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Gas chromatographic–mass spectrometric quantitation of urinary buprenorphine and norbuprenorphine after derivatization by direct extractive alkylation

A.M. Lisi*, R. Kazlauskas, G.J. Trout

Australian Sports Drug Testing Laboratory, P.O. Box 385, Pymble, N.S.W. 2073, Australia

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Abstract

A gas chromatographic–mass spectrometric procedure for the quantitation of buprenorphine and norbuprenorphine has been developed in which the analytes were converted, after enzyme hydrolysis, to their methyl derivatives by direct extractive alkylation using tetrahexylammonium hydrogen sulphate phase transfer reagent and iodomethane dissolved in *tert.*-butylmethyl ether. The procedure utilised a sample volume of 2 ml and gave a detection limit of 0.2 ng ml^{-1} for buprenorphine and norbuprenorphine. The buprenorphine and norbuprenorphine standard curves were linear in the concentration range of 1–100 ng ml^{-1} with $r=0.999$. The coefficients of variation for the intra-run precision were 1.3% for buprenorphine and 8.8% for norbuprenorphine ($n=10$). The coefficients of variation for the inter-run precision were 7.7% for buprenorphine and 10.1% for norbuprenorphine ($n=5$). The method recovery was 92% (C.V.=3.3%) for buprenorphine and 104% (C.V.=2.9%) for norbuprenorphine ($n=10$).

Keywords: Buprenorphine; Norbuprenorphine

1. Introduction

Narcotic analgesics, which have been banned in sport by the IOC Medical Commission, have the effect of reducing pain and producing feelings of euphoria. They also affect breathing and carry a risk of addiction.

Buprenorphine (BN; Fig. 1A), is administered in low doses intravenously or as a sublingual tablet and is metabolised by conjugation and N-dealkylation to norbuprenorphine (NBN; Fig. 1B). Urinary and plasma BN and NBN have been determined by radioimmunoassay (RIA), high-performance liquid chromatography (HPLC) and gas chromatography (GC). Debrabandere et al. [1] described an RIA technique for the determination of urinary and plasma BN. Hackett et al. [2] used HPLC with spectroscopic detection to determine urinary BN. The method involved the enzyme hydrolysis of urine samples, extraction of the BN on C_{18} bonded silica columns and purification of the extract by thin-layer chromatography before identification of the analyte. The technique required a sample volume of 10 ml. Debrabandere et al. [3] extracted BN and NBN from 2 ml of urine using toluene solvent and removing the urinary interferences by back extraction into dilute hydrochloric acid before re-extraction of the acid solution with toluene followed by HPLC analysis

*Corresponding author

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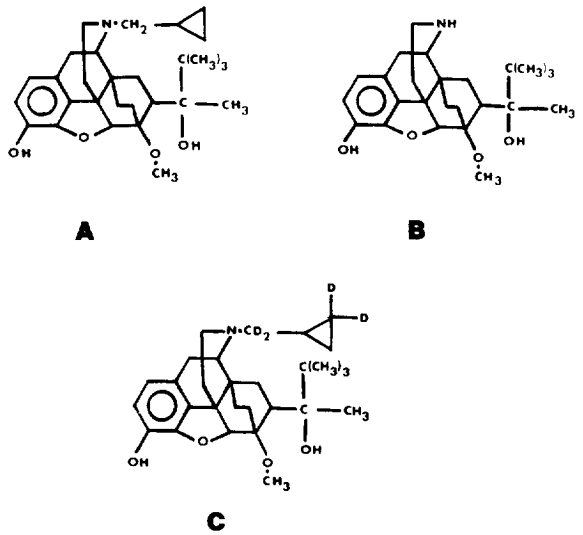

Fig. 1. Structures of (A) buprenorphine, (B) norbuprenorphine and (C) isotope labeled tetra-deuterated buprenorphine.

using an electrochemical detector. Schleyer et al. [4] also used HPLC with electrochemical detection. Their method used a valve-switching solid-phase trace-enrichment procedure and required 5 ml of sample. The procedure gave a detection limit for BN and NBN of 40 pg ml^{-1}.

Several GC methods of analysis using the acyl derivatives of BN and NBN have been presented. Cone et al. [5] used GC with an electron capture detector (ECD) to determine BN and NBN in urine and faeces after enzyme hydrolysis, clean-up by back extraction into dilute sulphuric acid and derivatization with pentafluoropropionic anhydride. With a 1 ml sample volume they achieved detection limits of 10 ng ml^{-1} for BN and 5 ng ml^{-1} for NBN. Ohtani et al. [7] extracted BN and NBN from plasma using a modification of the procedure described by Cone et al. [5] with detection by gas chromatography chemical ionization mass spectrometry. Blom et al. [6] determined BN and NBN in plasma and urine as their cyclic artifacts after acid hydrolysis. The samples were extracted with toluene–2-butanol, back-extracted into dilute sulphuric acid and heated to 110 °C to generate the cyclic artifacts of the analytes which were then extracted into dichloromethane–2-butanol and derivatized with pentafluoropropionic anhydride and analysed by gas chromatography–mass spectrometry (GC–MS). With 2 ml of plasma the detection limit was 150 pg ml^{-1}. Martinez et al. [8] determined BN in plasma and urine using GC with an ECD and a nitrogen–phosphorous detector (NPD). For the ECD the analytes were derivatized with heptafluorobutyric anhydride and for the NPD they were derivatized with acetic anhydride.

This communication describes the development of a extractive alkylation (EA) method for the gas chromatographic–mass spectrometric (GC–MS) determination of urinary BN and NBN as their methyl derivatives after enzyme hydrolysis.

