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Issues pertaining to the analysis of buprenorphine and its metabolites by gas chromatography–mass spectrometry

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ABSTRACT

"Substitution therapy" and the use of buprenorphine (B) as an agent for treating heroin addiction continue to gain acceptance and have recently been implemented in Taiwan. Mature and widely utilized gas chromatography–mass spectrometry (GC–MS) technology can complement the low cost and highly sensitive immunoassay (IA) approach to facilitate the implementation of analytical tasks supporting compliance monitoring and pharmacokinetic/pharmacogenetic studies. Issues critical to GC–MS analysis of B and norbuprenorphine (NB) (free and as glucuronides), including extraction, hydrolysis, derivatization, and quantitation approaches were studied, followed by comparing the resulting data against those derived from IA and two types of liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods. Commercial solid-phase extraction devices, highly effective for recovering all metabolites, may not be suitable for the analysis of free B and NB; acetyl-derivatization products exhibit the most favorable chromatographic, ion intensity, and cross-contribution characteristics for GC–MS analysis. Evaluation of IA, GC–MS, and LC–MS/MS data obtained in three laboratories has proven the 2-aliquot GC–MS protocol effective for the determination of free B and NB and their glucuronides.

1. Introduction

Exhibiting both partial agonist activity at the μ-opiate receptor and antagonist activity at the κ-opiate receptor, buprenorphine (B) (2S)-2-[(−)-(5R,6R,7R,14S)-9α-cyclopropylmethyl-4,5-epoxy-6,14-ethano-3-hydroxy-6-methoxymorphinan-7-yl]-3,3-dimethylbutan-2-ol/CAS 52485-79-7, has long been prescribed for pain relief and anesthetic induction [1]. In 1966, B (under the trade name of Subutex® for sublingual use) was adopted in France as a substitution agent for managing opiate-dependent individuals. In the US, Subutex® and Suboxone® (B/naloxone[1] combination product) were certified in 2002 for use in opioid detoxification and opioid substitution treatments [2]. Substitution therapy and the use of B as an agent for "treating" heroin addiction continue to gain acceptance and have been implemented in Taiwan in 2006. Yet significant numbers of B-related fatalities have been reported in France [4,5] and elsewhere [6].

Analysis of B and its metabolites in various biological matrices is an important component of pharmacokinetic/pharmacogenetic studies, compliance monitoring, and the implementation of deterrence testing strategy associated with the B-treatment policy. Since B is rapidly metabolized [7] to form pharmacologically active metabolite, norbuprenorphine (NB) (5α,6β,14β,18R)-18-[(1S)-1-hydroxy-1,2,2-trimethylpropyl]-6-methoxy-18,19-dihydro-4,5-epoxy-6,14-ethenomorphinan-3-ol/CAS 78715-23-8, while both B and NB readily form glucuronide conjugates, buprenorphine glucuronide (BG) and norbuprenorphine glucuronide (NBG), analytical studies should address all issues critical to the analysis of these four compounds.

With advances in instrumentation, there have been a substantial number of studies applying the latest liquid chromatography–tandem mass spectrometry (LC–MS/MS) technologies to the analysis of B and NB [8–20] and BG and NBG [15–30]. While these approaches hold certain merits (including simultaneous determinations of B, NB, BG, and NBG without

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[1] Naloxone is included in the Suboxone® formulation to prevent diversion of B for illicit intravenous use. With antagonist activity at the μ-opiate receptor but poor sublingual bioavailability, naloxone has little effect on the agonist activity of B when taken by the prescribed route. However, if the sublingual B/naloxone tablets are crushed and injected, the effect of naloxone predominates can result in the opioid withdrawal syndrome [3].

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prior derivatization and, reportedly, low limits of detection and quantitation), there are still considerable concerns, such as instrumentation cost, robustness, level of required operational skill, and perhaps precision and accuracy in quantitation. Many professionals are still skeptical in applying these technologies to regulatory and/or high-volume testing environment, such as workplace drug testing programs.

Interestingly, studies addressing issues critical to the analysis of B and its metabolites by the mature and now widely available gas chromatography–mass spectrometry (GC–MS) instrumentation are relatively rare [22–26]. Thus, we have conducted a thorough study on various derivatization approaches and concluded acetyl-derivatization to be the best option for the analysis of B and NB by GC–MS [27]. We have also completed an immunoassay (IA) study on parameters critical to its serving as the *preliminary* test methodology [28] in the 2-step test strategy, designed for a high-volume test environment. This current report further examines other issues critical to the utilization of the GC–MS technology to monitoring metabolites derived from B-treatment. Issues studied included: (a) effects of sample extraction approaches on the analysis of free B and NB; (b) deriving the concentrations of B, NB, BG, and NBG through a 2-step analytical protocol—without and with the hydrolysis step; and (c) correlating the GC–MS analyte concentrations to findings derived from preliminary test (IA) and LC–MS/MS methodologies.

