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Quantitative determination of butorphanol and its metabolites in human plasma by gas chromatography–electron capture negative-ion chemical ionization mass spectrometry

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Abstract

Two separate analytical methods have been developed for the determination of butorphanol and its metabolites in human plasma. One method is specific for butorphanol (I) while the other determines the metabolites, hydroxybutorphanol (II) and norbutorphanol (III). Both procedures incorporate solid-phase extraction, chemical derivatization and separation, and detection using gas chromatography–electron-capture negative-ion chemical ionization mass spectrometry (GC–ECNCI-MS). Both methods use the cyclopropyl analog of I (BC-2605, IV) as the internal standard and the procedures for extraction of the analytes from plasma are identical. However, following extraction, either the pentafluorobenzoyl ester of I or the *tris*- and *bis*-trifluoroacetyl esters of II and III, respectively, were prepared. The derivatives were analyzed by GC–ECNCI-MS with selected-ion monitoring of the molecular ions. The standard curves were linear over the concentration ranges of 20–2000, 20–1000 and 50–1000 pg/ml for I, II and III, respectively. All standard curves from the assay validation had r^2 values of $\geq$0.994, 0.991 and 0.985 for I, II and III, respectively. For all three compounds, the intra- and inter-assay precisions (C.V.) and inter-assay accuracy (deviation from nominal) were within 12% for plasma quality control samples. All derivatives were stable in the reconstitution solvent for at least 24 h. The assays are being used for the determination of plasma concentrations of I, II and III in humans following repeated administration of nasal spray.

Keywords: Butorphanol; Hydroxybutorphanol; Norbutorphanol

1. Introduction

Butorphanol [17-(cyclobutylmethyl)morphinan-3,14-diol, I, Fig. 1] is a synthetic opioid [1] which exhibits analgesic properties. Currently, it is marketed for the indication of relief of moderate to severe pain. At the therapeutic transnasal dose of 2 mg at 6-h dosing intervals, the steady state plasma C_{max} is approximately 2–3 ng/ml [2]. From data obtained in animal studies, the major metabolite hydroxybutorphanol (II, Fig. 1) was expected to be present in human plasma at levels comparable to

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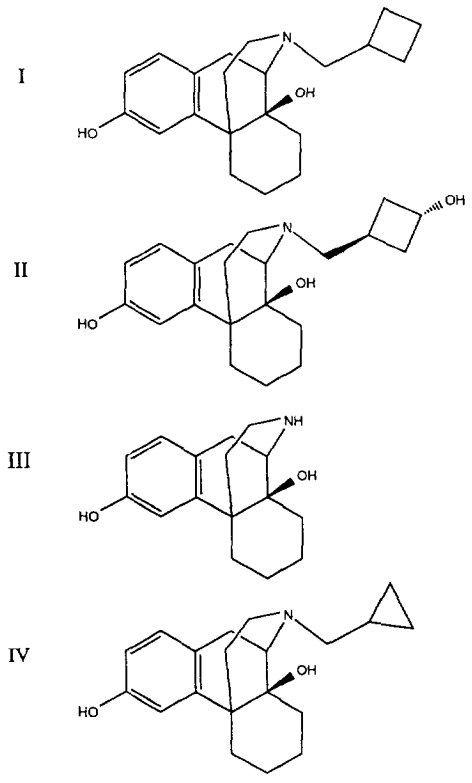

Fig. 1. Structures of butorphanol (I), hydroxybutorphanol (II), norbutorphanol (III) and the internal standard, BC-2605 (IV).

those of I, while the minor metabolite norbutorphanol (III, Fig. 1) was expected to exhibit plasma concentrations much lower than those of the parent. There currently exists a sensitive radioimmunoassay (RIA) which is specific for I [3] and the steady-state plasma pharmacokinetics of I have been investigated [2]. However, little is known about the steady state pharmacokinetics of the metabolites. Since activity of the metabolites has been observed, albeit much reduced, in receptor binding screens, it was desirable to develop a sensitive analytical method which could be used to determine the profiles of all three compounds. An HPLC method [4] was validated for the determination of levels of the parent drug and metabolites in human urine but the sensitivity is insufficient for determining these analytes in human plasma. Due to its inherent specificity and sensitivity, the ideal analytical technique would be gas chromatography–mass spectrometry with electron-capture negative-ion chemical ionization (GC–ECNCI-MS).

Compound I and its metabolites are opioid phenanthrene derivatives and are thus similar in structure to other opiate analgesics, including morphine, codeine, buprenorphine and nalbuphine. Many analytical methods have been developed for these and similar compounds [5–11], although an MS assay with the desired limit of quantitation of 20 pg/ml was not found in the literature. It was initially intended to develop a single assay for I and its metabolites, however, comparable sensitivity could not be obtained for all three compounds by a single procedure (see Section 3). Since high sensitivity was required for all three compounds, an assay was developed for I using one derivatization reagent while a separate assay was developed for the metabolites using a different reagent. The assays developed are identical in the analyte extraction procedure but differ in the derivatization and GC–MS conditions used.

2. Experimental

2.1. Materials and reagents

Tartrate salts of I, II, III and IV were supplied by Bristol-Myers Squibb (Syracuse, NY, USA). Methanol, ethanol, acetonitrile, toluene, ethyl acetate and methylene chloride were all AR grade and obtained from Mallinckrodt (Paris, KY, USA). Trifluoroacetic anhydride (TFAA) and pyridine were purchased from Pierce (Rockford, IL, USA) and pentafluorobenzoyl chloride (PFBC) and triethylamine (TEA) were obtained from Aldrich (Milwaukee, WI, USA). Ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA). Water was Milli-Q grade (Millipore, Bedford, MA, USA). Helium and methane (both ultra-high-purity grade) were supplied by Matheson (Twinsburg, OH, USA). Control human plasma (EDTA) was obtained from Biological Specialty (Lansdale, PA, USA). CN-N Bond-Elut solid-phase extraction (SPE) columns were purchased from Analytichem (Harbor City, NJ, USA).

