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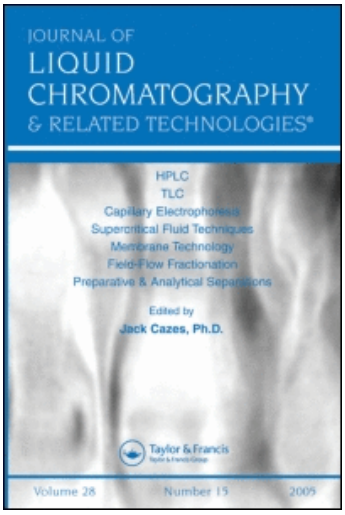

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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Online publication date: 28 October 2002

To cite this Article Caufield, William V. and Stewart, James T.(2002) 'RAPID DETERMINATION OF SELECTED DRUGS OF ABUSE IN HUMAN PLASMA USING A MONOLITHIC SILICA HPLC COLUMN AND SOLID PHASE EXTRACTION', Journal of Liquid Chromatography & Related Technologies, 25: 19, 2977 — 2998
To link to this Article: DOI: 10.1081/JLC-120015886
URL: http://dx.doi.org/10.1081/JLC-120015886

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JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES
Vol. 25, No. 19, pp. 2977–2998, 2002

RAPID DETERMINATION OF SELECTED DRUGS OF ABUSE IN HUMAN PLASMA USING A MONOLITHIC SILICA HPLC COLUMN AND SOLID PHASE EXTRACTION

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ABSTRACT

Two new high performance liquid chromatography (HPLC) assays were developed, which utilized a 100 × 4.6 mm I.D. monolithic silica column and binary mobile phase gradients, for the simultaneous determination of selected drugs of abuse in human plasma. Both methods used gradients consisting of 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate-acetonitrile (pH 2.9), and mixed mode solid phase extraction procedures. In the first method, cocaine (COC) and its metabolites benzoylecgonine (BE), norcocaine (NC), and cocaethylene (CE), were separated with a pump flow rate of 5.0 mL/min in a total run time of 5 min. All analyses were conducted at ambient temperature. The injection volume was 100 μL and the UV detector was operated at 231 nm. The method

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DOI: 10.1081/JLC-120015886
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was validated over the range of 50–5000 ng/mL for BE, COC, and CE, and 25–2500 ng/mL for NC. The method proved to be accurate (% bias for all calibration samples varied from −4.5 to 8.5%) and precise (within-run precision ranged from 1.5 to 12.8% and between-run precision ranged from 0.4 to 12.7%). The mean absolute recoveries were 93.5, 95.7, 105.4, and 98.8% for BE, COC, NC, and CE, respectively.

In the second method, morphine (MO), hydromorphone (HM), tolazoline (ISTD), codeine (CO), oxycodone (OC), and hydrocodone (HC) were separated with a pump flow rate of 8.0 mL/min in a total run time of 2 min. All analyses were conducted at 30°C temperature. The injection volume was 100 μL and the UV detector was operated at 208 nm. The method was validated over the range of 50–5000 ng/mL for the opiates studied. The method proved to be accurate (% bias for all calibration samples varied from −8.4 to 2.0%) and precise (within-run precision ranged from 1.7 to 16.9% and between-run precision ranged from 0.4 to 14.8%). The mean absolute recoveries were 95.6, 104, 103, 97.9, and 105% for MO, HM, CO, OC, and HC, respectively. The recovery for the internal standard was 99.6%. The assays should be suitable for use in routine determination of the selected drugs of abuse in human plasma.

Key Words: Solid-phase extraction; Human plasma; Cocaine; Opiates; Monolithic silica

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INTRODUCTION

In 1998, there were estimated to be 1.8 million Americans age 12 and older who were chronic cocaine (COC) users. Although, this represents a decrease from the 5.7 million users in 1985, the abuse of this addictive stimulant has become a persistent problem in the USA.[1] The euphoria that is associated with COC use is caused by an inhibition of neuronal reuptake of biogenic amines in the central nervous system (CNS). In addition to its addictive properties, COC has been shown to be toxic to both the CNS and the cardiovascular system.[2,3] Benzoylecgonine (BE), one of the major metabolites, is formed by de-esterification of COC. The human plasma elimination half lives of COC and BE are 30 to 90 min and 7.5 h, respectively.[4] Therefore, BE is often the analyte of choice for detecting COC use. Norcocaine (NC) is formed by *N*-demethylation of COC. Cocaethylene (CE) is a neurologically active compound that is formed when COC is coadministered with ethanol. It provides the same

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degree of euphoria as COC, but for longer periods of time and with more toxicity than COC alone.[5] Co-administration of COC and ethanol is the most common two-drug combination that results in drug-related death.[1]

In addition to the abuse of COC, an estimated 4 million people age 12 and over used prescription drugs for non-medical purposes in 1999.[6] The most commonly abused prescription drugs are opiates, CNS depressants, and stimulants. Opium alkaloids are very potent analgesics that bind to opiate receptors in the brain, spinal cord, and gastrointestinal tract, and block the transmission of pain signals. Commonly abused opiates include heroin, morphine (MO), hydromorphone (HM), codeine (CO), oxycodone (OC), and hydrocodone (HC). Heroin and CO are both metabolized, in part, to MO, although at different rates. Therefore, a ratio of the two drugs is often used to determine if the MO found was due to the consumption of heroin or MO itself. Morphine has an elimination half-life of 1.7 h and is largely metabolized to glucuronide conjugates. Hydromorphone is a synthetic derivative of MO that has an elimination half-life of 2.5 h and is 7–10 times more potent than MO. Oxycodone is a derivative of CO with an elimination half-life and potency similar to MO. Hydrocodone is very similar to CO and is converted in humans to HM.[7]