Thorough understanding of these analytical methods (preliminary and confirmatory tests) and sample preparation and quantitation approaches (extraction, hydrolysis, derivatization, and deriving the concentrations of B, NB, BG, and NBG from a 2-step test protocol) are essential to the development of a robust analytical protocol that can (a) be widely applied to pharmacokinetic/pharmacodynamic studies; and (b) meet the requirements of testing programs adapting similar rules mandated by the US Federal Guidelines for monitoring the exposure of the following drugs in the workplace: marijuana, cocaine, heroin, amphetamine/methamphetamine, and phencyclidine [29].

2. Experimental

2.1. Chemical and reagents

Reference materials to be used for preparing the standard solutions of the analytes and the ISs, including B, NB, BG, NBG, B-d_4, and NB-d_3 (in 0.1 mg/ml methanol solution), were purchased from Cerilliant Corporation (Austin, TX, US). Pre-formulated liquid–liquid extraction kit (Toxi-Tubes A) and solid-phase extraction cartridge (Bond Elut C_{18} SPE, 200 mg, 40 Å) were obtained from Varian (Walnut Creek, CA, US). β-Glucuronidases (*Helix pomatia*) were obtained from Sigma Aldrich Fine Chemicals (Saint Louis, MO, US). The derivatization reagent, acetic anhydride (AA), was purchased from Finechem (Wellington, Auckland, New Zealand). Other solvents and reagents are analytical or HPLC grade and were obtained from the following sources: ethyl acetate: Ferak (Berlin, Germany); acetic acid, potassium phosphate dibasic (K_2HPO_4), and phosphoric acid (H_3PO_4): Sigma Aldrich Fine Chemicals (Saint Louis, MO, US).

2.2. Extraction and derivatization procedure

Extraction methods studied included: (a) liquid–liquid extraction with a solvent system prepared in-house and with commercially pre-formulated Toxi-Tubes A (containing sodium carbonate and bicarbonate, pH 9.0, in a mixture of dichloromethane, dichloroethane, *n*-heptane and ethyl acetate); and (b) solid-phase extraction with Bond Elut C_{18}. For the former liquid–liquid extraction approach, 1-ml urine was added to the IS and 1 ml 0.1-M ammonium carbonate buffer (pH 10). The mixture was then extracted twice with 3-ml dichloromethane/isopropanol (9:1) mixture by mixing for 20 min, followed by centrifugation at 2500 rpm for 15 min. The extracts were combined and dried before proceeding to the derivatization step. The manufacturer's instructions were followed for the Toxi-Tubes A liquid–liquid extraction method. Specifically, 1-ml urine sample was added to a Toxi-Tubes A tube and the content was brought up to 5 ml with double-distilled water. After a 10-min mechanical shaking, the mixture was centrifuged at 2500 rpm for 10 min. The organic layer was transferred into a clean 16 mm × 100 mm glass tube and dried under a slow stream of nitrogen at 50 °C. For the Bond Elut C_{18} solid-phase extraction approach, 3-ml ammonium carbonate buffer was added to the sample (1 ml) and the mixture was gently vortexed, then passed through (1–2 ml/min) the SPE cartridge that had been conditioned with 3 ml methanol and 3 ml ammonium carbonate buffer. The loaded SPE cartridge was then washed with 3 ml water and dried for 5 min under low vacuum (35 kPa), followed by eluting the analytes with three consecutive 1-ml aliquots of methanol. The combined eluent was evaporated to dryness with nitrogen at 50 °C.

For acetyl-derivatization, the dried residue was re-dissolved in 100 μl AA, vortex mixed, capped, and incubated at 80 °C for 20 min. The reaction mixture was evaporated to dryness at 65 °C in a heating block under a slow stream of nitrogen. The residue was re-dissolved in ethyl acetate (typically 100 μl) and 1 μl was typically used for GC–MS analysis.

For the collection of standard spectra and the evaluation of ion intensity cross-contribution (CC) data, 10 μl standard solutions of the analyte and IS were placed in individual 16 mm × 100 mm glass tubes and evaporated to dryness under a stream of nitrogen at 50 °C. The same derivatization procedure described above was then followed.

2.3. Hydrolysis procedure

One-ml urine specimen (or BG and NBG standards) was put into a 15-ml centrifuge tube, followed by the addition of 1 ml 1.0-M acetate buffer and 1000 Fishman units of β-glucuronidase (100 μl 10,000-Fishman units/ml). The samples were capped and incubated at 60 °C for 4 h, which has been proven effective in the exploratory stage of this study [30].

2.4. GC–MS analysis

2.4.1. Instrumentation and operational parameters

An Agilent 6890N gas chromatograph/5975[5973N] mass selective detector system operating at 70 eV with ion source temperature set at 230 °C was used for this study. The gas chromatograph was equipped with a 12[30]-m HP-5 (Wilmington, DE, US) capillary column crosslinked 5% phenyl methyl siloxane with 200[250]-μm I.D. and 0.33[0.25]-μm film thicknesses. The injector temperature and GC–MS interface temperature were maintained at 280 °C. The sample was introduced into the gas chromatograph in splitless mode and the helium carrier gas flow rate was set at 1.2 ml/min. The initial oven temperature was held at 200 °C for 1[0.5] min, then raised to 300 °C at 30[40] °C/min, and held for 5[15] min. Data/information shown inside brackets ([]) were used in the GC–MS analysis conducted in another laboratory for comparative studies (see Section 3.2.1 for details).