2. Experimental

2.1. Reagents and chemicals

Buprenorphine hydrochloride, NBN and Temgesic (BN in tablet form) were kindly donated by Reckitt and Colman (Sydney, Australia). Isotope-labeled tetra-deuterated BN (BN-d_4), Fig. 1C, was purchased from Radian (Austin, TX, USA). HPLC grade *tert.*-butylmethyl ether (TBME) and β-glucuronidase enzyme type HP2 from *Helix pomatia* with a activity of 103 000 units ml^{-1} were purchased from Sigma (St. Louis, MO, USA). Iodomethane, nanograde diethyl ether, hexane, toluene, HPLC grade acetonitrile and analytical grade ethyl acetate were purchased from Malinckrodt (Paris, KY, USA). HPLC grade dichloromethane and methanol were purchased from EM Science (Gibbstown, NJ, USA). Analytical grade 36% hydrochloric acid was purchased from Rhone-Poulenc (Clayton, Australia). Tetrahexylammonium hydrogen sulphate (THA) was purchased from Fluka (Buchs, Switzerland) and analytical grade 200–400 mesh SM-7 resin was purchased from Bio-Rad, (Richmond, CA, USA). Silica gel 40 was purchased from Merck (Darmstadt, Germany).

2.2. Preparation of reagents

BN and NBN stock solutions (100 μg ml^{-1}) were prepared by dissolving 5.4 mg of buprenorphine hydrochloride and 5.0 mg of NBN in 50 ml of methanol. Iodomethane was redistilled as required and stored over silver at 4 °C. The redistilled iodomethane was kept for two weeks before redistilling a

fresh solution. A 0.2 *M* solution of THA was prepared by dissolving 4.5 g of the salt in 50 ml of acetonitrile. A 2 *M* potassium acetate buffer solution, pH 5.0, was prepared using a modification of the procedure recommended by the enzyme supplier. Potassium acetate (49 g) was dissolved in 200 ml of deionized water before adjusting the pH to 5.0 with 36% analytical grade HCl. The buffer solution was made to a final volume of 250 ml with deionized water.

2.3. Preparation of SM-7 resin columns

The fines were removed from the commercially available 200–400 mesh SM-7 sorbent by suspending it in methanol and decanting the supernatant. This procedure was repeated until the supernatant was clear. The suspended sorbent was packed as a slurry inside disposable glass pipettes (6 mm I.D.) that were fitted with plugs of silanised glass wool to act as bed supports. The sorbent was packed until a column with a length of 2.5–3.0 cm was produced. Each column was conditioned by washing with 2 ml of TBME and 2 ml of hexane.

2.4. Instrumentation

GC–MS measurements were performed on a Hewlett–Packard 5890 Series II GC equipped with a Hewlett–Packard 5970B electron-impact mass selective detector via a capillary direct interface. The capillary column was a HP ultra 2 with dimensions of 17 m×0.22 mm I.D. The carrier gas was helium and the column head pressure was maintained at 90 kPa. The linear velocity of the carrier gas through the capillary column was 27 cm s^{-1}. The sample was introduced into the GC column in split mode using a Hewlett–Packard 7673A automatic injector.

The flow through the split and purge valves were 7 ml min^{-1} and 3 ml min^{-1}, respectively. The injector and detector temperatures were set at 290°C. The oven temperature was programmed to start at 247°C and increase at the rate of 10°C min^{-1} to a final temperature of 310°C. The oven was held at the final temperature for 1 min. The mass selective detector was operated in the selected-ion mode (SIM) and set to monitor the nine fragment ions listed in Table 1 for the methyl derivatives of BN, NBN and BN-d_4.

2.5. Synthesis of the methyl derivatives of buprenorphine and norbuprenorphine

An 80-mg quantity of buprenorphine hydrochloride and NBN were added to separate 20-ml quickfit B14 test tubes (Mowbray Glass, Gosford, Australia) along with 10 ml of acetonitrile, 300 mg of analytical grade potassium carbonate and 1 ml of iodomethane. The tubes were sealed and the mixtures refluxed for 4 h on an aluminum block heated to 60°C before cooling to room temperature, evaporating the acetonitrile with a stream of nitrogen and extracting the residues with three 5 ml aliquots of ethyl acetate. The methyl derivatives were separated from unreacted material by column chromatography using a 10×1 cm column of silica gel 40. The methyl derivative of BN was eluted with a solvent system consisting of ethyl acetate–hexane (15:85, v/v) and the methyl derivative of NBN was eluted with ethyl acetate–hexane (25:75, v/v). The solvents were evaporated and the residues recrystallised from acetonitrile. Infra-red spectroscopy on the methyl derivative of BN indicated that acetonitrile was trapped in the crystal matrix, i.e. a peak at 2270 cm^{-1} was observed corresponding to the carbon–nitrogen triple bond of acetonitrile. The trapped acetonitrile was removed by heating the derivative under vacuum for 4 h at 55°C.

The methyl derivative of BN gave a melting point of 51–52°C and a composition of 74.4% carbon, 9.3% hydrogen and 3.2% nitrogen (the theoretical composition is 74.8% carbon, 9.0% hydrogen and 2.9% nitrogen). The methyl derivative of NBN gave a melting point of 185–186°C and a composition of 73.1% carbon, 8.9% hydrogen and 3.3% nitrogen

Table 1
Selected ions monitored for the methyl derivatives of BN, BN-d_4 and NBN

Derivative	Selected ions (*m/z*)
Methyl ether derivative of BN	392, 424, 481 (M^+)
Methyl ether derivative of BN-d_4	396, 428, 485 (M^+)
N,O-Dimethyl derivative of NBN	352, 384, 441 (M^+)

(the theoretical composition is 73.4% carbon, 8.9% hydrogen and 3.2% nitrogen).

