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Short communication

Development and validation of a highly sensitive GC/MS method for the determination of buprenorphine and nor-buprenorphine in blood

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ABSTRACT

A sensitive and specific GC/MS method for the determination of buprenorphine (BPN) and its main metabolite nor-buprenorphine (nor-BPN) in blood has been developed, optimized and validated. Sample preparation includes solid-phase extraction of both analytes and their derivatization with acetic anhydride in pyridine. BPN-d4 was used as internal standard for the determination of both analytes. Limits of detection and quantification for BPN and nor-BPN were 0.02 and 0.05 μg/L, respectively. The calibration curves were linear within the dynamic range of each analyte (0.05–30.0 μg/L) with a correlation coefficient higher than 0.996. Absolute recovery ranged from 90.2 to 97.6% for both analytes and their internal standard. Intra- and inter-day accuracy was found to be between −5.40 to 1.73% and −2.45 to 2.80%, respectively, while intra- and inter-day precision were less than 5.8 and 4.7%, for both analytes. The method was applied to real blood samples obtained from patients that follow BPN maintenance program. The developed method can be used in routine every day analysis by clinical and forensic laboratories, for pharmacokinetic studies, for therapeutic drug level monitoring in order to adjust BPN dosage of BPN maintained patients or for the investigation of forensic cases.

1. Introduction

Buprenorphine (BPN), a semi-synthetic opioid derived from thebaine [1] with a chemical structure similar to morphine [2], is about 30 times more potent than morphine [3]. BPN acts as a mixed opioid agonist-antagonist, with partial μ-type opioid agonist and potent κ-type opioid antagonist activity [4]. The combined agonist and antagonist action of BPN results in minimized withdrawal effects following discontinuation of its use [5]. Several different drug treatments have been proposed as substitution or replacement therapies, most notably methadone [6] and BPN [7]. BPN's benefits include increased safety for respiratory depression, suppressed heroin use, and possibility of longer dosing interval [8]. In combination with the increased medical use, BPN also occurs on the black market as an illicit drug [9], and fatalities due to poly-drug use have been reported [10]. Because of the small doses used and the resulting low blood concentrations, identification and monitoring of BPN presents a great challenge for clinical and forensic toxicology laboratories.

Several analytical methods based on GC/MS [2,5,11–13], LC/MS [14,15] or LC/MS/MS [1,13,16,17] have been published for the determination of BPN and nor-BPN in different biological matrices, mainly in blood [14–17], plasma [11–13] and urine [2,5,15,16]. The aim of our study was the development, optimization and validation of a simple, sensitive and rapid method, based on GC–EI-MS for the determination of BPN and its pharmacologically active metabolite, nor-BPN, in blood. It has to be mentioned here that many LC methods for BPN in blood or plasma do not fulfill the compound identification criteria as they monitor only a single ion [13,15,17] or only one characteristic transition ion [14]. To our knowledge, there are no validated GC methods published concerning the determination of BPN and nor-BPN in blood. Most of the available GC [11–13] methods are applied only to plasma samples that normally are not available in forensic cases. Moreover, the sensitivity or the linearity of these methods are not sufficient for pharmacokinetic studies or for therapeutic drug monitoring as BPN blood levels normally are rather low.

2. Experimental

2.1. Chemicals and reagents

Reference standards of BPN, nor-BPN and BPN-d4 at a concentration of 0.1 mg/mL in methanol were purchased from LGC Promochem (Molsheim, France). The solvents used (methanol, hexane, ethyl acetate, dichloromethane and iso-