2.2. Instrumentation and data systems

All analyses were performed using a Hewlett-Packard 5988A GC–MS instrument (Palo Alto, CA, USA) equipped with a split–splitless capillary inlet

and an HP-7673A autosampler. The analytical column used was a 15 m×0.25 mm I.D. DB-1 (0.25 μm film thickness) obtained from J & W Scientific (Folsom, CA, USA) equipped with a retention gap consisting of approximately 1.5 m of deactivated fused silica. The injection port liner was a deactivated, packed, single tapered direct injection liner obtained from Hewlett-Packard. Raw data were collected on an HP 59970 MS chemstation, with Pascal Rev. 3.2 operating software. Data were then uploaded via ChemLAN software to an HP 59940A UNIX workstation, with HP-UX software version

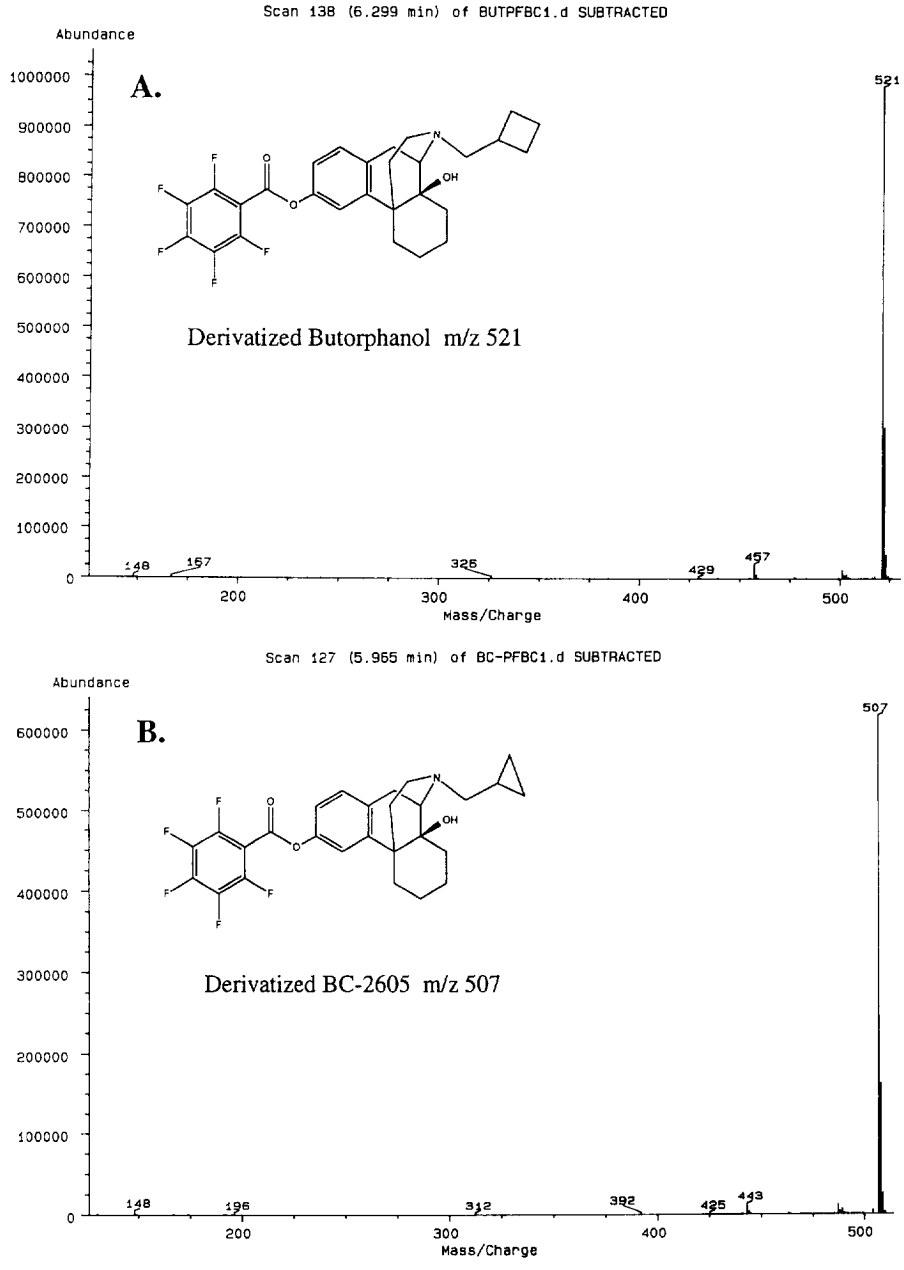

Fig. 2. Methane NCI mass spectra of PFBC derivatives of butorphanol (A) and BC–2605 (B).

A.01.04, where integration and data processing were performed. Integration results and sample information were then compiled in ASCII files from which injection number, sample identification, peak name and peak area were extracted by a program written in the UNIX vi screen editor. These data were then uploaded as editor files to an HP-1000 mainframe computer [12] via the program KERMIT for regression analysis and prediction of quality control and unknown sample concentrations. Standard curves were constructed using a linear regression weighted by the reciprocal of the standard concentration ($1/x$).

