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High-performance liquid chromatographic method for the quantitative determination of butorphanol, hydroxybutorphanol, and norbutorphanol in human urine using fluorescence detection

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Abstract

A sensitive, quantitative reversed-phase high-performance liquid chromatographic method has been established for the simultaneous determination of butorphanol, a synthetic opioid, and its metabolites, hydroxybutorphanol and norbutorphanol, in human urine samples. The method involved extraction of butorphanol, hydroxybutorphanol, and norbutorphanol from urine (1.0 ml), buffered with 0.1 ml of 1.0 *M* ammonium acetate (pH 6.0), onto 1-ml Cyano Bond Elut columns. The eluent was evaporated under nitrogen and low heat, and reconstituted with the HPLC mobile phase, acetonitrile–methanol–water (20:10:70, v/v/v), containing 10 m*M* ammonium acetate and 10 m*M* TMAH (pH 5.0). The samples were chromatographed on a reversed-phase octyl 5-μm column. The analysis was accomplished by detection of the fluorescence of the three analytes, at excitation and emission wavelengths of 200 nm and 325 nm, respectively. The retention times for hydroxybutorphanol, norbutorphanol, the internal standard, and butorphanol were 5.5, 9.0, 13.0, and 23.4 min respectively. The validated quantitation range of the method was 1–100 ng/ml for butorphanol and hydroxybutorphanol, and 2–200 ng/ml for norbutorphanol in urine. The observed recoveries for butorphanol, hydroxybutorphanol, and norbutorphanol were 93%, 72%, and 50%, respectively. Standard curve correlation coefficients of 0.995 or greater were obtained during validation experiments and analysis of study samples. The method was applied on study samples from a clinical study of butorphanol, providing a pharmacokinetic profiling of butorphanol.

1. Introduction

Butorphanol tartrate [levo-N-cyclobutylmethyl-6, 10aβ-dehydroxy-1, 2, 3, 9, 10, 10a-hexahydro - (4H) - 10, 4a - iminoethanophenanthrene tartrate] is a synthetic opioid, mixed agonist–antagonist, analgesic whose trade name is Stadol (Fig. 1). Both hydroxybutorphanol and norbutorphanol are metabolites of butorphanol. Butorphanol belongs to a group of compounds

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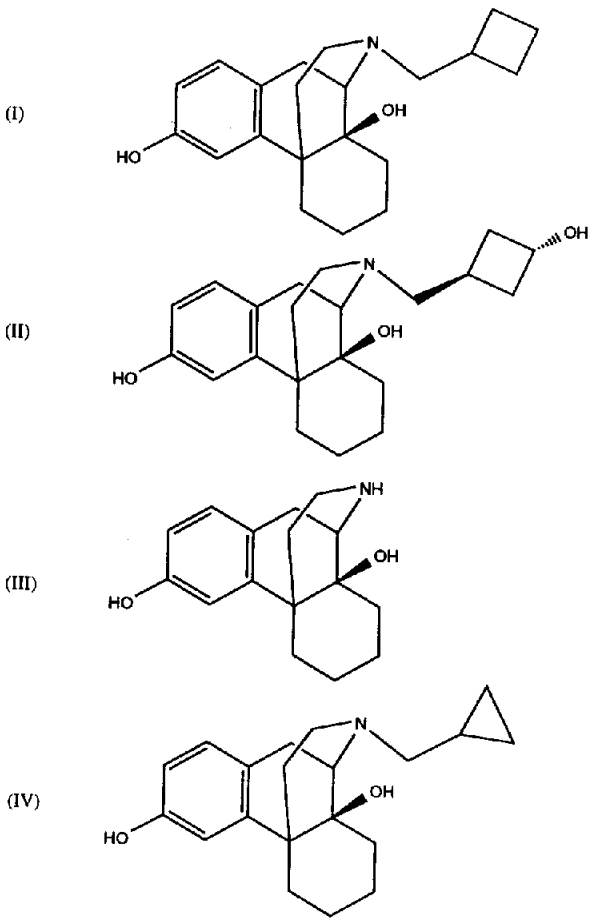

Fig. 1. Structures of butorphanol (I), hydroxybutorphanol (II), norbutorphanol (III), and BC-2605 (I.S.) (IV).