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propanol) were of analytical or HPLC grade and were purchased from Merck (Darmstadt, Germany). Analytical reagents were purchased as follows: pentafluoropropionic anhydride (PFPA) 99%, N,O-bis(trimethylsilyl)-trifluoracetamide (BSTFA) with 1% trimethylchlorsilane (TMCS), propionic anhydride 99% and propionylchloride 98% from Sigma-Aldrich (Steinheim, Germany), heptafluorobutiric anhydride (HFBA) 99% and trifluroacetic acid (TFAA) 99% from Fluka (Steinheim, Germany), acetic anhydride 97% from Mallinckrodt (St. Louis, USA), pyridine 99.5% from Ferak (Berlin, Germany) and triethylamine 99% from Merck (Hohenbrunn, Germany). Bond Elut LRC Certify I and II (Varian), as well as Nexus (Varian) SPE columns were used. Human blood was obtained after informed consent, from healthy donors and before its use it was screened by GC/MS for the presence of BPN and nor-BPN.

2.2. GC/MS analysis and apparatus

GC/MS analysis was performed on a Shimadzu GC-2010 equipped with a Shimadzu AOC-20i autosampler system and a Shimadzu QP 2010S mass spectrometer, using a cross-linked DB-1MS (Agilent, USA) capillary column (12 m × 0.20 mm i.d., 0.33 μm film thickness). Helium was used as carrier gas at a flow rate of 0.6 mL/min. The temperatures of injection port, ion source and interface were 280, 200 and 300 °C, respectively. Initial oven temperature of 150 °C (hold for 1 min) increased to 300 °C at a rate of 40 °C/min, with a final hold time of 6 min (total run time: 10.75 min). The mass spectrometer (MS) was operated in electron impact ionization/selective ion monitoring (EI/SIM) mode (70 eV). The mass fragments (dwell time of 10 ms) used for the qualitative analysis of the acetylated analytes were: **420**, 452 and 408 for BPN, **440**, 408 and 366 for nor-BPN, whereas the bold marked ions, as well as the *m*/*z* **424** for the internal standard BPN-d4, were used for their quantification.

2.3. Calibrators and controls

Working standard solutions containing BPN and nor-BPN at 1.00–600.0 μg/L, were prepared by mixing the appropriate volumes of the corresponding stock solutions of each compound and then by diluting with methanol. Spiked blood standards (calibrators and quality controls) were prepared by spiking 950 μL of blank blood with 50 μL of the mixed working standard solutions. The eight calibrators contained BPN and nor-BPN at equal concentrations of 0.05, 0.10, 0.30, 0.60, 1.50, 3.00, 10.0 and 30.0 μg/L, while blood QC samples contained 0.15, 5.00 and 20.0 μg/L of BPN and nor-BPN. A working internal standard solution containing 40.0 μg/L BPN-d4 in methanol was also prepared. Fresh working solutions were prepared daily.

2.4. Sample preparation

The following procedure was applied: addition of 50 μL of BPN-d4 (40.0 μg/L) to calibrator/QC/patient samples (1.00 mL), vortex for 15 s, pH adjustment with 4.00 mL of 0.1 M phosphate buffer pH 6.00, centrifugation at 3000 rpm for 5 min, conditioning of Bond Elut LRC Certify SPE with 3 mL of methanol, 3 mL of deionized water and 1 mL of phosphate buffer 0.1 M (pH 6.00), sample loading, washing of the SPE columns with 3 mL of deionized water, 1 mL of 0.1 M acetic buffer pH 4.00 and 3 mL of methanol, dryness under high vacuum (≥10 mmHg) for 5 min, twice elution of the analytes with 1.5 mL of a mixture of dichloromethane:isopropanol:ammonium hydroxide (85:15:2, v/v/v), evaporation to dryness (N_2 at 40 °C), acetylation by addition of 50 μL of pyridine and 50 μL of acetic anhydride, heating at 80 °C for 30 min, cooling, evaporation to dryness, reconstitution (30 μL ethyl acetate) and injection (1 μL) into the GC/MS system (splitless mode).

3. Results and discussion

3.1. Method development and optimization

A GC/MS method has been developed and optimized for the determination of BPN and nor-BPN in blood. The assay includes isolation of both analytes from the blood samples by SPE followed by derivatization.