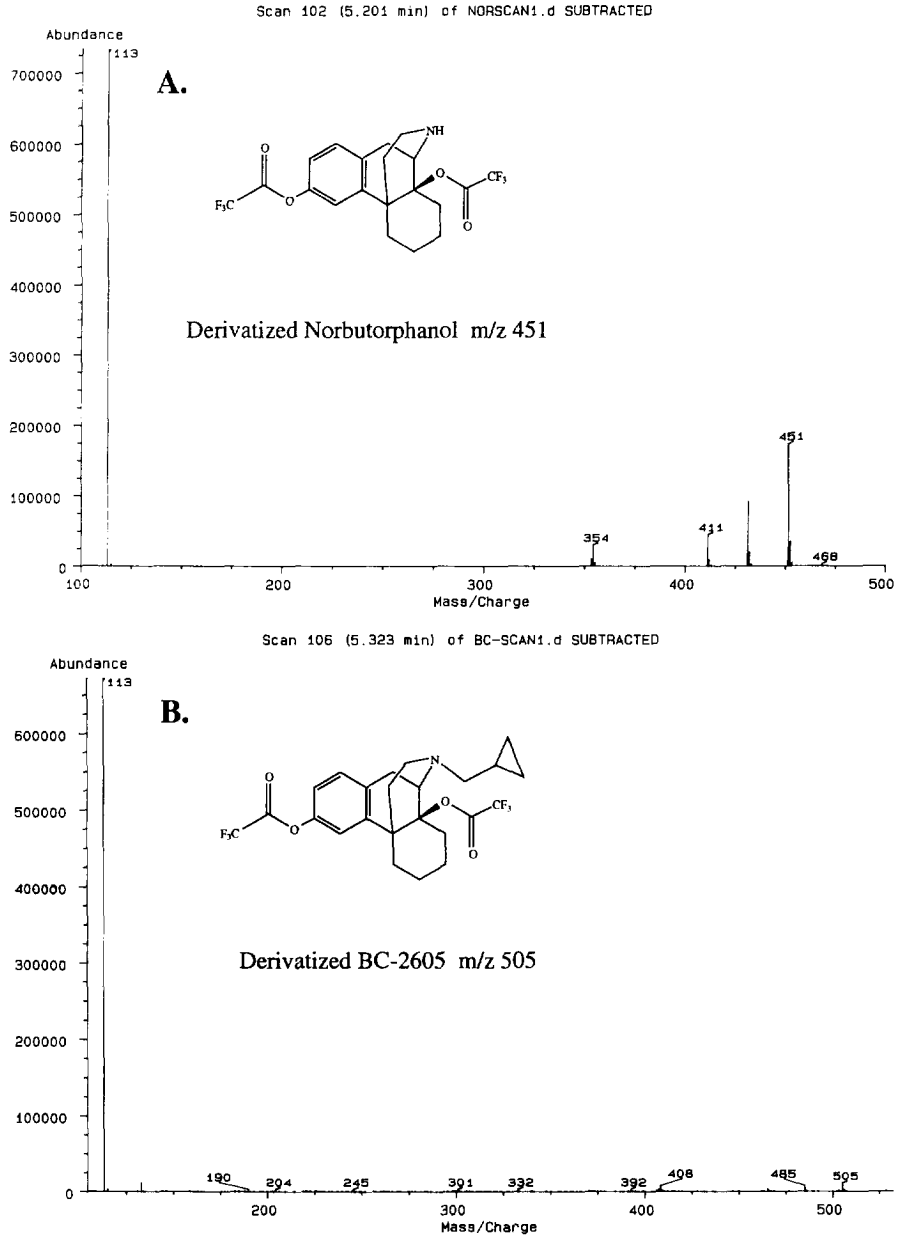

Fig. 3. Methane NCI mass spectra of the TFAA derivatives of norbutorphanol (A), BC-2605 (B) and hydroxybutorphanol (C).

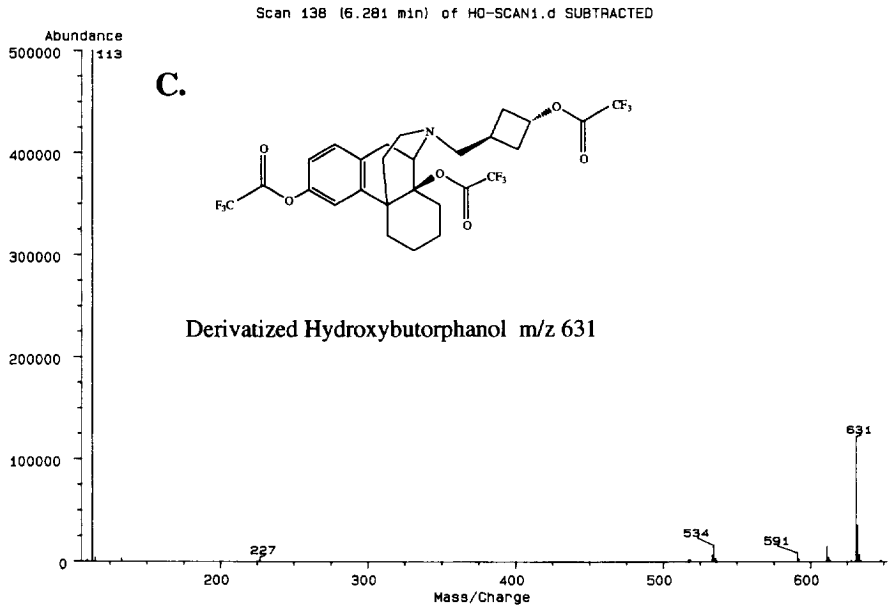

Fig. 3. (*continued*)