2.6. Preparation of the methylated cyclic artifacts of buprenorphine and norbuprenorphine

Aliquots (2 ml) of BN and NBN stock solutions (100 μg ml^{-1}) were added to separate 20 ml quickfit B14 test tubes and the methanol solvent evaporated to dryness under a stream of nitrogen before reconstituting the residues in 2 ml of deionized water and acidifying with 2–3 drops of 36% hydrochloric acid solution. The tubes were sealed and heated to 90°C for 6 h on an aluminium block. On cooling to room temperature the solutions were made alkaline with 6 *M* NaOH and the cyclic artifacts methylated by EA using toluene as the solvent and THA phase transfer reagent. To remove the excess THA the toluene was passed through pre-prepared columns of SM-7 resin, collected and evaporated to dryness. The methylated cyclic artifacts were dissolved in 5 ml methanol and stored at 4°C.

2.7. Hydrolysis

A 2-ml aliquot of urine was added to a 16×150 mm PTFE-lined screw-capped test tube, fortified with 10 μl of 2 μg ml^{-1} BN-d_4 (internal standard) and made to pH 5.0 by the addition of 300 μl of 2 *M* potassium acetate buffer. Type HP2 β-glucuronidase enzyme from *Helix pomatia* was added to the urine sample to give a urinary enzyme activity of 3000 units ml^{-1}. The urine was hydrolysed at 50°C for 1.5 h.

2.8. Extractive alkylation

The hydrolysed urine was allowed to cool to room temperature before the addition of 340 μl of 6 *M* NaOH, 5 ml of 0.7 *M* iodomethane in TBME and 75 μl of 0.2 *M* THA solution. The urine and the TBME phase were shaken at 25°C for at least 40 min before the addition of 1 ml of hexane and centrifugation at 600 *g* for 5 min. The TBME–hexane mixture was passed through a pre-prepared SM-7 resin column, collected in a disposable culture tube and evaporated to dryness under a stream of nitrogen at 35°C. The residue was reconstituted in 100 μl of ethyl acetate before injection of a 4-μl aliquot into the GC–MS system.

3. Results and discussion

3.1. Procedure

Following the enzyme hydrolysis of the urine the liberated BN and NBN were methylated by EA using THA phase transfer reagent and iodomethane dissolved in TBME. Figs. 2A and 3A show the full scan EI mass spectra of the methyl derivatives of BN and NBN. For each methyl derivative three ions were used for SIM analysis (Table 1). The molecular ion peak (M^+) is at *m/z* 481 for the methyl derivative of BN and at *m/z* 441 for the methyl derivative of NBN. The base peak (M^+-89) occurs at *m/z* 392 for BN and *m/z* 352 for NBN and results from the loss of CH_3OH and C_4H_9. Ions, *m/z* 424 for BN and *m/z* 384 for NBN, (M^+-57), are due to the loss of C_4H_9. The base peaks were used for quantitations while the remaining ions were used as qualifiers for confirmation of peak identity.

To avoid rapid deterioration of the capillary column from the excess phase transfer reagent it was necessary to remove the THA dissolved in the organic layer before the GC–MS analysis of the samples. The excess THA was removed by the solid-phase extraction procedure outlined by Lisi et al. [10] in which short columns of SM-7 resin were used to strip the THA from the organic phase. Before passing the TBME through a pre-prepared column of SM-7 resin a 1-ml aliquot of hexane was added to reduce the polarity of the organic phase so that the resin could absorb the more polar THA salts while allowing the less polar methyl derivatives of BN and NBN to elute from the columns.

3.2. Optimization of the extractive alkylation reaction

The yield of the methyl derivatives of BN and NBN under EA conditions was influenced by the concentration of the quaternary ammonium phase transfer reagent, the organic solvent, the mixing time of the aqueous and organic phases and the con-