Different derivatization reagents, such as PFPA, HFBA, TFAA, BSTFA with 1% TMCS, mixture of propionic anhydride with triethylamine (1:1, v/v), acetic anhydride in pyridine and propionylchloride were tested. It was found that BPN and nor-BPN were best derivatized by acetylation, using acetic anhydride in pyridine, judging by the peak areas of both derivatized analytes. Furthermore, the other derivatization reagents resulted in the formation of analyte-derived artifact products reducing the abundance of the derivatized BPN and nor-BPN and thus the sensitivity of the method. The results obtained in the development of the derivatization procedure were similar to Wu et al [18]. Subsequently, the derivatization conditions (volume of derivatization reagent, temperature and duration of the reaction) were optimized using standard solutions of the compounds.

Chromatographic conditions like injector temperatures (240, 260, **280**, 300 °C), interface temperatures (280, 290, **300**, 310, 320 °C), initial (60, 80, 100, 120, **150**, 180 °C) and final (280, 290, **300**, 310, 320 °C) column temperatures, as well as the column temperature rate (20, 30, **40**, 50 °C/min) and the carrier gas flow rate (0.5, **0.6**, 0.7, 0.8, 0.9, 1.0 mL/min) were optimized. The optimal conditions are in bold. The optimized GC separation of analytes was achieved within 10 min and the retention times of acetylated BPN and nor-BPN were 8.9 and 9.6 min, respectively.

In order to achieve high selectivity, specificity and sensitivity, solid phase extraction (SPE) of blood samples was selected, as the liquid–liquid extraction gave dirty samples unsuitable for derivatization and GC/MS analysis. During the optimization of the extraction procedure, three different types of SPE columns were tested: Nexus (Varian), Bond Elut LRC Certify (Varian) and Bond Elut LRC Certify II (Varian). Modifications like different washing methods of the column, as well as different solvent mixtures for the elution of the analytes were tried. By using Bond Elut LRC Certify SPE columns (mixed mode: non polar and cation exchange) high recovery results were achieved for all analytes and for the internal standard, without any interference from endogenous blood compounds. During the washing step of the SPE columns, acetic acid 1.0 M and acetate buffer 0.1 M (pH 4.0) were tested and the latter resulted in greater absolute recovery. The absolute recovery during SPE was also improved by using different mixtures of elution solvents, such as dichloromethane:isopropanol:ammonium hydroxide (85:15:2, v/v/v), ethyl acetate: ammonium hydroxide (100:2, v/v), dichloromethane: methanol: ammonium hydroxide (90:10:2, v/v/v), ethyl acetate: isopropanol: ammonium hydroxide (90:10:2, v/v/v), hexane: ethyl acetate: isopropanol: ammonium hydroxide (80:10:10:2, v/v/v/v). The mixture of dichloromethane: isopropanol: ammonium hydroxide (85:15:2, v/v/v) showed the best results for the elution of both analytes.

3.2. Method validation

The combination of SPE and acetylation proved to be useful for the determination of BPN and nor-BPN blood concentrations, as no interference from endogenous and exogenous compounds was observed. The **selectivity** of the method was

Table 1
Intra- and inter-day accuracy and precision of BPN and nor- BPN of blood quality control samples.

Analyte	Concentration expected (μg/L)	Intra-day ($n=6$)			Inter-day ($n=36$)		
		Concentration found, mean ± SD (μg/L)	Accuracy (% E_r)	Precision (% RSD)	Concentration found, mean ± SD (μg/L)	Accuracy (% E_r)	Precision (% RSD)
BPN	0.15	0.1526 (±0.0067)	1.73	4.4	0.1542 (±0.0057)	2.80	3.7
	5.00	4.90 (±0.23)	−2.00	4.7	5.10 (±0.20)	2.00	3.9
	20.0	19.69 (±1.15)	−1.55	5.8	20.07 (±0.93)	0.35	4.6
nor-BPN	0.15	0.1523 (±0.0088)	1.53	5.8	0.1519 (±0.0071)	1.27	4.7
	5.00	4.85 (±0.21)	−3.00	4.3	5.07 (±0.20)	1.40	3.9
	20.0	18.92 (±0.99)	−5.40	5.2	19.51 (±0.78)	−2.45	4.0