The analysis for the possible presence of drugs of abuse is usually a two-stage process in which an initial screening test is followed by a confirmation test. This approach requires that the test methods be fast and inexpensive. Currently, the preferred first stage screening method utilizes an enzyme immunoassay.[8] Samples that test positive are further analyzed by GC/MS due to its high sensitivity and selectivity, but the necessity of sample derivatization and the cost of the equipment, itself, restrict its applicability.[9,10] High performance liquid chromatography (HPLC) can be used for the direct analysis of a wide spectrum of compounds and is not dependent on solute volatility or polarity. This technique is slowly gaining acceptance as a confirmatory method for the analysis of drugs of abuse. However, as the workload in toxicology and forensic laboratories increases, the need for faster HPLC methods has become an important issue. In the past, efforts to decrease analysis times have focused on the use of short columns with particles that are smaller than the standard 5 μm. These columns offer good efficiency with higher flow rates, but also have a tendency to "plug" and backpressures tend to be high.[11] Recently, columns made of a single piece of monolithic silica were introduced as an alternative to particle-based columns. These columns possess a biporous structure consisting of larger macropores (2 μm) that permit high flow rates with low backpressure, and smaller mesopores (13 nm) that provide a high surface area for high efficiency.[12] Therefore, it is possible to perform analyses with high linear flow velocity but without significantly reduced separation efficiency. The utility of monolithic silica columns for high throughput bioanalysis in a drug discovery environment has

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been demonstrated.[13] The columns have also been used to analyze six hydroxylated debrisoquine isomers[14] and ochratoxin A in different wines.[15]

This paper describes the development and validation of two separate HPLC methods that are both rapid and sensitive for determining COC and three of its metabolites in human plasma, as well as five commonly abused opium alkaloids in human plasma. For sample pre-treatment, the methods utilize mixed mode solid phase extraction. Elution is performed by binary mobile phase gradients with 231 and 208 nm UV detection, respectively.

EXPERIMENTAL

Chemicals

Benzoylecgonine, benzoylnorecgonine hydrochloride (BN), cocaine hydrochloride, and cocaethylene fumarate were obtained from the National Institute of Drug Abuse (Bethesda, MD). Norcocaine and tolazoline hydrochloride (ISTD) were obtained from Sigma Chemical Co. (St Louis, MO). Morphine sulfate pentahydrate, hydromorphone hydrochloride, codeine phosphate hemihydrate, Oxycodone hydrochloride, and hydrocodone bitartrate were obtained from the United States Pharmacopeial Convention Inc. (Rockville, MD). Monobasic sodium phosphate, phosphoric acid, pentanesulfonic acid, monohydrate sodium salt, HPLC grade methanol, and acetonitrile were from J.T. Baker Inc. (Phillipsburg, NJ 08865). Trifluoroacetic acid was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI 53201). Water was purified with a cartridge system (Continental Water System, Roswell, GA). Drug free human plasma was obtained from Bioreclamation Inc. (Hicksville, NY 11801).

Instrumentation

The chromatographic separation of COC and the selected metabolites was performed on a Model 1090 HPLC system (Hewlett Packard Co., Palo Alto, CA). This system included a pump, an autosampler equipped with a 250 μL loop, and a Model 117 variable wavelength UV detector (Gilson, Middleton, WI). 0.010″ I.D. tubing was used before and after the column and was kept at a minimum length. Turbochrom (Perkin Elmer, Norwalk, CT) chromatography software was used for data integration. Separations were performed on a reversed phase monolithic silica column (Chromolith Performance RP-18e, 100 mm × 4.6 mm I.D., Merck KgaA, Darmstadt, Germany).

The chromatographic separation of the selected opiates was performed on an HPLC system that consisted of two Model 515 pumps and a Model 996

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photodiode array detector with Millenium-32 chromatography software (Waters, Milford, MA). Samples were injected in triplicate with a Model 728 autosampler (Alcott, Norcross, GA) with a 100 μL loop. The monolithic column that was used on the Hewlett Packard system above was also used for the separation of the selected opiates.

Chromatographic Conditions for Analysis of Cocaine and Selected Metabolites

Cocaine and the selected metabolites were separated using a binary mobile phase gradient. Mobile Phase A consisted of 86 : 14 v/v 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate-acetonitrile. The buffer pH was adjusted to 2.9 with trifluoroacetic acid prior to mixing with the acetonitrile. Mobile Phase B consisted of 76 : 24 v/v 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate (pH 2.9)-acetonitrile. The gradient program was as follows: 0–2.7 min, linear change from 100% Mobile Phase A to 100% Mobile Phase B; 2.7–3.5 min, linear change from 100% Mobile Phase B to 100% Mobile Phase A; 3.5–5 min, equilibration at 100% Mobile Phase A. The HPLC pump flow rate was 5.0 mL/min and all analyses were conducted at ambient temperature. The injection volume was 100 μL and the UV detector was operated at 231 nm.

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Chromatographic Conditions for Analysis of Selected Opiates

The five opiate drugs and internal standard were also separated using a binary mobile phase gradient. Mobile Phase A consisted of 95 : 5 v/v 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate-acetonitrile. The buffer pH was adjusted to 2.9 with trifluoroacetic acid prior to mixing with the acetonitrile. Mobile Phase B consisted of 80 : 20 v/v 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate (pH = 2.9)-acetonitrile. The gradient program was as follows: 0–1.4 min, linear change from 60% Mobile Phase A to 60% Mobile Phase B; 1.4–1.5 min, linear change from 60% Mobile Phase B to 60% Mobile Phase A; 1.5–2 min, equilibration at 60% Mobile Phase A. The HPLC pump flow rate was 8.0 mL/min and the column was thermostated at 30°C. The injection volume was 100 μL and the diode array detector was operated at 208 nm. All mobile phase was filtered through a 0.45 μm nylon-66 filter (MSI, Westborough, MA) and degassed in an ultrasonic bath for 15 min before use.