2.4.2. Collection and evaluation of mass spectra and ion-pair intensity cross-contribution data

Typically, full-scan mass spectra of the analytes of interest were obtained by injecting individual derivatization products into the GC–MS system. Full-scan mass spectra were collected starting at *m/z* 50 and ended at a mass higher than the molecular weights of the derivatized products, rounded to the next 50, i.e., 350, 400,

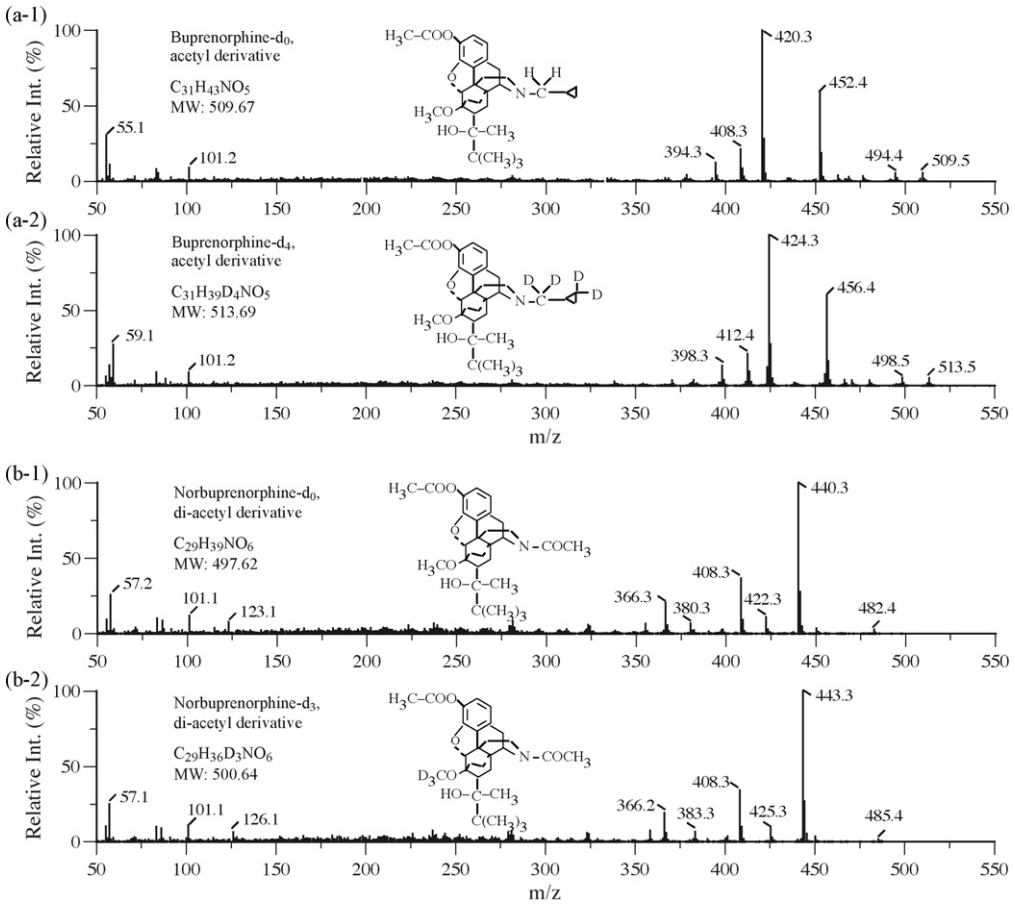

Fig. 1. Mass spectra of acetyl derivative of buprenorphine (a-1) and buprenorphine-d_4 (a-2); and di-acetyl derivative of norbuprenorphine (b-1) and norbuprenorphine-d_4 (b-2).

450, etc. A separate run was repeated for each isotopic analog of each analyte. Retention time and mass spectrometric data derived from these runs were used to characterize the analytes and their isotopic analogs. Full-scan mass spectrometric data were stored as digital files that were then converted into mass spectra of a more desirable format for systematic presentation. This conversion was carried out using the DelraGraph software (DeltaPoint: Seattle, WA, US) run on an Apple iMac G5 computer (Cupertino, CA, US). Shown in Fig. 1 are full-scan mass spectra and structural information of acetyl-derivatized B and NB along with their deuterated analogs, B-d_4 and NB-d_3.

Full-scan mass spectrometric data were reviewed to select ions that may potentially be used to identify the analytes and their ISs in quantitative GC–MS protocols. The derivatization products were again injected into the GC–MS system under selected ion monitoring (SIM) mode, using ions selected from the full-scan mass spectrometric data. General criteria adapted for SIM ion selection were: (a) the ion's relative intensity in the full-scan mass spectrum was ≥10%; and (b) the full-scan intensity data indicated <10% CC. Ions with lower intensity would have been included if there were less than three pairs of ions that met the above criteria. The resulting SIM data were then used to derive more accurate CC data for the pairs of ions that may potentially be used to designate the analyte/IS pair. Details of the methodology have been described in our earlier publications [31,32].