adequate with minimal matrix effects for all blank samples ($n=6$). The **specificity** study documented that blood concentrations of 1000 μg/L of the drugs selected (morphine, codeine, 6-acetyl-morphine, methadone, Δ^9-tetrahydrocannabinol, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, cocaine, ecgonine methylester, benzoyloecgonine, diazepam, nordiazepam, bromazepam, alprazolam, 7-amino-flunitrazepam, phenobarbital, amitriptyline, clomipramine, amphetamine, methamphetamine, MDMA, ephedrine and ketamine) do not interfere with the accurate determination of BPN and nor-BPN in blood. The ***LOD and LOQ*** for each analyte were determined as the lowest concentration yielding signal-to-noise ratios of at least 3:1 (0.02 μg/L) and 10:1 (0.05 μg/L), respectively, for both analytes. ***Linearity*** was excellent with correlation coefficients (R^2) exceeding 0.996 and the linear dynamic range was 0.05–30.0 μg/L for both analytes. The % RSD of the slopes was found to be 2.9 and 3.6%, for BPN and nor-BPN, respectively. ***Precision and accuracy*** of the method (intra-day $n=6$ and inter-day $n=36$) were calculated by analyzing three QC levels within analytes' linear range (0.15, 5.00 and 20.0 μg/L for both BPN and nor-BPN) and the results are presented in Table 1. ***Absolute recovery*** for three QC levels was found to be between 90.2 and 95.6% for BPN, 94.1–97.6% for nor-BPN and higher than 93.8% for BPN-d4. SIM chromatogram of a spiked sample at the low QC concentration is shown in Fig. 1. ***Robustness*** of the entire method was studied by changing several parameters of the procedure (pH of samples was adjusted to 6.50 instead of 6.00, derivatization temperature 75 °C instead of 80 °C, and the ratio of the solvents in the elution mixture 80:20:2, v/v/v instead of 85:15:2, v/v/v) as well as GC parameters (flow rate of carrier gas: 0.97 mL/min, injector temperature: 277 °C and 3% lower detector voltage). Neither a single parameter nor a combination of the ones changed, showed a significant influence on the results of the method, which proved to be sufficiently robust. ***Stability study*** was assessed by analyzing spiked blood with BPN and nor-BPN, at low and high QC levels, and the loss for both analytes was less than 5.7, 6.5 and 4.4% at room temperature for 16 h, at −20 °C for 1 month and after three freeze–thaw cycles, respectively.