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Preparation of Standard Stock Solutions

Standard stock solutions of COC, BE, NC, and CE were prepared by dissolving appropriate amounts of each drug in deionized water to obtain final drug concentrations of 196, 90, 115, 51, and 102 μg/mL, respectively. Working solutions were prepared by further diluting these stock solutions with pooled human plasma. The pooled human plasma was supplemented with sodium fluoride (NaF) to a final concentration of 0.064 M to inhibit esterases, thereby, preventing hydrolysis of COC and NC.

Standard stock solutions of MO, HM, CO, OC, and HC were prepared by dissolving appropriate amounts of each drug in deionized water to obtain final drug concentrations of 5.30, 5.13, 5.36, 5.30, and 5.28 mg/mL, respectively. Working solutions were prepared by further diluting these stock solutions with pooled human plasma. The internal standard (ISTD) stock solution was prepared by dissolving an appropriate amount of the drug in deionized water to obtain a final concentration of 208 μg/mL. A working internal standard solution was prepared by further diluting this stock solution with deionized water to yield a concentration of 2491 ng/750 μL.

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Sample Preparation Procedure

Calibration standards and quality control samples were prepared by making appropriate dilutions of the working standard solution with pooled human plasma. The extraction methods used in these studies were based on the generally accepted mixed mode solid phase extraction (SPE) protocols.[16]

Extraction of Cocaine and Metabolites

Solid phase extraction cartridges (Varian Inc., Bond Elut Certify, 3 cc 130 mg) were placed on a vacuum elution manifold (Alltech, Deerfield, IL 60015) and rinsed with 3 mL of methanol, followed by 3 mL of 100 mM sodium phosphate dibasic (pH 6.0). Care was taken that the cartridges did not run dry. 1.1 mL of each standard or sample was transferred to a 1.5 mL polypropylene microcentrifuge tube and centrifuged at 13,000 rpm for 15 min. Following centrifugation, 1 mL of each standard or sample was transferred to a 13 mm test tube and mixed with 3 mL of 100 mM sodium phosphate dibasic (pH 6.0). The entire spiked plasma samples were then transferred to the SPE cartridges. Vacuum was then applied to obtain a flow through the cartridges of 1–2 mL/min. The cartridges were then washed with 6 mL deionized water and 3 mL 1 M acetic acid. The cartridges were then dried under vacuum for 5 min. This was followed

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by a final rinse with 6 mL of methanol. The analytes were eluted from the cartridges with two 1 mL aliquots of methylene chloride/2-propanol/ammonium hydroxide (78 : 20 : 2 v/v). Extracts were dried under a stream of nitrogen in a 40°C water bath. The extracts were then reconstituted in 500 μL of Mobile Phase A, vortex mixed, and 100 μL was injected onto the liquid chromatograph.

Extraction of Selected Opiates

Solid phase extraction cartridges (Varian Inc., Bond Elut Certify, 3 cc 130 mg) were placed on a vacuum elution manifold and rinsed with 2 mL of methanol followed by 2 mL of 100 mM sodium phosphate dibasic (pH 8.5). Care was taken that the cartridges did not run dry. Each standard or sample (1.1 mL) was transferred to a 1.5 mL polypropylene microcentrifuge tube and centrifuged at 13,000 rpm for 15 min. Following centrifugation, 1 mL of each standard or sample was transferred to a 13 mm test tube and mixed with 750 μL of internal standard and 2 mL of 100 mM sodium phosphate dibasic (pH 8.5). The entire spiked plasma samples were then transferred to the SPE cartridges. Vacuum was then applied to obtain a flow through the cartridges of 1–2 mL/min. The cartridges were then washed with 2 mL deionized water, 2 mL of 0.1 M sodium acetate buffer (pH 4.0), and 2 mL of methanol. The cartridges were then dried under vacuum for 5 min. The analytes were eluted from the cartridges with two 1 mL aliquots of methanol/ammonium hydroxide (98 : 2 v/v). Extracts were dried using a vacuum centrifuge (SC110A SpeedVac Plus and RVT400 refrigerated vapor trap, Savant Inc., Farmingdale, NY). The extracts were then reconstituted in 750 μL of Mobile Phase A, vortex mixed, and 100 μL was injected onto the liquid chromatograph.

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Specificity

The specificity of the assay was checked by analyzing four independent blank human plasma samples. The chromatograms of these blank plasma samples were compared with chromatograms obtained by analyzing human plasma samples spiked with the analytes.

Linearity

Calibration plots for the analytes in plasma were prepared by diluting stock solutions with pooled human plasma to yield five concentrations over a range of 50–5000 ng/mL. For NC, the range of the calibration standards was

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25–2500 ng/mL. Calibration standards at each concentration were extracted and analyzed in triplicate. Calibration curves of COC, and the selected metabolites, were constructed using the observed analyte peak area vs. nominal concentrations of the analytes. Calibration curves of the selected opiates were constructed using ratios of the observed analyte peak height to internal standard vs. nominal concentrations of analyte. Weighted ($W = 1/x$) least squares linear regression analysis of the data gave slope, intercept, and correlation coefficient data. From this data, a first order polynomial model was selected for each analyte.