known as morphinans [1] and has a similar structure to that of morphine. Administered by intravenous, intramuscular, and epidural routes, butorphanol has been successful in providing analgesia after surgical procedures and is recommended for the relief of moderate to severe pain [2]. In addition, transnasal butorphanol has been proven efficacious and safe for the relief of moderate to severe pain after surgery. Mixed agonist–antagonist opioids have been shown to offer analgesic efficacy and safety with lower abuse potential compared to pure agonist opioids such as morphine [3]. Since further pharmacokinetic and biopharmaceutic studies of butorphanol and its metabolites would be beneficial, a rapid and sensitive HPLC method for the simultaneous determination of butorphanol, hydroxybutorphanol, and norbutorphanol in biological fluids was developed. This method has advantages over previously published assays described for morphine and morphine-related compounds [4–10]. It has a lower limit of quantitation, capability for the simultaneous determination of butorphanol and its metabolites, a rapid and simple extraction procedure that allows the reuse of bond elut columns, and no interference from either endogenous urine constituents or common concomitant medications. Very few procedures have been published for the simultaneous determination of morphine and/or morphine-related compounds and their metabolites in human urine. An electrochemical assay employing solid-phase extraction was developed, initially for the purpose of simultaneous determination of butorphanol and its two metabolites, hydroxybutorphanol and norbutorphanol, in urine (unpublished results). The high optimum voltage for butorphanol, +0.7 volts compared to +0.5 volts for morphine, led to substantial interference at the retention times of the two metabolites that would not allow their quantitation. As a result, this attempt failed, and only butorphanol could be quantitated.

2. Experimental

2.1. Chemicals and reagents

Butorphanol tartrate, hydroxybutorphanol tartrate, norbutorphanol tartrate, and BC-2605, the internal standard (I.S.), were obtained from the References Standard Laboratory of Bristol-Myers Squibb. Acetonitrile and methanol (both HPLC grade), and ammonium acetate were obtained from J.T. Baker (Phillipsburg, NJ, USA). Ethanol was from Quantum Chemical (Newark, NJ, USA). Glacial acetic acid (Certified A.C.S. reagent), and sodium acetate (Certified A.C.S. chemical), were both obtained from Fisher Scientific (Fair Lawn, NJ, USA). Tetramethylammonium hydroxide (TMAH) was obtained from Sigma (St. Louis, MO, USA). Distilled water was deionized and filtered

through a Millipore (Bedford, MA, USA) Milli-Q system. End-capped Cyano (100 mg) disposable Bond Elut columns were obtained from Analytichem International (Harbor City, CA, USA). Control human urine was obtained from volunteers in the Department of Metabolism and Pharmacokinetics, Bristol-Myers Squibb, Syracuse, NY, USA.

2.2. Preparation of standards and quality control samples

Standards were prepared from 100.0 μg/ml stock solutions of butorphanol, hydroxybutorphanol, norbutorphanol, and BC-2605 in ethanol. Each stock solution was diluted to 10.0 μg/ml in deionized water. The high standard (100 ng/ml for butorphanol and hydroxybutorphanol, and 200 ng/ml for norbutorphanol) was prepared by diluting a portion of each 10.0 μg/ml solution (in deionized water) with control urine. The lower standards (1, 2, 5, 10, 20, 50, and 80 ng/ml for butorphanol and hydroxybutorphanol, and 2, 4, 10, 20, 40, 100, and 160 ng/ml for norbutorphanol) were prepared, immediately prior to each analytical run, by diluting the high standard with control urine. The human urine standards were prepared immediately prior to each analytical run. Quality control samples, at 9.29, 92.9, and 813 ng/ml for each analyte, were prepared similarly from different ethanolic stock solutions prior to study sample analysis.