2.5. LC–MS/MS (triple quadrupole configuration) analysis

The LC–MS/MS system consists of an Agilent 6410 triple quadrupole mass spectrometer (Santa Clara, CA, US) fitted with an electrospray interface and an Agilent 1200 rapid resolution LC system (Santa Clara, CA, US). Chromatographic separation was achieved using an Agilent Zorbax SB-Aq (100 mm × 2.1 mm I.D., 1.8-μm particle) analytical column operated at 50 °C. The mobile phase consisted of 0.1% formic acid (v/v) in water (A) and methanol (B), operated at a flow rate of 0.32 ml/min. The initial gradient composition (90% A/10% B) was held for 1.5 min, then decreased to 0% A in 8.5 min and held for 2 min. For re-equilibration, the initial gradient composition was restored by increasing solvent A to 90% in 1 min, held for 2 min, and allowed to equilibrate for 5 min. A 5-μl aliquot of the samples was injected. The electrospray source was operated at 350 °C with an ionization voltage of 4000 V. Gas source (nitrogen) was via an Agilent oxygen analyzer. The nebulizer gas pressure and the drying gas flow rate were set at 40 psi and 10 l/min, respectively. Mass spectrometric analysis was performed in positive-ion mode with 200 ms dwell time, applying multiple reaction monitoring (MRM) using appropriate collision energy for each precursor ion. Transitions and other mass spectrometric parameters adopted for the analysis of B, BG, NB, and NBG are summarized in Table 1.

3. Results and discussion

The premise of this study is the establishment of a robust GC–MS methodology for the quantitations of free and conjugated forms of B and NB. Thus, issues studied are mainly on the development and validation of various analytical steps and the application of the established methodology to the analysis of test specimens. The contents of these studies are discussed in the following sections.

Table 1
Transitions and MS–MS parameters for LC–MSMS (triple quadrupole) analysis of buprenorphine (B), buprenorphine glucuronide (BG), norbuprenorphine (NB) and norbuprenorphine glucuronide (NBG).

Compound	Precursor ion	Product ion	Frag. votage (V)	Collision energy (V)
B	468.4	414.4	240	35
BG	644.5	468.4	240	40
B-d_4	472.5	400.4	240	45
NB	414.4	340.4	240	35
NBG	590.5	414.4	240	40
NB-d_3	417.4	399.3	240	40

3.1. Method development and validation

Method development and validation studies included (a) effective calibrations for the quantitation of B and NB, with and without the incorporation of the hydrolysis step; (b) evaluation of hydrolysis completeness and extraction recoveries; and (c) determining the analyte concentrations (B, NB, BG, and NBG) in test specimens using a 2-aliquot approach.

3.1.1. Quantitation approach and the effect of the hydrolysis step on the quantitation of B and NB

Internal standard method utilizing the *isotopically labeled analog of the analyte* as the IS is now considered the most effective approach for quantitative determination of drugs (and their metabolites) in biological specimens [33]. With this approach, one of the most important concerns is the intensity CC between the ions designating the analyte and the IS, that may affect the quality of the quantitation data [34]. Since ^{2}H-labeled (but not ^{13}C-labeled) analogs of B and NB are both available, these deuterated analogs were adopted as the ISs, respectively, for the quantitation of these two analytes.

Our earlier work [27] concluded that, among the alkylation, acylation, and silylation approaches studied, acetylation of B and NB achieved the best over-all results when all of the following factors are considered together: derivatization yields, ionization efficiency, chromatographic characteristics, and CC between ions designating the analyte and the IS. Based on the full-scan mass spectra data of the acetyl-derivatized analytes and ISs shown in Fig. 1, the intensities of ion-pairs with potential for designating these two analytes and their ISs were monitored under SIM mode. Resulting CC data for the ion-pairs designating the B/B-d_4 and NB/NB-d_3 systems, along with these ions' relative intensities in their respective full-scan mass spectra, were determined based on a method reported earlier [35] and summarized in Table 2. These data indicate the three ion-pairs that are most suited for designating the B/B-d_4 and NB/NB-d_3 systems are m/z 420/424, 452/456, 408/412 and 440/443, 422/425, 441/444, respectively.

Having selected the most suitable ion-pairs to designate B and NB and their respective ISs, two sets of standard solutions were analyzed with and without the incorporation of the hydrolysis step, each in duplicates. The resulting analytical parameters are shown in Table 3, indicating the effect of the hydrolysis step on the achievable LOD and LOQ. Our earlier study [27] has indicated that more favorable limits of detection (LOD) and quantitation (LOQ), especially for NB, can be achieved using B-d_4 as the IS for NB and narrower calibration ranges to avoid biased toward the higher concentration end in establishing the calibration line by linear regression. However, since the hydrolysis step is needed only for the analysis of total B and NB, which are normally present at high concentration levels, no further refinements were pursued.