Precision and Accuracy

The within-run and between-run accuracy and precision of the assays in plasma were determined by assaying four quality control samples in triplicate over a period of three days. The concentrations represented the entire range of the calibration curves. The lowest level was at the expected limit of quantification (LOQ) for each analyte (49.9, 44.6, 44.9, and 50.1 ng/mL for BE, COC, NC, and CE and 103, 53.2, 53.2, 53.2, and 53.2 ng/mL for MO, HM, CO, OC, and HC, respectively). The second level was within three times the LOQ (125, 112, 62.7, and 125 ng/mL for BE, COC, NC, and CE and 153, 152, 152, 152, and 153 ng/mL for MO, HM, CO, OC, and HC, respectively), and the third level was at the mid-point of the calibration curves (2501, 2233, 1252, and 2501 ng/mL for BE, COC, NC, and CE and 2519, 2519, 2517, 2519, and 2519 ng/mL for MO, HM, CO, OC, and HC, respectively). The fourth level was at 80% of the upper boundary of the calibration curves (4001, 3752, 2003, and 4000 ng/mL for BE, COC, NC, and CE and 3978, 3978, 3976, 3977, and 3978 ng/mL for MO, HM, CO, OC, and HC, respectively). Calibration curves were prepared and analyzed daily, and linear models were used to determine concentrations in the quality control samples. The nine measured concentrations per concentration level (triplicates from three runs) were subjected to analysis of variance (ANOVA) to estimate the within-run and between-run precision. Percent accuracy was determined (using the data from the precision assessment) as the closeness of spiked samples to the nominal value of in-house standards. Precision was reported as % relative standard deviation (%RSD).

Limit of Detection and Limit of Quantification

Decreasing concentrations of the analytes were prepared by diluting stock solutions with pooled human plasma, and then analyzed. The limit of detection (LOD) was defined as the concentration that yields a signal-to-noise ratio of 3. The LOQ was calculated to be the lowest analyte concentration in plasma that could be measured with a between-run RSD of $<20\%$ and an accuracy between 80 and 120%.

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Recovery

The absolute recoveries of the analytes from plasma were assessed at two concentrations (within three times the LOQ and 80% of the upper boundary of the calibration curves). The recovery of the internal standard from plasma was assessed at the working concentration of 2491 ng/750 μL. For each level three samples were extracted and analyzed in triplicate. Three replicates of each concentration, prepared in the eluent, were directly injected. The assay absolute recovery for each compound, at each concentration, was computed using the following equation: absolute recovery = (peak area of extract)/(mean peak area of direct injection) × 100.

RESULTS AND DISCUSSION

Assay of Cocaine and Selected Metabolites

The chemical structures for COC and the selected metabolites are shown in Fig. 1. The goals in developing this method were low ng/mL sensitivity, a run time of less than 5 min, and a simple extraction method that could be easily automated. Initial experiments utilized isocratic conditions with a 25 mM

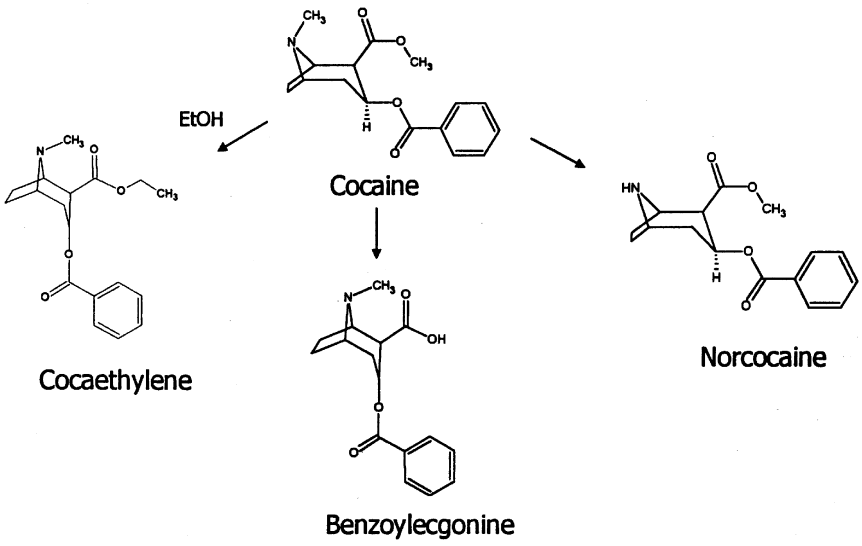

Figure 1. The chemical structures of COC and the selected metabolites.

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phosphate buffer (pH 2.9)–acetonitrile mobile phase. However, under these conditions it was not possible to achieve baseline separation of BE from the structurally similar BN. The next approach was to improve the resolution by the addition of an ion pair reagent to the aqueous phase. Under acidic conditions, the basic amine group of COC and its metabolites is protonated and will, therefore, react with an alkylsulfonate in a cation exchange process. Several alkylsulfonates, including octane-, heptane-, and pentane-sulfonic acid sodium salt were tried, with the latter giving the shortest run time with baseline resolution between BE and BN. The large difference in lipophilicity between BE, COC, and CE posed the greatest challenge in the development of the separation. The more hydrophilic BE tended to elute with endogenous substances in the plasma extract, whereas COC and CE tended to elute much later in the run. Ordinarily, a gradient separation would not be desirable, due to the additional time required for a particle based reversed phase column to re-equilibrate to the initial conditions. This is particularly so in the case of ion pair separations, where gradient elution is usually not recommended. However, this was not an issue with the monolithic silica column used in these studies. Due to the high degree of porosity of the monolithic column, it was possible to re-equilibrate the column in 1.5 min at a flow rate of 5 mL/min. At this flow, the system pressure was only 120 bar. Ultimately, it was possible to separate COC and the selected metabolites, using a binary mobile phase gradient consisting of 86 : 14 v/v 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate-acetonitrile. The buffer pH was adjusted to 2.9 with trifluoroacetic acid prior to mixing with the acetonitrile. Trifluoroacetic acid was added to lower the mobile phase pH and to minimize the retention of endogenous sample components. The initial conditions were followed by a linear increase in the acetonitrile concentration to 24% v/v in 3.5 min. These conditions were found to give good selectivity and sensitivity in a 5 min run.

