest concentration giving a chromatographic peak at the S/N 2. The LOD value for both compounds was 0.1 ng/mL. The LOQ was evaluated as the lowest concentration giving a chromatographic peak at S/N = 3 (Table I).

Polettini et al. (11) developed a method with an LOQ of 0.1 ng/mL; however, our procedure with an LOQ of 0.2 ng/mL also allowed for the determination of buprenorphine after administration high and low therapeutic doses.

The intra-assay precision was evaluated by analyzing four serum samples spiked with analytes to achieve three different levels (0.5, 1.0, and 10.0 ng/mL) in one series (Table I). The relative standard deviation ranges from 9.5% to 11.9% for buprenorphine and 9.0% to 10.4% for norbuprenorphine. Day-to-day reproducibility data (intra-assay precision) was determined by analyzing four spiked serum samples on the same level as during study intra-assay precision. The results obtained are present in Table I.

Accuracy of the method was checked on the basis of certified material. Determined concentrations of buprenorphine and norbuprenorphine were inside the range of concentration declared by the manufacturers of the certified materials (Table I).

**Determination of buprenorphine in patients in a buprenorphine-maintenance program**

The developed method was applied for determination of buprenorphine and nor-buprenorphine in serum of patients receiving buprenorphine during therapy of opiates addiction. In serum of studied patients, the concentration of buprenorphine ranged from 0.36 to 4.60 ng/mL and of norbuprenorphine ranged from 0.21 to 2.50 ng/mL with doses of 0.8–6.0 mg (2.8 ± 1.57 mg). A correlation between the concentration of buprenorphine and buprenorphine dose was 0.4583. The concentration of norbuprenorphine did not correlate with the dose of parent drug (Figure 4).

**Conclusions**

The developed method of the buprenorphine and norbuprenorphine quantification in serum with the use of the deuterated analogues for internal standards is characterized by high sensitivity and appropriate validating parameters, linearity, precision, and accuracy, allowing for routine monitoring of the buprenorphine therapy. The method may be also used in clinical and forensic toxicology in the cases of intoxication with this drug. Concurrent quantification of norbuprenorphine may facilitate studying of the drug’s pharmacokinetics. The work demonstrates that the HPLC–ESI–MS technique applied is a sensitive and selective technique and does not require derivatization of the sample, which is the case in the GC–MS analysis (14,15).

**References**