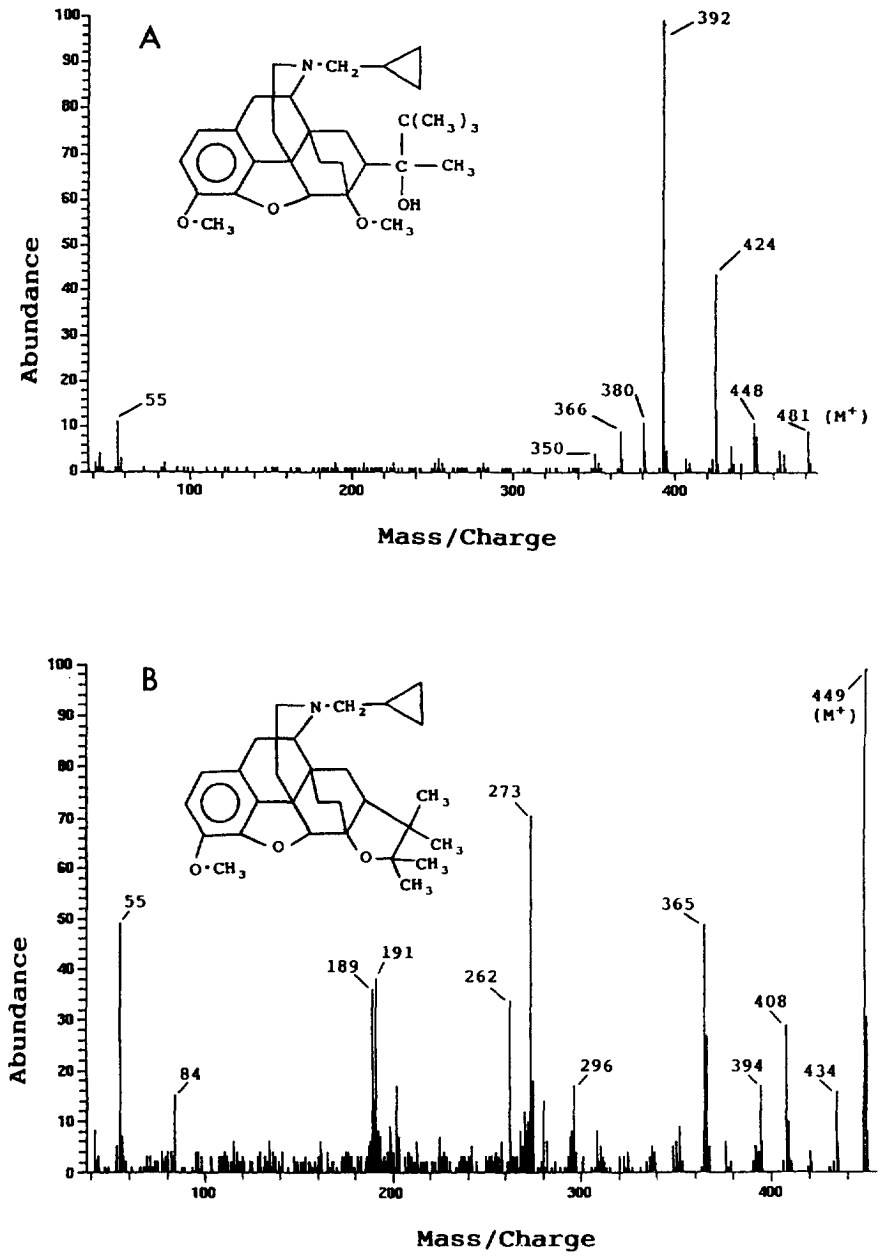

Fig. 2. Electron-impact mass spectrum of the methyl ether derivatives of (A) BN and (B) the cyclic artifact of BN.

centration of iodomethane in the organic phase. Figs. 4 and 5 demonstrate the influence of the organic phase THA concentration and mixing time on the yield of the methyl derivatives of BN and NBN. The limiting reaction was the methylation of NBN which required an organic phase THA concentration of 3.0 m*M* and a minimum reaction time, at 25°C, of 40 min to obtain the maximum yield. For BN the maximum yield of the methyl derivative was obtained with an organic phase THA concentration of 0.25 m*M* and a minimum reaction time, at 25°C, of 20 min.