The SPE method utilized in these studies has been used extensively in the analysis of COC and metabolites in biological fluids.[16–18] The method utilizes a copolymeric phase combining a C8 and a strong cation exchange phase to achieve a mixed mode separation mechanism. The 3 cc, 130 mg Bond Elut Certify cartridge was found to give high recoveries for COC and the metabolites in this study, while at the same time removing endogenous interferences. The method sensitivity was improved by a factor of two, by extracting 1 mL of plasma and reconstituting in 0.5 mL of mobile phase.

Specificity

The analytical figures of merit for this method are shown in Table 1. Benzoylecgonine, COC, NC, and CE were well separated under the HPLC

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Table 1. Analytical Figures of Merit for COC and Selected Metabolites

Analyte	k	Tailing Factor[a]	R_s	α
Benzoylecgonine	1.5	1.1	N/A	N/A
Cocaine	5.9	1.0	22.0	3.9
Norcocaine	6.5	1.0	2.6	1.1
Cocaethylene	8.0	1.0	6.7	1.2

[a]Calculated at 5% peak height.

conditions applied. Retention times were 0.9, 2.4, 2.6, and 3.1 min for BE, COC, NC, and CE, respectively. No interferences were observed in drug free human plasma samples. Figures 2 and 3 show chromatograms of a blank plasma sample and a calibration sample, respectively.

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Linearity

The calibration curves showed good linearity in the range of 50–5000 ng/mL for BE, COC, and CE, and 25–2500 ng/mL for NC. The correlation coefficients (r) of calibration curves of each drug were higher than 0.99, as determined by least squares analysis.

Precision and Accuracy

A summary of the accuracy and precision results is given in Table 2. The method proved to be accurate (% bias for all calibration samples varied from −4.5 to 8.5%) and precise (within-run precision ranged from 1.5 to 12.8% and between-run precision ranged from 0.4 to 12.7%). The acceptance criteria (within-run and between-run %RSDs of <15% and an accuracy between 85 and 115%) were met in all cases.

Limit of Detection and Limit of Quantification

The LOD, as defined in the Experimental section, were 11.6, 16.9, 18.5, and 22.0 ng/mL for BE, COC, NC, and CE, respectively. The LOQ of each calibration graph was 49.9, 44.6, 25.1, and 50.1 ng/mL. Limit of detection and

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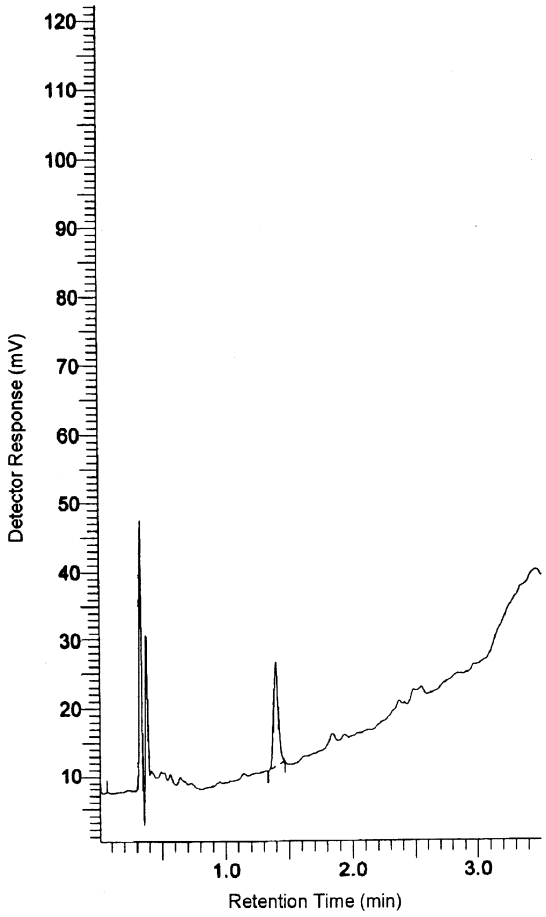

Figure 2. Chromatogram of blank pooled human plasma using the method for the analysis of COC and the selected metabolites.

LOQ data are shown in Table 3. Accuracy and precision data for the LOQ were also acceptable and are reported in Table 2.

Recovery

The results of the recovery experiments were acceptable. The mean absolute recoveries were 93.5, 95.7, 105.4, and 98.8% for BE, COC, NC, and CE, respectively.

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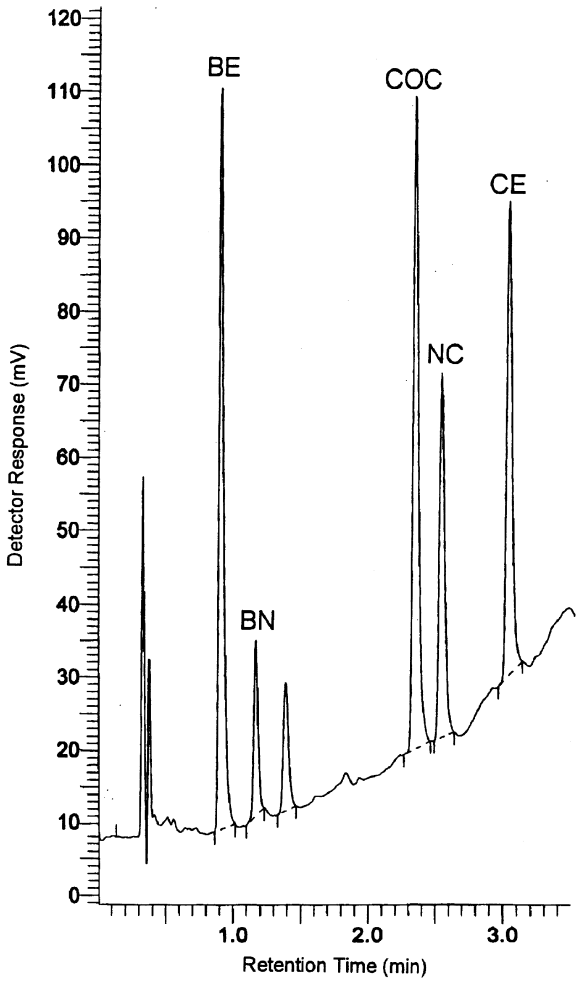

Figure 3. Chromatogram of pooled human plasma spiked with 2500 ng/mL BE, BN, COC, CE, and 1250 ng/mL NC.