2.3. Sample processing

Standard, quality control, and clinical study samples were processed as a batch. All samples (1.0 ml) were buffered with 0.1 ml of 1.0 *M* ammonium acetate (pH 6.0) and spiked with 50 μl of the 0.5 μg/ml internal standard, BC-2605. After mixing on a multi-tube vortex-mixer (Model 2600, Scientific Manufacturing Industries, Emeryville, CA, USA) for 20 s at a setting of 4, the analytes were extracted from urine by solid-phase extraction on 1-ml Cyano Bond Elut columns (Analytichem International, Harbor City, CA, USA). The Bond Elut columns were reused for consecutive batches until the flow-rate substantially decreased. The columns were first conditioned with 2 ml of methanol, followed by 2 ml of 0.01 *M* ammonium acetate (pH 6.0). The columns were not allowed to dry. The samples were loaded onto individual Bond Elut columns and washed with 2 ml of 0.01 *M* ammonium acetate (pH 6.0), followed by 2 ml of acetonitrile. The columns were dried under vacuum for 1 min. Butorphanol, hydroxybutorphanol, norbutorphanol, and BC-2605 were eluted from the columns into glass collection tubes using 2 × 1 ml of 1.0% triethylamine (TEA) in acetonitrile.

The eluents were evaporated to dryness under nitrogen gas at 30°C in an N-Evap (Organomation Associates, Northborough, MA, USA). The residues were reconstituted in 150 μl of acetonitrile–methanol–water (20:10:70, v/v/v), containing 10 m*M* ammonium acetate and 10 m*M* TMAH (pH 5.0 with glacial acetic acid), and vortex-mixed on a multitube vortex-mixer at a setting of 4 for 30 s. The reconstituted samples were transferred to WISP vials containing limited volume inserts. The vials were randomized in the autosampler, and 75 μl was injected from each sample onto the HPLC column.

2.4. HPLC instrumentation and conditions

Chromatography was performed using a Waters Model 510 solvent pump and a Model 712 WISP autosampler (Waters Associates, Milford, MA, USA). Detection was performed with a Kratos 980 fluorescence detector (Kratos, Ramsey, NJ, USA). The HPLC column was an octyl 5-μm 250 mm × 4.6 mm I.D. column (Jones Chromatography, Littleton, CO, USA). Chromatograms were recorded on a Model 585 flatbed recorder (Linear Instruments, Irvine, CA, USA) and on a Model 3357 Laboratory Automation System computer (Hewlett-Packard, Palo Alto, CA, USA) for peak integration and quantitation.

The HPLC mobile phase was prepared by mixing 20 ml of a 1.0 *M* TMAH–1.0 *M* ammonium acetate solution (pH 6.0 with glacial acetic acid) with 1380 ml of deionized water, 200 ml of methanol, and 400 ml of acetonitrile. The resulting mixture was filtered through a 0.22-μm

Durapore filter (Millipore Corp., Milford, MA, USA). The flow-rate was 1.0 ml/min. The excitation and emission wavelengths of the detector were 200 nm and 325 nm, respectively. The retention times of hydroxybutorphanol, norbutorphanol, BC-2605, and butorphanol were approximately 5.5, 9.0, 13.0, and 23.4 min, respectively.

2.5. Data processing

Detector output was recorded on a strip-chart recorder and by a Hewlett-Packard HP 3357 laboratory automation system computer (Hewlett-Packard) for peak integration and quantitation. The data management system used to acquire and process data for this work has been previously described [11].

2.6. Validation procedures

Limit of detection/limit of quantitation (LD/LLQ)

Urine samples (1.0 ml) from twelve different volunteers were obtained and divided into 4 sets; each set contained 1 urine sample from each volunteer. One set was spiked with deionized water and used as blanks. The second set was spiked to 0.5 ng/ml of each of the three analytes. The third set was spiked to 1 ng/ml of each of the three analytes. The fourth set was spiked to 2 ng/ml of each of the three analytes. Samples were processed according to the stated procedure and subjected to HPLC analysis. The precision (%R.S.D.) and accuracy (%Dev) obtained from these samples were used to evaluate the LD/LLQ.

Intra- and inter-assay accuracy and precision

The intra-assay accuracy and precision of the method were determined by analyzing ten to twelve aliquots of three different concentrations (1, 9.29, and 92.90 ng/ml for butorphanol and hydroxybutorphanol, and 2, 9.29, and 92.90 ng/ml for norbutorphanol) on the same day. The inter-assay accuracy and precision were determined by assaying twelve samples of two different concentrations (9.29 and 92.90 ng/ml) on three different days.