2.3. Instrumental conditions

For both assays, the MS was operated in the NCI mode, the buffer gas was methane (0.75–0.85 Torr), the GC injection port temperature was 285°C and the carrier gas was helium at a linear velocity of 50 cm/s at 150°C. For the I assay, the ion source temperature was 200°C, the electron energy was 240 eV, the interface temperature was 300°C and the injection port purge time was 0.8 min. After sample injection, the oven temperature was maintained at 200°C for 1 min then ramped at 25°C/min to 310°C for 2.1 min. The pentafluorobenzoyl derivatives were monitored in a single group by selected-ion monitoring (SIM) of ions 507 (IV) and 521 (I) with a dwell time of 100 ms. For the metabolite assay, the ion source temperature was 120°C, the electron energy was 140 eV, the interface temperature was 285°C and the injection port purge time was 1 min. The autosampler tray was cooled by flowing water (10°C) which greatly enhanced the stability of the derivatives. Following sample injection, the oven temperature was maintained at 150°C for 1 min then ramped at 25°C/min to 320°C for 0.2 min. The bis-trifluoroacetyl esters of III and IV were monitored in a single group by SIM of ions 451 and 505 respectively, with a dwell time of 100 ms. The tris-trifluoroacetyl ester of II was monitored in a separate group by SIM of ion 631 with a dwell time of 100 ms.

2.4. Preparation of standard stock solutions, plasma standards and quality control samples

Separate stock solutions of I, II, III and IV were prepared by dissolving 1–2 mg of reference standard in 10 ml of ethanol. Methanol (1 ml) was first added to III followed by sonication, due to its decreased solubility in ethanol. For the I assay, an ethanolic working solution containing 200 ng/ml I, II and III was prepared from the stocks and this was used for the preparation of plasma standards. Plasma standards (1.25 ml) were prepared in duplicate at concentrations of 20, 50, 200, 500, 1000, 1500 and 2000 pg/ml. Plasma quality control (QC) samples were prepared at concentrations of 170, 1100 and 1680 pg/ml. An ethanolic internal standard spiking solution was prepared at a concentration of 10 ng/ml from the IV stock.

For the metabolite assay, an ethanolic working solution was prepared containing 100 ng/ml of I, II and III which was used for the preparation of plasma

standards. Additional stock solutions were prepared which contained either 20 ng/ml of I and II or 20 ng/ml of III. These were used to prepare the lower three concentrations of the standard curve, since the concentrations of I, II, and III are different within standard level for these standards. Plasma standards (1.25 ml) were prepared in duplicate at II/III concentrations of 20/50, 50/100, 100/175, 250/250, 500/500, 750/750 and 1000/1000 pg/ml. Plasma QC samples were prepared at concentrations of 192, 580 and 868 pg/ml of each analyte. An ethanolic internal standard spiking solution was prepared at a concentration of 100 ng/ml from the IV stock. It should be noted that all plasma standards contained I, II and III, even though all three compounds were not analyzed in either assay. The purpose of this was to simulate actual sample composition and to prove the absence of assay interference of one compound upon another.

2.5. Extraction procedure

For the I assay, 0.5-ml aliquots of standard, QC or sample were added to 100×13 mm borosilicate glass test tubes, followed by 50 μl of internal standard spiking solution (10 ng/ml) and 0.5 ml of 10 m*M* ammonium acetate, pH 6.0. An identical procedure was followed for the metabolite assay except that 30 μl of the internal standard spiking solution (100 ng/ml) were added. For both assays, the following extraction procedure was used. Extraction columns were conditioned with 2 ml of methanol followed by 2 ml of 10 m*M* ammonium acetate, pH 6.0. After the samples were mixed by vortexing, the entire sample was transferred to the SPE column by transfer pipette. After aspiration of the sample through the column, it was washed with two 1-ml aliquots of 10 m*M* ammonium acetate, pH 6.0 and 1 ml of 70% (v/v) acetonitrile in water. The column was allowed to dry under full vacuum for 2 min. The analytes were eluted from the column into 100×13 mm screw-cap borosilicate glass test tubes using two 1-ml aliquots of 1% (v/v) triethylamine in acetonitrile. The contents were evaporated in a 40°C water bath under a stream of nitrogen.

2.6. Derivatization procedure

For the I assay, derivatization was accomplished by adding 20 μl of 1% (v/v) pyridine in methylene chloride, 25 μl of 0.1% (v/v) PFBC in methylene chloride, capping the tube with a teflon-lined cap, vortexing and heating the tube in a dry block at 40°C for 1 h. For the metabolite assay, the compounds were derivatized in a glove bag under a dry nitrogen atmosphere by adding 100 μl of 5% (v/v) TFAA in dry (4 Å molecular sieve) ethyl acetate, capping the tube with a teflon-lined cap, vortexing and allowing the tubes to remain at ambient temperature for 1 h. For both assays, the contents were then evaporated at ambient temperature under nitrogen. The derivatized extracts were then reconstituted in 30 μl of toluene and 3 μl were injected onto the GC–MS system.