The quantitation approach hereby developed has been applied to the analysis of 3 standard solutions (containing 30, 300, 500 ng/ml of B; 30, 300, 600 ng/ml of NB) in 3 days. The means and CV derived from these analyses were found satisfactory: 35.4, 1.85%; 279, 4.79%; 511, 1.62% for B and 32.7, 6.79%; 285, 12.8%; 584, 1.96% for NB.

When the GC–MS method described above and the triple quadrupole LC–MS/MS method were used in another laboratory (see details in Section 3.2.1) for comparative studies, narrower cal-

Table 2
Relative intensity and cross-contribution data of ions with potential for designating the analyte and the adapted internal standard.

Derivatization group	Analyte			Internal standard		
	Ion (m/z)	Rel. int.	Analog's cont.	Ion (m/z)	Rel. int.	Analog's cont.
Buprenorphine/buprenorphine-d_4						
Acetyl	452	51.6	0.76	456	61.1	0.13
	420	100	0.77	424	100	0.50
	408	18.8	1.38	412	21.7	0.85
	394	15.3	2.04	398	17.1	1.01
Norbuprenorphine/norbuprenorphine-d_3						
$[Acetyl]_2$	422	10.6	2.66	425	9.53	1.84
	440	100	1.20	443	100	0.68
	441	24.7	1.79	444	30.2	0.23

Relative intensities are based on full-scan data, while analog's contributions (cross-contribution) are derived from selected ion monitoring data.

Table 3
Analytical parameters derived from linear calibration of buprenorphine (B) and norbuprenorphine (NB) (all concentrations are in ng/ml).

Hydrolysis	Buprenorphine				Norbuprenorphine			
	Conc.	r^2	LOD	LOQ	Conc.	r^2	LOD	LOQ
Yes	0–1000	0.9984	20	30	0–1500	0.9993	20	50
		0.9997	20	30		0.9995	20	20
No	0–500	0.9990	4	10	0–1000	0.9998	2	20
		0.9987	4	10		0.9972	2	20

Protocols with and without the hydrolysis step were intended for the analysis of total and free B and NB, respectively. LOD is defined as the lowest concentration at which the two independent ion intensity ratios of the three ions monitored are within ±20% of that observed in the calibration standard with the same concentration as the IS. LOQ is defined as the lowest concentration that meets the LOD requirement and the observed concentration is also within ±20% of the expected value [33]. The exact concentrations of B and NB in these standards in the protocols with and without the hydrolysis step are: "0, 20, 30, 80, 150, 300, 600, 1000 ng/ml" and "0, 20, 50, 100, 200, 400, 800, 1500 ng/ml"; and "0, 4, 10, 30, 80, 150, 300, 500 ng/ml" and "0, 2, 20, 60, 150, 300, 600, 1000 ng/ml", respectively. The highest concentration of the analyte in each calibration study was found within the achievable upper limit of linearity.

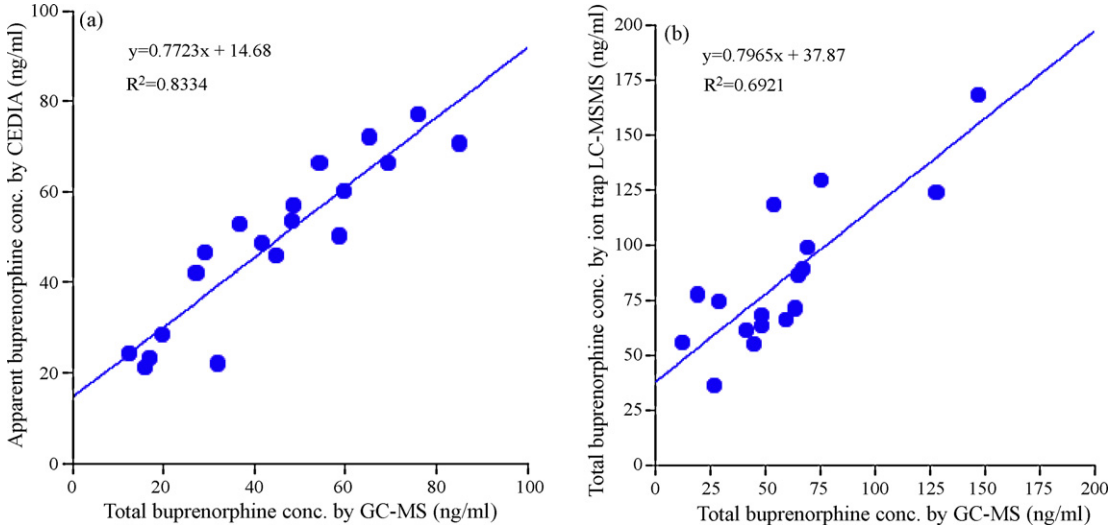

Fig. 2. Correlation of total buprenorphine concentration determined by GC–MS against (a) apparent buprenorphine concentration resulting from CEDIA buprenorphine assay; and (b) total buprenorphine concentration as determined by ion trap LC–MS/MS. The first GC–MS data shown in Table 5 for specimens 10–13, 40, and 53 were used for plotting (a), while the second data were used in (b). Data for specimens 10 and 20 were excluded in (b).