Recovery

The extraction efficiency of the analytes was determined by comparing the slope of a processed human urine standard curve to that of a standard curve prepared in acetonitrile–methanol–water (20:10:70, v/v/v) without processing.

$$\%\text{Recovery} = \frac{\text{slope of processed standard curve}}{\text{slope of non-processed standard curve}} \times 100$$

Stability

(a) The stability of butorphanol, hydroxybutorphanol, and norbutorphanol in frozen human urine was determined by analyzing quality control (QC) samples (10.69, 84.09, and 932.92 ng/ml), at selected intervals, stored at −20°C for 1 month.

(b) The stability of reconstituted samples was determined by spiking control human urine with butorphanol, hydroxybutorphanol, and norbutorphanol, yielding a concentration of 100 ng/ml for each analyte. Thirty 1.0-ml aliquots were removed and processed. The reconstituted samples were pooled, thoroughly mixed, and injected in sets of four over a 68-h time interval.

Selectivity

(a) The selectivity of this method was determined by examining the separation of butorphanol, hydroxybutorphanol, and norbutorphanol from endogenous urine constituents in blank urine.

(b) The effect of concomitant medications on the performance of the assay was also determined by examining the separation of butorphanol, hydroxybutorphanol, and norbutorphanol from interfering concomitant medications. Ethanolic solutions of eleven possible concomitant medications were injected to determine their retention times.

3. Results and discussion

Fig. 2 compares the chromatograms of a 0 ng/ml quality control (QC) sample and a sample spiked with butorphanol, hydroxybutorphanol, and norbutorphanol. The chromatogram profile indicated no interference from endogenous urine substances. In addition, the peak shapes of the 1 ng/ml butorphanol and hydroxybutorphanol and 2 ng/ml norbutorphanol sample were well resolved. A significant difference was observed between the response at 1 ng/ml and 0 ng/ml for butorphanol and hydroxybutorphanol, and at 2 ng/ml and 0 ng/ml for norbutorphanol. As a result, the lower limit of quantitation for butorphanol and hydroxybutorphanol was set at 1 ng/ml, and at 2 ng/ml for norbutorphanol.

The assay was linear over a concentration range of 1 to 100 ng/ml for butorphanol and hydroxybutorphanol, and 2 to 200 ng/ml for norbutorphanol. Correlation coefficients of 0.995 or better were obtained throughout the validation (Table 1). For a typical eight-point standard curve extracted in duplicate, %R.S.D. were 5% or less, and %Dev 10% or less, at all concentrations. As in most previous methods, the use of an internal standard (BC-2605 in this method) enables each analyte to be measured reliably.

The intra-assay precision (R.S.D.) was less than 8% and accuracy (Dev) was within 11% for all values (Table 2). The inter-assay precision results show that mean predicted concentrations were within 10% of the nominal values, with the R.S.D. less than 10%, except for the low QC for norbutorphanol which had a 18.4% R.S.D. (Table 3).

The extraction efficiencies of butorphanol, hydroxybutorphanol, and norbutorphanol were 93%, 72%, and 50%, respectively, based on the ratio of the slope of a processed standard curve to that of a non-processed standard curve.

The stability of butorphanol, hydroxybutorphanol, and norbutorphanol in frozen human urine was established at three different concentrations, 10.69, 84.09, and 932.92 ng/ml, for one month. After analysis on both occasions, mean observed analyte concentrations had Dev within ±11%, except for a 21% Dev in one instance, and R.S.D. within 11% of the nominal concentrations, except two instances where the R.S.D. was 16.5%. There does not appear to be a concentration dependence in the stability of butorphanol, hydroxybutorphanol, or norbutorphanol.

Reconstituted samples are stable in the autosampler for at least 68 h, where there was no significant decrease in mean peak height with time. This finding was important in optimizing the number of samples that could be analyzed during an analytical run without sacrificing sample integrity.

None of the eleven possible concomitant medications tested, including compounds routinely co-administered with butorphanol in the clinic, interfered with the quantitation of butorphanol or its metabolites (Table 4). Three compounds, cimetidine, ibuprofen, and *n*-acetyl procainamide, were not detected when injected as ethanolic solutions. In addition, no interference was found when the other concomitant medications were injected. This finding demonstrates the applicability of this assay for the analysis of clinical study samples from patients who are usually treated with concomitant medications.