2.7. Assay validation procedure

The assay methods were validated by procedures defined by the departmental standard operating procedures which were written in accordance with guidelines established by the U.S. Food and Drug Administration [13]. The standard curve range was determined by first establishing the lower limit of quantitation (LLQ) for each analyte. This involved spiking plasma samples from ten different subjects at analyte concentrations of 0, 20 and 40 pg/ml of I or 0, 20 and 50 pg/ml of the metabolites. These were assayed versus a standard curve which encompassed the desired concentration range. Precision (% C.V.) and accuracy (% deviation from nominal) were assessed at each non-zero level. The LLQ was established as that level at which at least 80% of the samples exhibited deviations from nominal of ≤25%. The blank samples were included to assess the chromatographic interference from the matrix. To assess assay precision and accuracy, QC samples were prepared at concentrations which were within the lower, upper, and middle quartiles of the standard curve range. The QC samples were assayed in replicates of five on three separate days versus a standard curve and from the predicted concentrations, the assay variability was calculated using a one-way ANOVA. Additionally, long-term storage stability of the analytes in plasma was assessed as well as the stability of processed samples. Extraction efficiency was determined from the ratio of the slopes of extracted versus non-extracted standard curves, with the internal standard added post-extraction.

3. Results and discussion

The initial goal of this project was to develop an assay which could give high sensitivity for I, II, and III; however, this was not feasible for several reasons. While excellent sensitivity was observed for the derivatization of I using PFBC with pyridine catalysis, only mixtures of mono- and diacylated products were observed for III. PFBC derivatives of II were not isolable in significant quantity. On the other hand, although excellent sensitivity was observed for derivatization of II and III using TFAA, the *bis*-trifluoroacetyl ester of I was found to fragment extensively in the source of the MS (see below). Other acylating reagents were examined (pentafluoropropionic anhydride, pentafluoropropionyl imidazole and their heptafluorobutyryl analogs); however, none was found to be satisfactory for all three compounds.

Due to the superior stability of alkyl ethers compared to esters, an attempt was made to derivatize the phenolic hydroxyl groups of II, III and IV using pentafluorobenzyl bromide. While the procedure was successful in the monoalkylation of all of the analytes, the product obtained following sample workup was considerably less clean than that obtained from either of the derivatizations used in this method. As a result, the ion current background was much higher, resulting in a sensitivity loss. The injection port liner also degraded much more quickly, making batch analysis impossible. The advantage of preparing TFA derivatives of II, III and IV was that the derivatized extracts were very clean, resulting in low background and enabling batch analysis.

Full scans obtained from the pentafluorobenzoyl esters of I and IV are presented in Fig. 2. In both cases, the base peak is due to the molecular ion and fragmentation is minimal. The scans obtained from the trifluoroacetyl derivatives of II, III and IV are presented in Fig. 3. The structure of the derivatized III was not confirmed and the molecular structure may be the diacylated N-TFA product. It is evident in all three cases that the molecular ions are considerably less stable than those of the PFBC derivatives since the base peak in all three spectra is due to the trifluoroacetate anion (m/z 113). This fragmentation is much more extensive for IV, for which the molecular ion is barely visible, accounting for the fact that it was necessary to add the internal standard at a concentration of 6 ng/ml to obtain a reasonable signal. In spite of this extensive fragmentation, excellent reproducibility was observed for the internal standard over all analytical runs. The TFAA derivative of I also exhibited this extensive fragmentation (spectrum not shown) which eliminated TFAA as a potential derivatization reagent for this compound. In contrast, the molecular ions of both II and III exhibited significant responses. Additional fragmentation is observed for all three compounds due to the sequential loss of HF (20 a.m.u.) from the molecular ions. Both types of fragmentation were decreased at lower source temperatures but operating below 120°C was impractical due to long equilibration times of the source temperature, which increases several degrees between injections, due to the oven temperature program.

Ion chromatograms obtained from the analysis of plasma standards of I at 0, 20 and 2000 pg/ml are presented in Fig. 4. A small peak has been observed in plasma samples from many different donors which is coincident with the ion and retention time of I. Since this peak has been found to be consistently approximately 1/3 the area of the low standard, it is not of sufficient size to adversely affect the standard curve at the low end. Selected-ion chromatograms obtained from the analysis of plasma standards at concentrations of 0, 20 and 1000 pg/ml of II and 0, 50 and 1000 pg/ml of III are compared in Fig. 5. No chromatographic interference with the low standard was observed for either of the metabolites.

Standard curve data obtained from the four accuracy and precision assays are presented in Table 1. The standard curves were linear over the ranges 20–2000, 20–1000 and 50–1000 pg/ml for I, II and III respectively. For all standard curves from the assay validations, the r^2 values were $\geq$0.994, 0.994 and 0.989 for I, II and III respectively.

A summary of the overall precision and accuracy data obtained from the analysis of QC samples is presented in Table 2. For I, all precision and accuracy values were within 12% for QC samples at 170, 1100 and 1680 pg/ml. Precision and accuracy values were within 9% for II and 8% for III QC samples at concentrations of 192, 580 and 868 pg/ml.