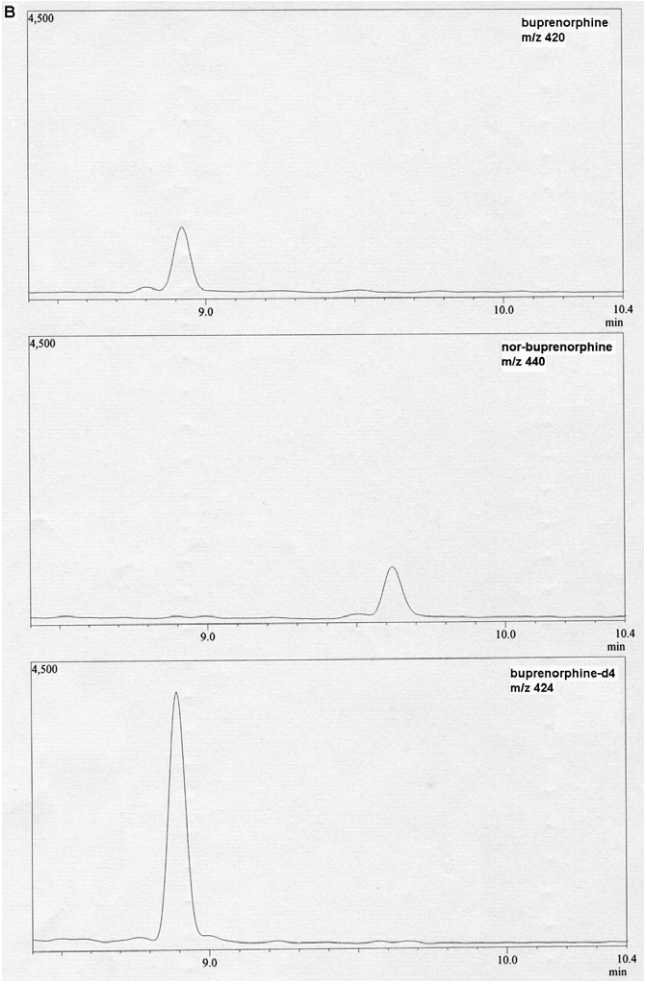

Fig. 1. Chromatogram of a spiked at the low QC concentration (0.15 μg/L) sample for both BPN and nor-BPN.

3.3. Method application

The developed method was successfully applied to the drug therapeutic monitoring of patients participated in BPN maintenance program. The blood samples were collected from BPN maintained patients before and 3 h after BPN intake, and were stored at −20 °C until their analysis. In this manuscript, five representative cases are presented (Table 2). The patients were receiving a single BPN daily dose of 5 mg (patient A), 6 mg (patient B) and 7 mg (patient C), or a dose of 16 mg BPN (patients D and E) every 2 days. BPN blood levels of these patients were within the therapeutic range and their response was judged satisfactory by the clinical doctors.

Table 2
Concentration of BPN and nor-BPN (μg/L) in blood samples obtained from five BPN maintained patients, before and 3 h after oral administration.

Concentration (μg/L)	Patient A		Patient B		Patient C		Patient D		Patient E	
	Before	3 h after	Before	3 h after	Before	3 h after	Before	3 h after	Before	3 h after
BPN	0.207	0.791	0.324	1.407	0.494	2.020	0.418	2.658	0.269	1.519
nor-BPN	0.438	0.717	0.536	0.758	2.251	3.531	0.235	1.155	0.200	0.631

4. Conclusion

The developed method is the first GC/MS method that determines simultaneously BPN and nor-BPN in blood providing full validation data and showing such a low LOQ. When compared with previously described GC or LC methods for the determination of BPN and nor-BPN in blood or plasma, it shows comparable and in some cases enhanced validation results. The developed method shows improved sensitivity, accuracy and precision, higher extraction recoveries and significant robustness. More specifically, previously published relative methods show higher [12–14,16] or same LOQ [11] (which concerns plasma) for BPN or nor-BPN. The wide linear range (0.05–30.0 μg/L) for both BPN and nor-BPN, starting from LOQ concentration [in contrast to the only previously published GC/MS method [11] for BPN in plasma that has the same LOQ (0.05 μg/L) for BPN with ours but its linearity curve starts from 0.10 μg/L] allows the analysis of most clinical and forensic specimens.

BPN dosage, as well as BPN and nor-BPN blood levels, in usual clinical cases are very low, demonstrating the need for a highly sensitive method for their determination. The proposed GC/MS method can solve already existing analytical problems in such cases and it satisfies sensitivity requirements by using elementary equipment, available at common clinical or forensic laboratories that perform everyday routine analysis, at a significantly low cost. The developed method can be used in the determination of BPN and nor-BPN in blood samples for pharmacokinetic studies, for therapeutic drug level monitoring in order to adjust BPN dosage of BPN maintained patients or for the investigation of forensic cases.

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