Typical processed human urine chromatograms are shown in Fig. 2 with 0, 1, and 100 ng/ml standard samples, and a representative clinical study sample.

While the present assay is unsuitable for plasma, an assay quantitating butorphanol has been validated in human plasma [12].

4. Conclusion

The results from the validation of this procedure demonstrate that the lower limit of quantitation is lower than that for any of the published procedures for urine [4–10]. With this heightened sensitivity and inclusion of the metabolites, this method provided better evaluation of study samples from butorphanol clinical studies. The procedure reported here is a accurate, precise, and sensitive method that allows the quantitation of butorphanol and hydroxybutorphanol concentrations of 1–100 ng/ml, and nor-

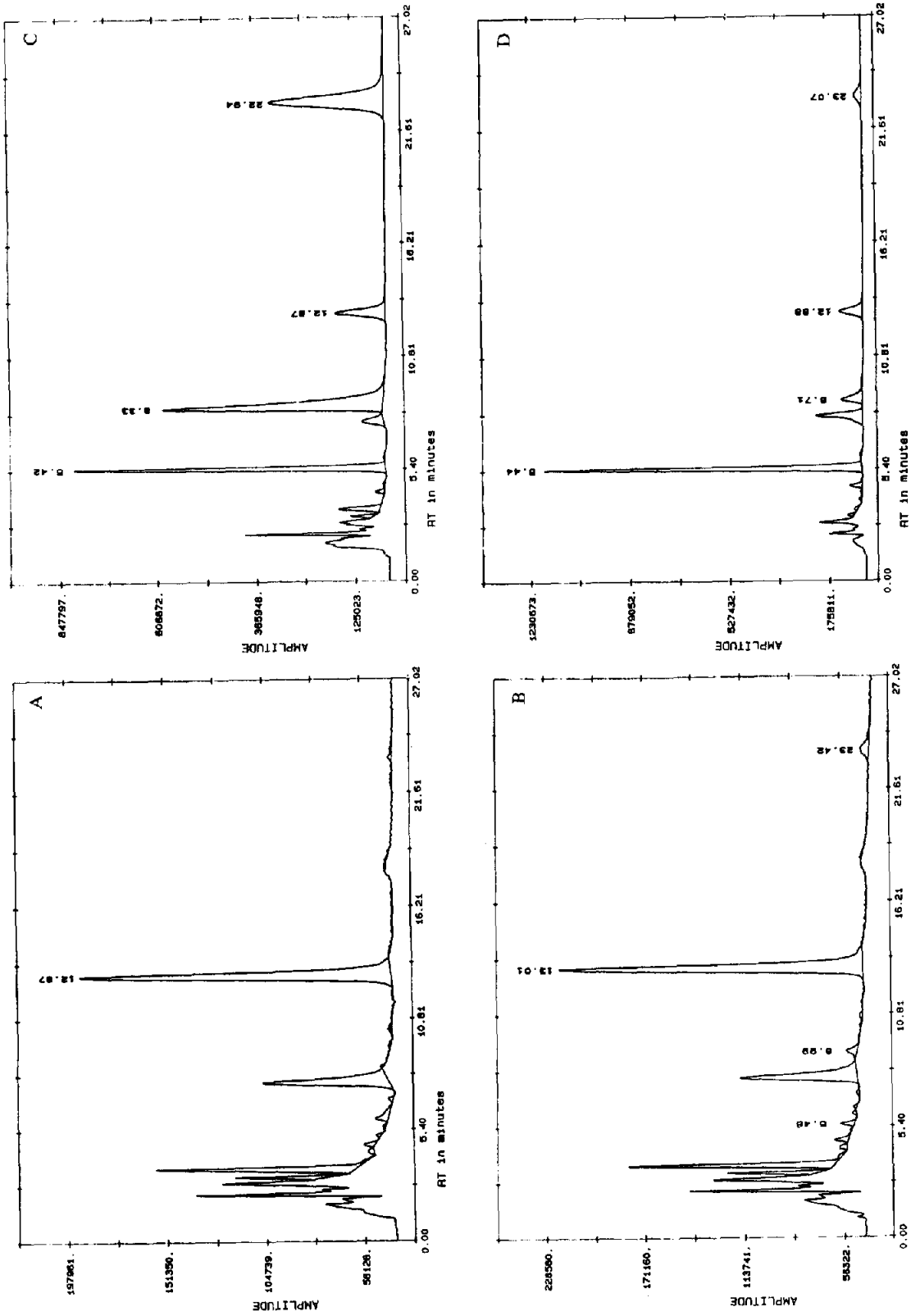

Fig. 2. Chromatograms of processed human urine samples. (A) 0 ng/ml quality control; (B) 1 ng/ml butorphanol and hydroxybutorphanol, 2 ng/ml norbutorphanol standard; (C) 100 ng/ml butorphanol and hydroxybutorphanol, 200 ng/ml norbutorphanol standard; (D) 2–4 h clinical sample. Retention times: hydroxybutorphanol, 5.42–5.48 min; norbutorphanol, 8.33–8.99 min; BC-2605 (I.S.), 12.87–13.01 min; butorphanol, 22.94–23.42 min.

Table 1
Representative standard curve data for butorphanol, norbutorphanol, and hydroxybutorphanol in human urine ($n = 2$)

Analyte	Concentration (ng/ml)		Dev (%)	r^2
	Added	Measured		
Butorphanol	1.00	1.06	6.0	
	50.0	48.62	−2.8	
	100.0	100.88	0.9	0.998
Norbutorphanol	2.00	1.90	−5.0	
	100.0	107.09	7.1	
	200.0	200.26	0.1	0.996
Hydroxybutorphanol	1.00	0.98	−2.0	
	50.0	53.38	6.8	
	100.0	101.33	0.91	0.994

Table 2
Intra-assay accuracy and precision for the butorphanol human urine assay ($n = 12$)

Analyte	Concentration (ng/ml)		Deviation (%)
	Added	Measured	
Butorphanol	9.29	10.07	8.40
	92.90	99.71	7.33
	813.00	846.43	4.11
Hydroxybutorphanol	9.29	9.86	6.14