The extraction efficiencies were 90%, 73% and 64% for I, II and III respectively. It should be noted

that a significant matrix effect was observed during the recovery experiment. The experiment was initially performed by comparing extracted and neat standard curves. Unexpectedly, larger responses were obtained from the extracted than from the non-extracted samples. This phenomenon is suspected to be due to the enhancing effect of extracted matrix constituents upon sample volatilization in the injection port. This problem was circumvented by spiking with analyte the elution solvent obtained

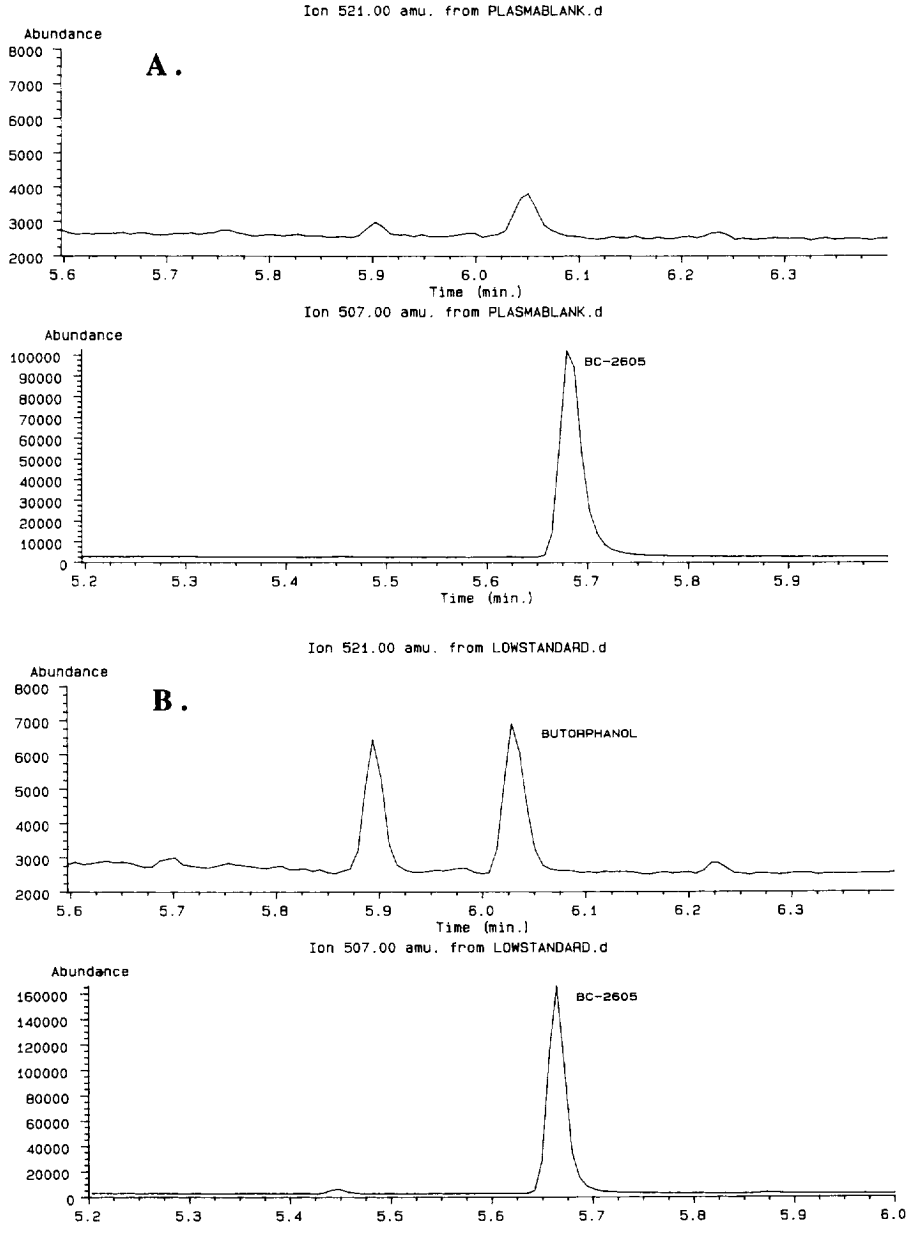

Fig. 4. Ion chromatograms obtained from processed plasma samples spiked with 0 pg/ml (A), 20 pg/ml (B) and 2000 pg/ml (C) of butorphanol.

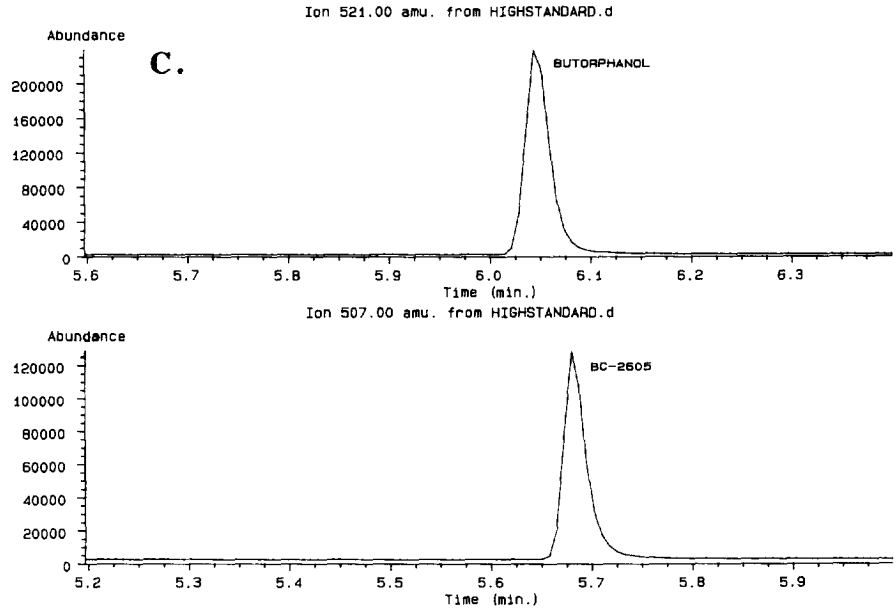

Fig. 4. (*continued*)

from extracted blank plasma and then processing these samples as non-extracted standards. The expected results were obtained; extracted standards showed lower responses than non-extracted standards.