ibration ranges were adopted: 0.5, 1.5, 2.5, 5, 10, 25, 50, 80 ng/ml for B, NB, BG, and NBG. The GC–MS method achieved more favorable LOD and LOQ than the calibration described in Table 3: both 1.5 for B and 2.5 ng/ml for NB. Both of the LOD and LOQ achieved by the triple quadrupole LC–MS/MS method were 1.5, 0.5, 0.5, and 0.5 ng/ml for B, BG, NB, and NBG, respectively. All correlation coefficients observed from these calibrations were better than 0.999.

3.1.2. *Extraction and hydrolysis methods for the determination of free and total buprenorphine and norbuprenorphine*

Since B and NB present in biological specimens are in *free* and *conjugate* forms, the analyst should first decide whether the analytical task is for the determination of the analytes (a) in free forms; (b) in both free and conjugate forms; or (c) the total concentrations. The predetermined objective will then guide the selection of extraction and hydrolysis methods with the most desirable characteristics.

With deuterated B and NB now readily available to serve as the ISs, the concentrations of *free B* and *NB* in a sample can be accurately determined even if the adopted extraction method does not achieve 100% recoveries of the analytes. It is very important, however, that the method does not co-extract the conjugates (BG and NBG), especially if a following sample preparation step may result in the hydrolysis of the co-extracted conjugates. Secondly, if the analytical objectives include finding the exact concentrations of *BG* and *NBG* in the specimen, an extraction method that can achieve the highest recoveries of these two analytes would be very desirable, especially if they are to be directly analyzed—as in LC–MS/MS protocols. Thirdly, when finding the total concentrations of B and NB in the specimen is the objective of the analysis, the ability of the adopted hydrolysis method to achieve 100% conversion becomes the most important consideration.

With these concerns in mind, we have designed several series of experiments assessing methods that would be most suitable for the analysis of B and NB in their *free* forms and the *total* concentrations in a specimen. Shown in Table 4 are the recovery data (in percentage) of B/NB achieved by three extraction approaches. Two sets of standards containing B/NB and BG/NBG, respectively, were used in this series of study. These data indicate: (a) the liquid–liquid extraction procedure using the solvent system prepared in-house achieved the lowest recoveries of B/NB with no detectable BG/NBG; (b) the Bond Elut C_{18} solid-phase approach was very effective in extracting both B/NB and BG/NBG; (c) the derivatization procedure following the extraction step must have converted very significant proportions of the extracted BG/NBG into B/NB that were then derivatized and determined as B/NB at the GC–MS analysis step; and (d) the Toxi-Tubes A liquid–liquid extraction procedure extracted B/NB effectively, but also co-extracted small amounts of BG/NBG which were finally determined as B/NB.

Under compliance monitoring and workplace testing environment, total concentrations of B and NB serve as the most convenient analytical parameters. To accurately determine the total concentrations of B and NB, the adopted hydrolysis step must convert BG and NBG to B and NB completely. An enzymatic hydrolysis approach was reportedly capable of achieving this requirement for both BG and NBG [23,26] and had been verified in our laboratory [30]. We have therefore incorporated this hydrolysis method into our analytical protocol where the hydrolysis step was needed.

3.1.3. *Analysis of free and total buprenorphine/norbuprenorphine and buprenorphine glucuronide/norbuprenorphine glucuronide in urine specimen*

In addition to *free* and *total* B/NB, the concentrations of BG/NBG are often of interest in pharmacokinetic/pharmacogenetic studies. An analytical scheme capable of providing the following three sets of analyte concentrations was established: free B/NB, total B/NB, and BG/NBG. A specimen would be analyzed once if only the total *or* the free B/NB concentrations are needed. Specifically, the analytical protocol would and would not include the hydrolysis step. On the other hand, if the concentrations of BG and/or NBG are also of interest, a specimen would have to be analyzed twice, i.e., once without and once with the hydrolysis step. Shown below are the analytical steps and calculation for deriving the concentrations of BG/NBG through a 2-aliquot analytical protocol.

Table 4
Comparison of three extraction methods for their recoveries (%) of buprenorphine (B) and norbuprenorphine (NB) from urine samples containing B/NB and buprenorphine glucuronide (BG)/norbuprenorphine glucuronide (NBG).

Extraction method	B/NB in sample		BG/NBG in sample	
	B	NB	BG	NBG
Bond Elut C_{18}	97.9	95.2	46.2	51.3
Toxi-Tubes A	83.2	99.2	5.47	1.87
Liquid–liquid extraction	68.3	77.7	0.0	0.0

The hydrolysis step was not included in this series of experiments. The high recoveries of B and NB by the Bond Elut C_{18} method is explained in the text.