	92.90	96.64	4.03
	813.00	823.08	1.34
Norbutorphanol	9.29	10.29	10.76
	92.90	101.83	9.61
	813.00	865.62	6.47

Table 3
Inter-assay precision and accuracy for the butorphanol human urine assay ($n = 12$)

Analyte	Concentration (ng/ml)		Deviation (%)
	Added	Measured	
Butorphanol	9.29	10.09	8.61
	92.90	99.10	6.67
	813.0	866.75	6.61
Hydroxybutorphanol	9.29	9.57	3.01
	92.90	93.02	0.13
	813.0	831.24	2.24
Norbutorphanol	9.29	10.15	9.26
	92.90	93.56	0.71
	813.0	832.88	2.45

Table 4
Chromatographic retention times for butorphanol, hydroxybutorphanol, norbutorphanol and possible concomitant medications

Compound	Retention time (min)
Acetaminophen	2.9
Antipyrine	3.4
Procainamide	4.2
Acetyl salicylic acid	5.4
Hydroxybutorphanol	5.6
Lidocaine	8.6
Norbutorphanol	9.8
BC-2605 (I.S.)	13.6
Furosemide	15.4
Dextrorphan	23.0
Butorphanol	24.0
Dextromethorphan	26.2

butorphanol concentrations of 2–200 ng/ml in human urine. Its sensitivity surpasses that of previous assays specific to morphine-related compounds and its simplicity allows the analysis of at least 100 samples per day. Additionally, semi-automation of the solid-phase extraction procedure facilitates greater efficiency of sample analysis. Moreover, the capability of reusing Bond Elut columns saves time and is cost-efficient.

Unlike previous methods [4–10] which required lengthy sample preparation or the use of volatile solvents, this method involves a rapid four-step solid-phase extraction procedure including (1) conditioning of extraction cartridges,

(2) addition of sample to cartridges, (3) washing endogenous materials through cartridges, and (4) eluting the compounds from the cartridge. In addition, this method requires only a 22-min run time. The simplicity of this method allows the analysis of at least 100 samples per day. Additionally, this procedure does not require the use of volatile or dangerous solvents.

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