Assay of Selected Opiates

The chemical structures for the selected opiates are shown in Fig. 4. As in the previous method, the goals in developing this method were low ng/mL sensitivity, a run time of less than 5 min, and a simple extraction method that could be easily automated. Since the opiates also contain basic tertiary amines,

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Table 2. Within-Run and Between-Run Accuracy and Precision and Absolute Recoveries for the Analysis of COC and Metabolites in Human Plasma ($n = 15$)

	Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Bias (%)	Within-Run RSD (%)	Between-Run RSD (%)	Recovery (%)
BE	49.9	50.1	0.4	4.1	1.1	N/A
	125	119.3	−4.5	5.5	1.7	89.5
	2,501	2,599	3.9	2.3	0.8	97.0
	4,001	3,983	−0.5	2.6	1.7	93.9
COC	44.6	44.4	−0.5	7.7	1.7	N/A
	112	120.3	7.9	7.4	6.7	93.2
	2,233	2,230	−0.2	3.3	1.1	95.2
	3,752	3,749	5.0	1.8	0.4	98.6
NC	25.1	25.4	1.1	12.8	5.3	N/A
	62.7	64.8	3.6	9.8	10.1	109.5
	1,252	1,224	−2.2	1.9	2.4	101.8
	2,003	2,173	8.5	2.8	2.5	105.0
CE	50.1	50.0	−0.1	10.0	1.3	N/A
	125	130	3.8	5.5	12.7	96.6
	2,501	2,494	−0.3	3.1	1.4	98.7
	4,000	4,137	3.4	1.5	2.5	101.1

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the initial experiments utilized the method parameters developed for the separation of COC and the selected metabolites (see above). However, under these conditions it was not possible to achieve baseline separation of MO from the structurally similar HM. However, by increasing the percentage of the aqueous phase to 89% v/v the two peaks were well resolved. The gradient conditions and flow rate were then modified to obtain good resolution of the other opiates in the shortest possible run time. Experiments were also conducted to

Table 3. Range of Calibration Curves, LOD and LOQ of COC and Metabolites in Spiked Human Plasma

Drug	Range of Calibration Curves (ng/mL)	Limit of Detection (ng/mL)[a]	Limit of Quantitation (ng/mL)
BE	49.9–4,999	11.6	49.9
COC	44.6–4,475	16.9	44.6
NC	25.1–2,504	10.4	25.1
CE	50.1–4,983	22.0	50.1

[a]$S/N = 3$.

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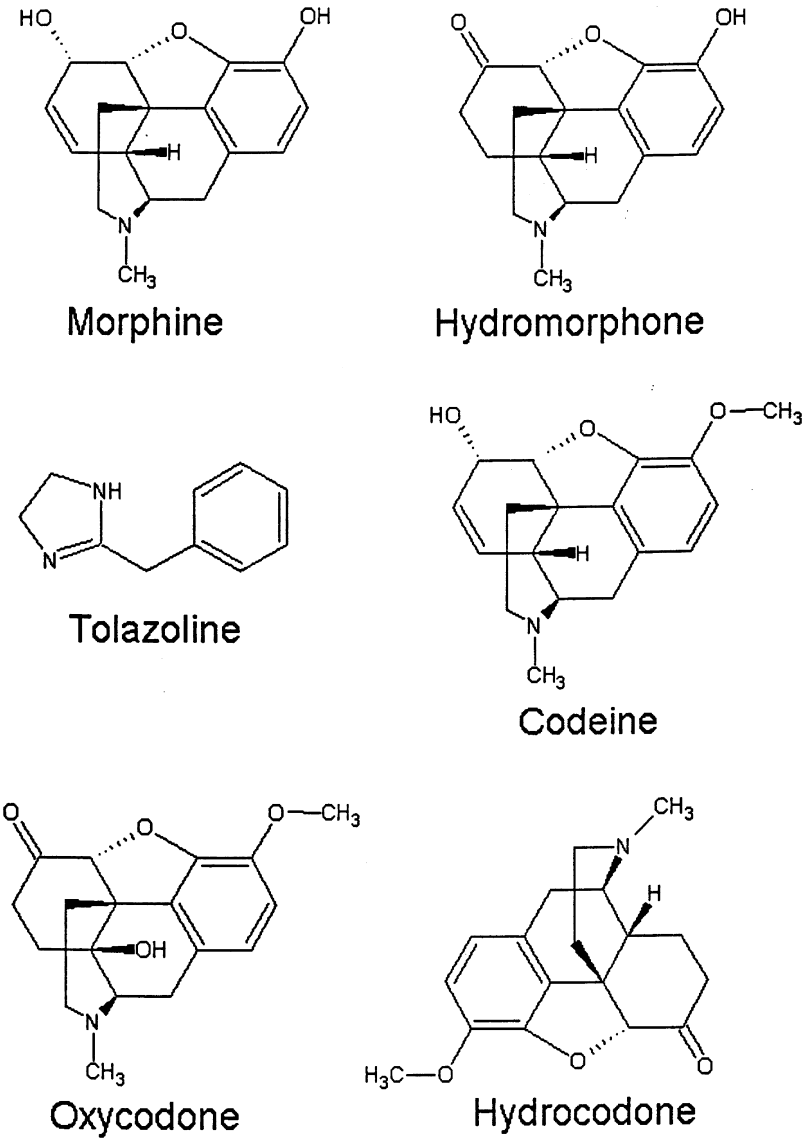

Figure 4. The chemical structures of the selected opiates.