The concentrations of *free* and *total* B/NB and BG/NBG in a urine sample were determined with the analysis (without and with hydrolysis) of two aliquots and the applications of two correction factors. The first aliquot was processed with the following steps: extraction (Toxi-Tubes A), derivatization, and GC–MS. The resulting concentrations of B and NB were considered "apparent" concentrations of *free* B and NB, designated as [FB′] and [FNB′], respectively. The second aliquot was analyzed with hydrolysis that included the following steps: hydrolysis (β-glucuronidases), extraction (Toxi-Tubes A), derivatization, GC–MS. The resulting concentrations of B and NB were considered *total* concentrations of B and NB, designed as [TB] and [TNB], respectively.

Using B and BG as the example, their concentrations in the specimen are calculated as follows.

$$[FB] = [FB'] - 0.0547[B'] \quad (1)$$

$$[B'] = [TB] - [FB] \quad (2)$$

where [FB] = the concentration of *free* B; [FB′] = apparent concentration of FB, or the observed concentration of free B resulting from the analysis of the first aliquot (without hydrolysis); [B′] = the concentration of B derived from the BG present in the urine sample (see Table 4); 0.0547 (or 5.47%) = the percentage of BG (in term of B) in the sample that would be extracted and detected as B using the Toxi-Tubes A extraction protocol; and [TB] = the concentration of B as determined by the analysis of the second aliquot (with hydrolysis).

Eqs. (1) and (2) can be combined:

$$[FB]=[FB']-0.0547([TB]-[FB])=[FB']-0.0547[TB]+0.0547[FB] \text{ or} \quad (3)$$

$$[FB] = \frac{[FB'] - 0.0547[TB]}{0.945} \quad (4)$$

Similarly, the concentration of free NB ([FNB]) can be derived using the following equation:

$$[FNB] = \frac{[FNB'] - 0.0187[TNB]}{0.981} \quad (5)$$

where the definitions of [FNB], [FNB′], 0.0187, and [TNB] are equivalent to [FB], [FB′], 0.0547, and [TB], as defined above for their B analogs.

Thus, Eqs. (4) and (5) are used to derive the concentrations of free B and NB ([FB] and [FNB]). The concentrations of BG and NBG ([BG] and [NBG]) can then be calculated using the following two equations:

$$[BG] = \frac{[TB] - [FB]}{0.726} \quad (6)$$

$$[NBG] = \frac{[TNB] - [FNB]}{0.701} \quad (7)$$

where 0.726 and 0.701 are the ratios of the molecular weights of B/BG and BG/NBG, respectively.

3.2. Applications and comparative studies

The over-all analytical scheme established above was applied to the analysis of approximately 50 urine specimens collected from patients (following the hospital's IRB protocols) under B-treatment. These same urine specimens have also been analyzed by CEDIA IA as described in our earlier study [28], while 19 of them have also been analyzed at another institution using the ion trap LC–MS/MS methodology [19]. Analytical data for these 19 specimens resulting from these three methods obtained in two laboratories are shown in Table 5. The total B concentrations of these specimens as determined by GC–MS are plotted against the corresponding data generated by the CEDIA and ion trap LC–MS/MS methodologies as shown in Fig. 2a and b, respectively.

Table 5
Comparison of total buprenorphine concentrations (ng/ml) as determined by CEDIA immunoassay, GC–MS, and ion trap LC–MSMS.

Specimen	CEDIA	GC–MS	LC–MSMS
1	77.0	75.9	129
3	66.3	69.3	98.7
5	48.6	41.5	61.3
6	58.7	50.2, 502[a]	537[a]
15	52.9	36.8, 147	168
18	21.2	15.9, 63.8	71.3
26	22.1	32.0, 128	124
30	45.8	44.8	55.1
33	60.0	59.8	66.2
35	57.1	48.5	63.4
36	24.2	12.6	55.9
38	53.5	48.3	68.3
39	72.1	65.1	86.3
40	85.0	70.7, 707[a]	783[a]
44	42.0	27.2	36.2
45	28.4	19.6	77.5
50	46.7	29.0	74.1
51	66.4	54.3	118
53	23.3	16.9, 67.5	88.7

CEDIA reagent exhibits approximately the same degree of responses to B and BG and much lower responses [28] to other metabolites (NB and NBG). Buprenorphine was used as the calibrator in the analytical protocol, while the responses generated by these specimens were converted to the equivalent concentrations of B. For GC–MS analysis, specimens were hydrolyzed for quantitative determination of total B without dilution. Two GC–MS concentrations are shown in the table for some specimens. The second figures were the results of GC–MS analysis, while the first figures were obtained by dividing the GC–MS concentrations by the respective dilution factors used to prepare these specimens for CEDIA analysis. For example, specimen #15 was diluted by a factor of 4 prior to the CEDIA analysis to obtain an apparent B concentration of 52.9 ng/ml.

[a] Buprenorphine concentrations in these 2 specimens were significantly higher. They were excluded from the GC–MS vs. ion trap LC–MS/MS plot (Fig. 2b) to avoid bias of the regression analysis.