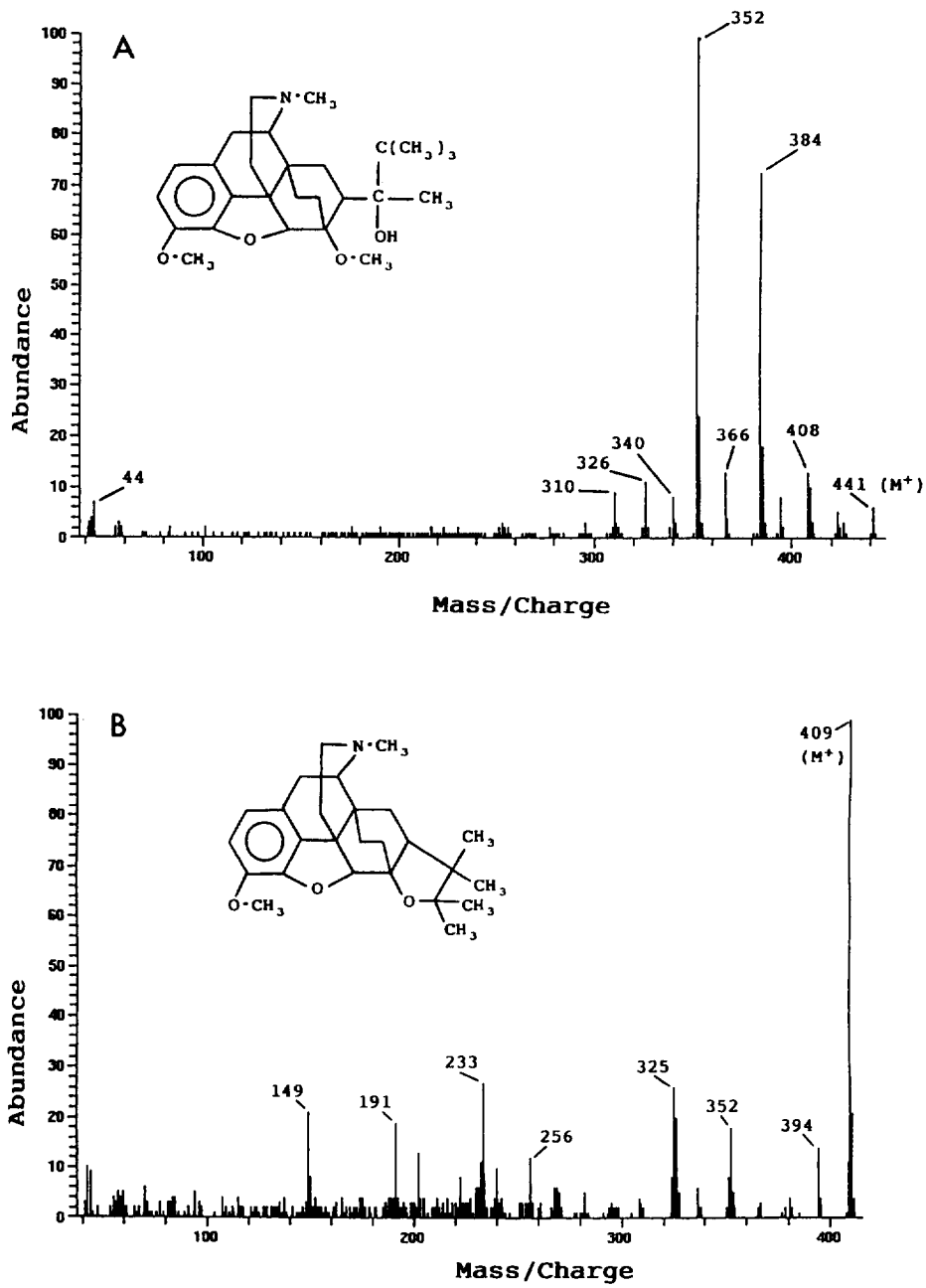

Fig. 3. Electron-impact mass spectrum of the N,O-dimethyl derivatives of (A) NBN and (B) the cyclic artifact of NBN.

Table 2 demonstrates the influence of the organic solvent on the yields of the methyl derivatives of BN and NBN. Maximum yields for the two analytes were obtained with TBME.

The rate of alkylation of BN and NBN was also dependent on the concentration of iodomethane in the organic phase. Fig. 6 demonstrates the influence of the organic phase iodomethane concentration on the yields of the methyl derivatives of BN and NBN. The limiting reaction was the methylation of NBN

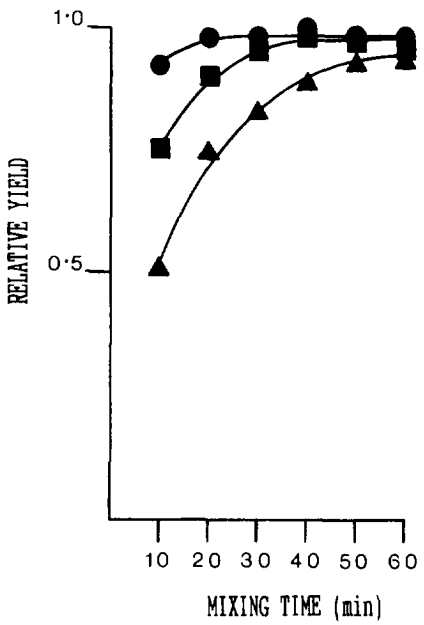

Fig. 4. Time-course showing the effect of the organic phase concentration of THA on the methylation of BN. Aqueous phase: 2 ml of urine. Organic phase: 5 ml of 0.1 *M* iodomethane in TBME. Concentration of BN: 50 ng ml^{-1}. Concentration of THA in the organic phase: ▲=0.05 m*M*; ■=0.15 m*M*; ●=0.25 m*M*. Temperature: 25°C.

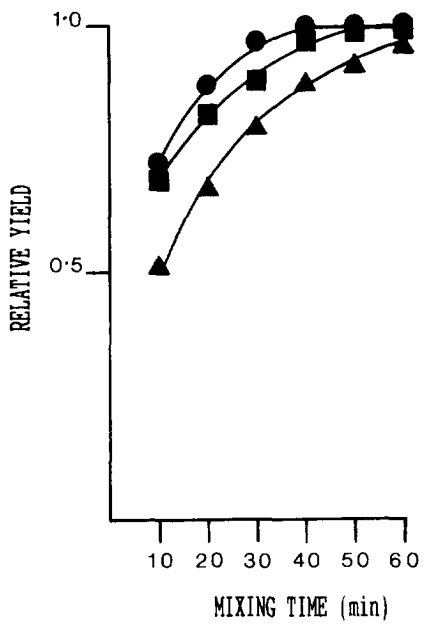

Fig. 5. Time-course showing the effect of the organic phase concentration of THA on the methylation of NBN. Aqueous phase: 2 ml of urine. Organic phase: 5 ml of 0.7 *M* iodomethane in TBME. Concentration of NBN: 50 ng ml^{-1}. Concentration of THA in the organic phase: ▲=1.0 m*M*; ■=2.0 m*M*; ●=3.0 m*M*. Temperature: 25°C.

which required a minimum iodomethane concentration of 0.6 *M* for optimum yields. With BN the optimum yields were obtained with a minimum organic phase iodomethane concentration of 0.1 *M*.

3.3. Optimization of the enzyme hydrolysis

The optimum conditions for the enzyme hydrolysis of the glucuronide conjugates of BN and NBN at 50°C were determined using the urine collected from a volunteer who ingested a single 0.2 mg sublingual dose of BN. The urine samples were prepared as described under hydrolysis in the experimental section except that urinary β-glucuronidase activities in the range of 500–3000 units ml^{-1} and hydrolysis times in the range of 0.5–3.0 h were investigated. On cooling to room temperature the liberated BN and NBN were methylated by EA using the experimentally determined conditions. The influence of the urinary β-glucuronidase activity and the hydrolysis time are demonstrated in Figs. 7 and 8. The hydrolysis of NBN was the limiting step requiring a urinary β-glucuronidase activity of 3000 units ml^{-1} and a minimum hydrolysis time of 1.5 h to achieve the maximum yield. BN gave maximum yields after a minimum hydrolysis time of 1.5 h and a urinary β-glucuronidase activity of 1500 units ml^{-1}.