Reanalysis of QC samples stored at −20°C showed I and the metabolites to be stable in plasma for at least 123 and 87 days respectively. The reconstituted PFB-derivatives of I were shown to be stable for at least 42 h by injecting a standard curve, reinjecting it 42 h later and comparing the slopes. By a similar procedure, the TFA-derivatives of the metabolites were demonstrated to be stable for at least 24 h. Degradation was observed after 72 h but this was minimized by cooling the autosampler tray with tap water.

Table 1
Summary of the standard curve parameters obtained from the assay of butorphanol and its metabolites on four separate occasions

Analyte	Slope	Intercept	r^2	*P* value (lack of fit)
Butorphanol	0.000987	0.00986	0.998	0.780
	0.001144	0.14750	0.995	0.571
	0.001151	0.13386	0.994	0.087
	0.001013	0.03064	0.998	0.431
Hydroxybutorphanol	0.0006568	−0.000996	0.995	0.773
	0.0006812	−0.000960	0.998	0.218
	0.0005595	0.003075	0.994	0.646
	0.0007874	0.002790	0.996	0.350
Norbutorphanol	0.002426	0.049590	0.989	0.235
	0.002327	0.027990	0.993	0.123
	0.001842	0.005772	0.995	0.469
	0.001809	0.146873	0.989	0.302

In an effort to compare the present I assay with the RIA, plasma samples from a clinical patient transnasally dosed with 1 mg of I were assayed by both procedures. The RIA had been previously performed and the data reported [14]. Fig. 6 compares the I plasma concentrations determined by the RIA and GC–ECNCI-MS assays. Comparable data were obtained from the RIA and GC–MS assays with regard

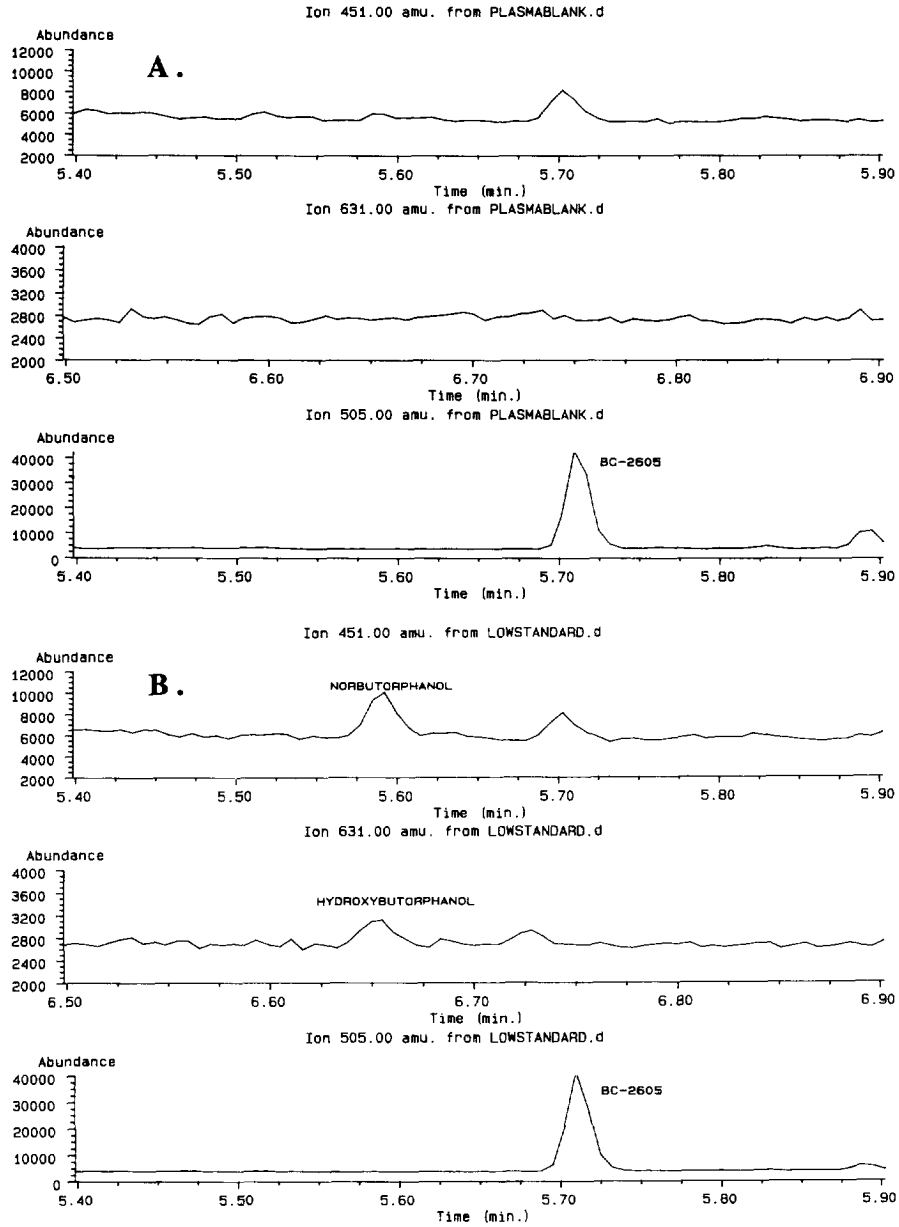

Fig. 5. Ion chromatograms obtained from processed samples spiked with hydroxybutorphanol and norbutorphanol at 0 pg/ml (A), 20 and 50 pg/ml, respectively (B) and 1000 pg/ml (C).