3.2.1. Comparison of analytical data obtained in different laboratories using different methods

For the most part, three sets of data (CEDIA, GC–MS, ion trap LC–MS/MS) shown in Table 5 are compatible. The concentrations reported by the GC–MS methodology (Lab 1) are generally lower, but very close, to those reported by the CEDIA methodology (Lab 1). This strongly suggests that these two sets of data are more reliable than those derived from the ion trap LC–MS/MS methodology (Lab 2) for two reasons: (a) standard solution used for calibration for the CEDIA and the GC–MS methods came from two different sources, i.e., CEDIA reagent manufacturer and standard solutions prepared in the authors' laboratory using reference materials from Cerillant Corporation; and (b) CEDIA reagent is known to respond to B and BG similarly with low CC to other major metabolites (NB and NBG) [28]; thus, what CEDIA reagent detects are basically what are targeted by GC–MS (B and hydrolyzed BG), plus its low level of responses toward other metabolites present in the specimen. This would explain why the CEDIA data are slightly higher.

Data generated by the ion trap LC–MS/MS methodology (Lab 2) are also slightly higher, but compatible in general. This is probably a reflection of a slight variation in the calibration standards used in these two laboratories. However, the concentrations of total B found in 5 of these 19 specimens by the ion trap LC–MS/MS (Lab 2) method are significantly higher; we have thus conducted further comparative studies on the analysis of these 5 specimens in yet a third laboratory (Lab 3) using both GC–MS and *triple quadrupole* LC–MS/MS instrumentations. The resulting data for these 5 specimens are summarized along with the CEDIA, GC–MS and *ion trap* LC–MS/MS data previously obtained in Lab 1 and Lab 2 (Table 6).

Table 6
Comparison of analytical data (all in ng/ml) obtained from three laboratories using GC–MS and two configurations of LC–MS/MS.

Specimen	CEDIA	GC–MS		LC–MS/MS	
	Lab 1	Lab 1	Lab 3	Lab 3	Lab 2
Total buprenorphine (B)					
1	77.0	75.9	88.4	67.9	129
36	24.2	12.6	18.0	19.2	55.9
45	28.4	19.6	25.2	17.2	77.5
50	46.7	29.0	39.1	31.0	74.1
51	66.4	54.3	53.1	46.0	118
Total norbuprenorphine (NB)					
1	–	769	913	832	816
36	–	271	291	315	359
45	–	375	388	349	324
50	–	388	448	387	292
51	–	388	363	360	368

The LC–MS/MS instrumentation used by Lab 2 and Lab 3 are both from Agilent Technologies, but with ion trap and triple quadrupole configurations, respectively. Total concentrations of B and NB by the LC–MS/MS methods were calculated using the following formula: total [B] = free [B] + [BG] × [molecular weight of B (467.65)/molecular weight of BG (643.77)]; total [NB] = free [NB] + [NBG] × [molecular weight of NB (413.56)/molecular weight of NBG (589.67)]. Since CEDIA also exhibit low level of cross-reactivities to other metabolites, the observed total B concentrations are "apparent" values. Without using NB as the target analyte in CEDIA, total NB concentration could not be derived.

Data shown in Table 6 reveal that four sets of *total NB* concentrations obtained in three laboratories using three different types of instrumentations are practically the same—they all practically fall within ±20% of the means. However, the total B concentrations for these 5 specimens derived from the ion trap LC–MS/MS instrument are significantly higher than the other three sets of data, which are statistically indistinguishable. An application note [36], by the manufacturer (Agilent Technologies) of both LC–MS/MS systems used in this study, strongly suggested that the triple quadrupole configuration was superior over the ion trap platform in providing accurate quantitation data: "For quantification, the QQQ [triple quadrupole] was the best. . . The ion trap [LC–MS/MS]. . . can be hampered by the presence of coeluting interferences, not making it the best choice for quantification."

Interestingly, a recent study [37] stated that their "comparison between the direct detection of [B] and its metabolites with the analysis. . . using the hydrolysis method is reported for the first time. . ." and found that "the direct method gave slightly higher concentrations for [B] metabolites compared to the hydrolysis method." The authors proposed incomplete hydrolysis of the metabolites (present at high concentrations) as the cause of this observed difference. Our data have not shown this difference and wonder if the differences thereby reported were caused by not factoring in the molecular weight differences (between B/NB and their respective glucunonides) in concentration calculations.

In conclusion, we believe the GC–MS based approach has provided the accurate analytical data.

4. Conclusions

This study has demonstrated the widely available technology, GC–MS, can be effectively applied to compliance monitoring and pharmarcokinetic/pharmacogenetic studies associated with the use of B as an agent for treating heroin addiction. Studies on issues critical to GC–MS analysis of B and its three metabolites, including extraction, hydrolysis, derivatization, and IS, concluded: (a) acetyl-derivatization generates the products with most favorable chromatographic and ion intensity characteristics in GC–MS analysis; (b) commercial solid-phase extraction devices highly effective for recovering all metabolites may not be suitable for the analysis of free B and NB; and (c) a 2-aliquot protocol is an effective GC–MS approach for the analysis of B, NB, BG, and BNG in urine specimens.

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