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evaluate the utility of replacing the mobile phase gradient with a flow gradient. Although good resolution was achieved for all components, band broadening of the later eluting peaks resulted in decreased sensitivity. The final method used a binary gradient in which Mobile Phase A contained 95 : 5 v/v 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate-acetonitrile. The buffer pH was adjusted to 2.9 with trifluoroacetic acid prior to mixing with the acetonitrile. Mobile phase B contained 80 : 20 v/v 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate-acetonitrile. The gradient program was as follows: 0–1.4 min, linear change from 60% Mobile Phase A to 60% Mobile Phase B; 1.4–1.5 min, linear change from 60% Mobile Phase B to 60% Mobile Phase A; 1.5–2 min, equilibration at 60% Mobile Phase A. The flow rate throughout the gradient was 8 mL/min. At this flow, the system pressure was 190 bar. These conditions were found to give good selectivity and sensitivity in a 2 min run.

As with the COC and metabolites method, a well-established solid phase extraction method was selected for the extraction of the opiates from plasma.[16] The method again utilized the mixed mode Certify cartridge and yielded high recoveries for all of the opiates tested, while at the same time removing endogenous interferences. The method sensitivity was improved by a factor of 1.3, by extracting 1 mL of plasma and reconstituting in 0.75 mL of mobile phase.

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Specificity

The analytical figures of merit for this method are shown in Table 4. Morphine, HM, ISTD, CO, OC, and HC were well separated under the HPLC conditions applied. Retention times were 0.4, 0.5, 0.7, 0.8, 1.1, and 1.3 min for MO, HM, ISTD, CO, OC, and HC, respectively. No interferences were observed in drug free human plasma samples. Figures 5 and 6 show chromatograms of a blank plasma sample and a calibration sample, respectively.

Table 4. Analytical Figures of Merit for Selected Opiates

Analyte	k	Tailing Factor[a]	R_s	α
Morphine	1.3	1.0	N/A	N/A
Hydromorphone	2.0	1.1	1.6	1.5
INT STD	2.9	1.0	1.4	1.5
Codeine	3.6	1.0	2.8	1.2
Oxycodone	5.2	1.1	2.8	1.4
Hydrocodone	6.2	1.0	1.8	1.2

[a]Calculated at 5% peak height.

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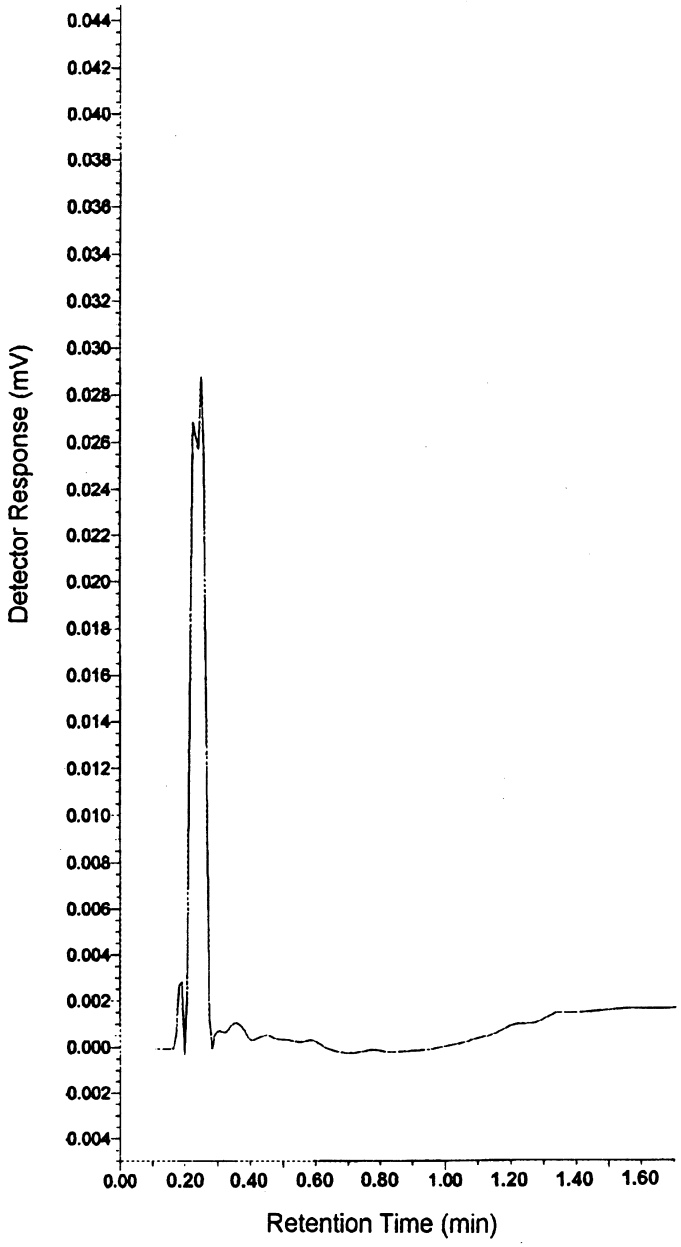

Figure 5. Chromatogram of blank pooled human plasma using the method for the analysis of the selected opiates.