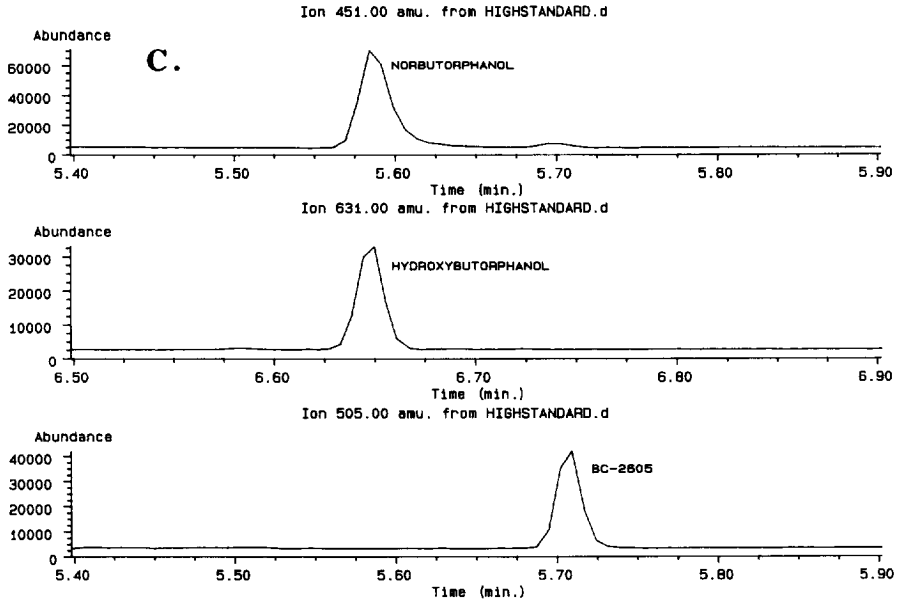

Fig. 5. (*continued*)

to half-life and AUC_{INF} (area under the plasma concentration–time curve from time 0 to infinity) which differed by only 5% and 10% respectively. Data obtained from the analysis of plasma samples from the I steady state study are presented in Fig. 7. The patient received a single 2-mg transnasal dose of I on days 1 and 6 and four 2 mg doses at 6 h intervals on days 2 through 5. In this study, very low levels of III (C_{max}<300 pg/ml, generally much lower) have been observed, making the determination of pharmacokinetics for this analyte impossible by this method. In these same samples, II has

Table 2
Summary of the accuracies and precisions for the analysis of QC samples containing butorphanol, hydroxybutorphanol and norbutorphanol

Nominal QC concentration (pg/ml)	Mean observed concentration (pg/ml)	Deviation from nominal (%)	Intra-assay precision (% R.S.D.)	Inter-assay precision (% R.S.D.)
Butorphanol (n=3)				
170.0	173.0	1.7	11.7	3.5
1100.0	1068.1	−2.9	4.9	3.3
1680.0	1697.5	1.0	6.4	4.4
Hydroxybutorphanol (n=3)				
192.0	191.4	−0.3	6.2	3.0
580.0	553.9	−4.5	6.8	4.5
868.0	829.9	−4.4	8.4	4.5
Norbutorphanol (n=3)				
192.0	183.6	−4.4	4.6	7.8
580.0	565.3	−2.5	6.6	0.0
868.0	815.6	−6.0	4.5	1.1

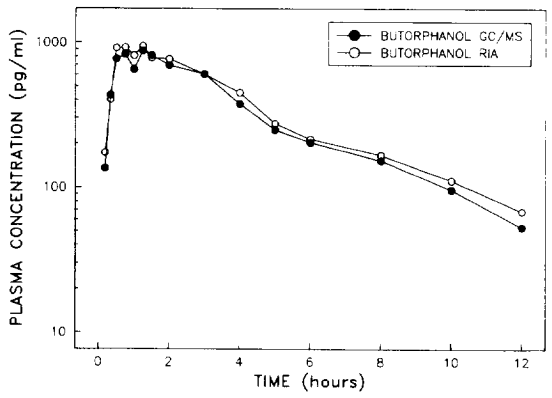

Fig. 6. Plasma levels of butorphanol as determined by RIA and GC–MS in a patient following a single transnasal dose.

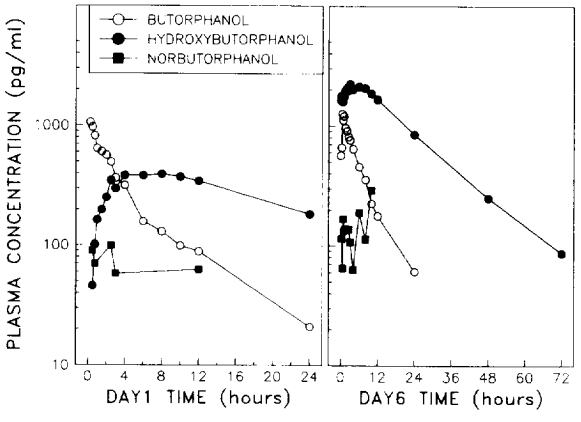

Fig. 7. Plasma levels of butorphanol, hydroxybutorphanol and norbutorphanol on days 1 and 6 in a patient from the butorphanol steady state study.

been observed at levels greater than 2 ng/ml. For most patients, it was necessary to perform sample dilutions at some time points in order to allow quantification of I and II.

It can be concluded from the data presented that the assays are sufficient to determine the steady state pharmacokinetic profiles of I and its major metabolite, II.

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