Cone et al. [9] demonstrated that under extreme conditions of heat and low pH, BN and NBN

Table 2
Relative recovery of the methyl derivatives of BN and NBN in four solvents

Solvent	Relative recovery[a] for BN (%)	Relative recovery[a] for NBN (%)
tert.-Butyl methyl ether	100	100
Diethyl ether	68	96
Toluene	77	92
Dichloromethane	89	83

[a] The results are reported relative to the yields obtained for *tert.*-butylmethyl ether.

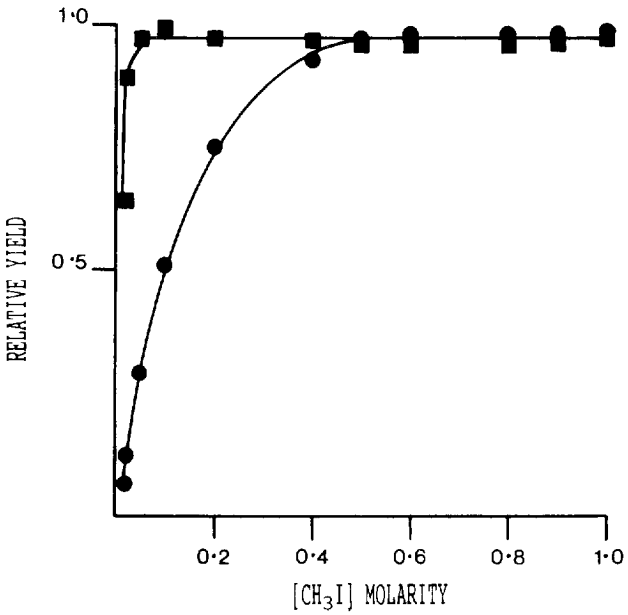

Fig. 6. Effect of the organic phase iodomethane concentration on the methylation of BN (■) and NBN (●). Concentration of THA in the organic phase: 3.0 m*M*. Aqueous phase: 2 ml of urine. Concentration of BN and NBN: 50 ng ml^{-1}. Mixing time: 40 min. Temperature: 25°C.

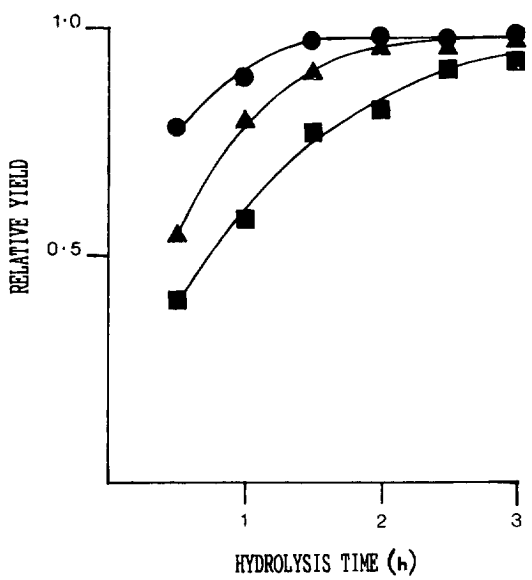

Fig. 7. Time-course for the enzyme hydrolysis of BN-glucuronide. Aqueous phase: 2 ml of a urine sample collected from a volunteer who ingested a 0.2-mg sublingual dose of buprenorphine. Hydrolysis temperature: 50°C. Urinary enzyme activity: ■=500 units ml^{-1}; ▲=1000 units ml^{-1}; ●=1500 units ml^{-1}.

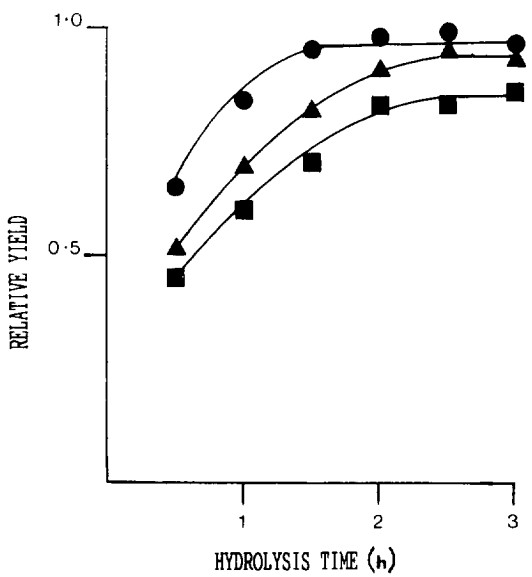

Fig. 8. Time-course for the enzyme hydrolysis of NBN-glucuronide. Aqueous phase: 2 ml of a urine sample collected from a volunteer who ingested a 0.2-mg sublingual dose of buprenorphine. Hydrolysis temperature: 50°C. Urinary enzyme activity: ■=1500 units ml^{-1}; ▲=2000 units ml^{-1}; ●=3000 units ml^{-1}.

undergo an acid catalysed cyclic rearrangement reaction with the net loss of a molecule of methanol and formation of a new tetrahydrofuran ring. To determine if the cyclic artifacts of BN and NBN were generated under the hydrolysis conditions, ten 2-ml aliquots of urine were spiked to a concentration of 100 ng ml^{-1} with BN and NBN, made to pH 5.0 with 300 μl of 2 *M* potassium acetate buffer, heated for 3 h in a 50°C water bath and methylated by EA. Fig. 2B Fig. 3B demonstrate the full scan EI mass spectra of the methyl derivatives of the cyclic artifacts of BN and NBN. The ions chosen for SIM analysis were *m/z* 449 and 365 for the BN artifact and *m/z* 409 and 325 for the NBN artifact. The two artifacts were not detected indicating that BN and NBN did not undergo cyclic rearrangement under the hydrolysis conditions used for this work.

3.4. Linearity, precision and accuracy

Standard curves were prepared by fortifying duplicate 2 ml aliquots of drug free urine to 1, 2, 5, 10, 25, 50, 75 and 100 ng ml^{-1} of BN and NBN. Each sample was also fortified to 10 ng ml^{-1} with BN-d_4 (internal standard). The standard curves were constructed from the peak-area ratio versus concentration using least-squares regression analysis and forced through zero. Of the nine ions selected for SIM analysis (Table 1) *m/z* 392 of the BN derivative, *m/z* 352 of the NBN derivative and *m/z* 396 of the BN-d_4 derivative were used as quantifier ions. The area ratios between the quantifier ions of the analytes and the internal standard (*m/z* 392/396 for BN and *m/z* 352/396 for NBN) were used for the regression analysis. The inter-run standard curves shown in Table 3 and Table 4 were linear in the concentration range of 1–100 ng ml^{-1} with $r=0.999$.

To determine the inter-run precision five trials were carried out over a period of two months in which a 2-ml aliquot of a urine known to contain BN and NBN was assayed by EA and injected into the GC–MS system in duplicate. The assay results are shown in Tables 3 and 4. The inter-run coefficient of variation (C.V.) for BN was 7.7% and for NBN it was 10.1% ($n=5$).

The intra-run precision was evaluated by the same day assaying of 2-ml aliquots of a urine known to contain BN and NBN. The assay results are shown in Table 5. The intra-run C.V. for BN was 1.3% and for NBN it was 8.8% ($n=10$).

Table 3
Inter-run precision and linear regression curves for the determination of urinary BN

Trial	[BN] (ng ml^{-1})	BN regression curves	*r*
1	2.06	$y=0.100x+0.071$	0.9995
2	2.47	$y=0.117x+0.041$	0.9998
3	2.10	$y=0.109x+0.057$	0.9997
4	2.12	$y=0.106x+0.066$	0.9996
5	2.12	$y=0.092x+0.053$	0.9997
Mean	2.17		
C.V.[a]	7.7%		

[a] Coefficient of variation.

The accuracy was determined by assaying 2-ml aliquots of urine that were fortified to a concentration of 18 ng ml^{-1} BN and 4 ng ml^{-1} NBN. The assayed urinary BN concentration was 18.24±0.30 ng ml^{-1} and the assayed NBN concentration was 4.11±0.34 ng ml^{-1} ($n=10$).

3.5. Detection limit and recovery

The detection limit, defined as the concentration of analyte that gave a signal-to-noise ratio of 3, was determined using the base peaks *m/z* 392 for BN and *m/z* 352 for NBN. The detection limit for BN and NBN was 0.2 ng ml^{-1}. The limit of quantitation for total urinary NBN was 1.0 ng ml^{-1} due to traces of

Table 4
Inter-run precision and linear regression curves for the determination of urinary NBN

Trial	[NBN] (ng ml^{-1})	NBN regression curves	*r*
1	2.43	$y=0.106x+0.020$	0.9998
2	2.65	$y=0.127x+0.038$	0.9998
3	2.73	$y=0.108x+0.077$	0.9996
4	2.86	$y=0.122x+0.029$	0.9999
5	3.21	$y=0.097x+0.044$	0.9995
Mean	2.77		
C.V.[a]	10.1%		

[a] Coefficient of variation.

Table 5
Intra-run precision for the determination of urinary BN and NBN

Aliquot	[BN] (ng ml^{-1})	[NBN] (ng ml^{-1})
1	19.39	2.88
2	19.93	3.04
3	20.07	3.63
4	19.92	3.17
5	20.15	3.00
6	20.07	3.42
7	19.68	3.13
8	20.16	3.59
9	20.19	3.54
10	20.01	3.63
Mean	19.95	3.30
S.D.	0.25	0.29
C.V.[a]	1.3%	8.8%

[a] Coefficient of variation.

an interfering compound originating from the enzyme used in the hydrolysis procedure.

The method recovery was determined by fortifying 2-ml aliquots of drug free urine to a concentration of 15 ng ml^{-1} of BN and 5 ng ml^{-1} of NBN and assaying by the EA procedure described in Section 2.8. At the conclusion of the procedure the residues were reconstituted in 100 μl of ethyl acetate containing the methyl derivative of the cyclic artifact of NBN as the internal standard. The in-house synthesised methyl derivatives of BN and NBN were used to prepare calibration curves in the concentration range of 2.5–50 ng ml^{-1} with the pre-prepared methyl derivative of the cyclic artifact of NBN acting as internal standard. The quantifier ions used were *m/z* 392 of the BN derivative, *m/z* 352 of the NBN derivative and *m/z* 409 of the derivative of the cyclic artifact of NBN (internal standard). The area ratios between ions *m/z* 392/409 for BN and *m/z* 352/409 for NBN were used to construct the calibration curves. The recovery for BN was 92% (C.V.=3.3%) and for NBN it was 104% (C.V.=2.9%) for $n=10$.

3.6. Determination of BN and NBN in urine

The method that has been outlined was used to determine the urinary concentrations of free and total BN and NBN after administration of a single 0.2 mg sublingual dose of Temgesic to a volunteer. Urinary levels of the analytes were measured for 42.5 h post dosing and were not corrected for volume or specific gravity. Fig. 9 shows the SIM GC–MS profile obtained after enzyme hydrolysis and EA on a urine sample with an assayed BN concentration of 20 ng ml^{-1} and an assayed NBN concentration of 3.3 ng ml^{-1}. Fig. 10 demonstrates the urinary concentration of total BN and NBN. BN was detected in the urine from 1 h post dosing. The urinary concentration of total BN reached a maximum of 33 ng ml^{-1} after 2.5 h and was detectable to 42.5 h post dosing. NBN was detected in the urine from 2.5 h post dosing with the concentration of total NBN ranging from 2.0 to 6.3 ng ml^{-1} over the time period that was investigated.

Fig. 11 demonstrates the urinary concentrations of free BN and NBN. Free BN was detected at very low levels to 12 h post dosing with the concentration ranging from 0.2–0.5 ng ml^{-1}. The very low levels of free BN are in agreement with the work of Cone et al. [11] who could not detect free urinary BN in their investigation. Free NBN was detected from 2.5

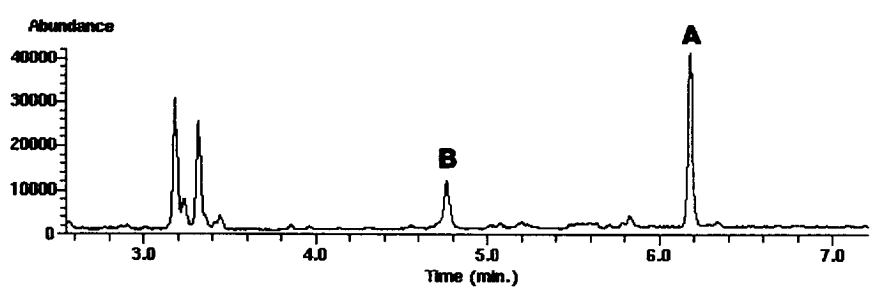

Fig. 9. SIM GC–MS profile of a urine sample with an assayed BN concentration of 20 ng ml^{-1} and an assayed NBN concentration of 3.3 ng ml^{-1}. The urine was hydrolysed with *Helix pomatia* enzyme at 50°C before EA. Peaks: A=methyl ether derivative of BN; B=N,O dimethyl derivative of NBN.

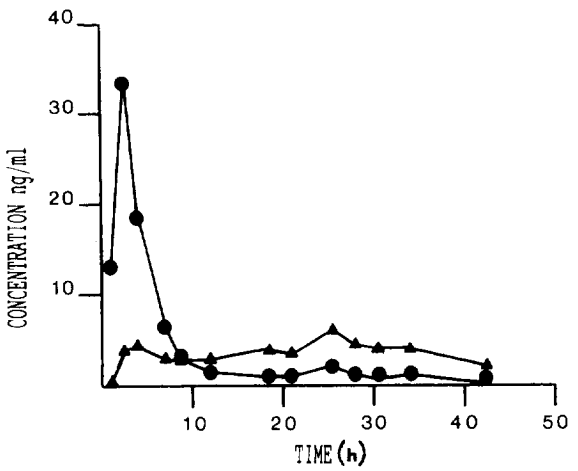

Fig. 10. Urinary concentrations of total BN (●) and NBN (▲) after the sublingual administration of a single tablet of Temgesic containing 0.2 mg of BN. At $t=1.0$ h post dosing the total concentration of NBN was below the method detection limit.

to 42.5 h post dosing except at $t=8.5$ and 12.0 h. The concentration range of free NBN was 0.4 to 1.7 ng ml^{-1}.

The urine matrix did not interfere with the determination of BN or NBN but the matrix of the helix pomatia enzyme that was used in the hydrolysis procedure gave traces of interference on the m/z 352 and 384 ions of NBN with the interference being strongest on the m/z 384 ion. The quantitation of total NBN required a blank correction to compensate for the enzymatic interference. The limit of quantitation for total NBN was 1.0 ng ml^{-1}.

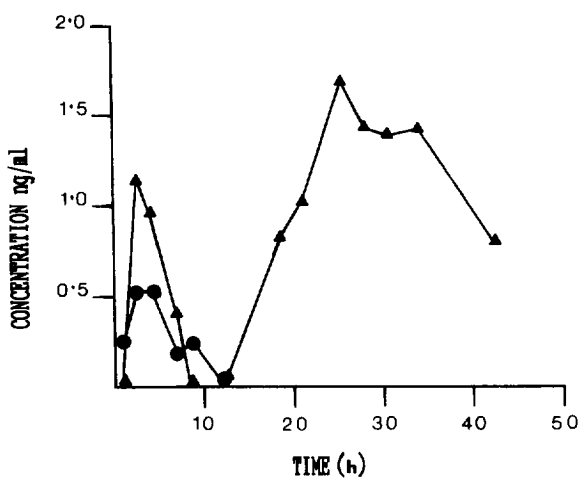

Fig. 11. Urinary concentrations of free BN (●) and NBN (▲) after the sublingual administration of a single tablet of Temgesic containing 0.2 mg of BN. From $t=12$ h post dosing the concentration of free BN was below the method detection limit. The concentration of free NBN was below the method detection limit at $t=8.5$ and 12.0 h.

4. Conclusion

The method that has been described is suitable for the monitoring of urinary levels of BN and NBN after therapeutic doses and for sport doping analysis.

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