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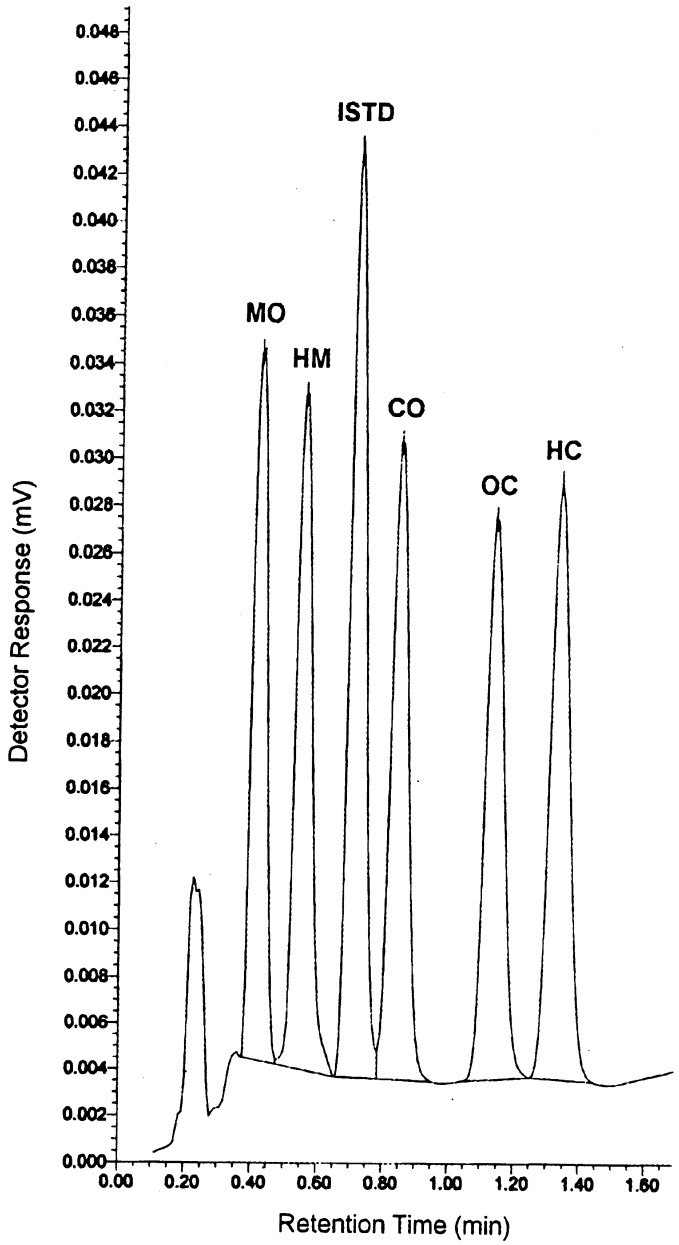

Figure 6. Chromatogram of pooled human plasma spiked with 600 ng/mL MO, HM, ISTD, CO, OC, and HC.

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Linearity

The calibration curves showed good linearity in the range of 50–5000 ng/mL for all of the opiates studied. The correlation coefficients (r) of calibration curves of each drug were higher than 0.997, as determined by least squares analysis.

Precision and Accuracy

A summary of the accuracy and precision results is given in Table 5. The method proved to be accurate (% bias for all calibration samples varied from −8.4 to 2.0%) and precise (within-run precision ranged from 1.7 to 16.9% and between-run precision ranged from 0.4 to 14.8%). The acceptance criteria

Table 5. Within-Run and Between-Run Accuracy and Precision for the Analysis of Selected Opiates in Human Plasma ($n = 15$)

	Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Bias (%)	Within-Run RSD (%)	Between-Run RSD (%)	Recovery (%)
MO	103	103	0.0	12.1	1.9	N/A
	153	145	−5.0	6.3	4.7	87.4
	2,519	2,502	−0.7	2.1	2.4	99.3
	3,978	3,962	−0.4	1.9	2.4	100
HM	53.2	52.5	−1.4	16.9	5.2	N/A
	152	146	−4.4	7.6	2.7	108
	2,519	2,491	−1.1	3.0	1.6	101
	3,978	3,936	−1.0	3.0	2.6	103
CO	53.2	53.4	0.4	12.4	0.7	N/A
	152	140	−8.4	5.3	14.8	112
	2,517	2,496	−0.9	1.7	2.0	97.9
	3,976	3,908	−1.7	2.0	1.3	100
OC	53.2	53.7	0.9	13.2	3.0	N/A
	152	151	−1.0	5.0	2.6	98.6
	2,519	2,487	−1.3	2.5	1.6	96.4
	3,977	3,891	−2.2	2.5	3.3	98.8
HC	53.2	54.3	2.0	10.6	1.5	N/A
	153	147	−4.4	2.6	4.9	113
	2,519	2,459	−2.4	2.5	0.4	100
	3,978	3,831	−3.7	2.4	1.5	103

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(within-run and between-run %RSDs of <15% and an accuracy between 85 and 115%) were met in all cases.

Limit of Detection and Limit of Quantification

The LODs, as defined in the Experimental section, were 38.6, 12.0, 8.7, 20.9, and 25.2 ng/mL for MO, HM, ISTD, CO, OC, and HC, respectively. The LOQ was 103 ng/mL for MO and 53.2 ng/mL for all of the other opiates studied. Limit of detection and LOQ data are shown in Table 6. Accuracy and precision data for the LOQ were also acceptable and are reported in Table 5.

Table 6. Range of Calibration Curves, LOD and LOQ of Selected Opiates in Spiked Human Plasma

Drug	Range of Calibration Curves (ng/mL)	Limit of Detection (ng/mL)[a]	Limit of Quantitation (ng/mL)
MO	103–5,002	38.6	103
HM	53.2–5,002	12.0	53.2
CO	53.2–4,999	8.7	53.2
OC	53.2–5,001	20.9	53.2
HC	53.2–5,003	25.2	53.2

[a]S/N = 3.

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Recovery

The results of the recovery experiments were satisfactory. The mean absolute recoveries were 95.6, 104, 103, 97.9, and 105% for MO, HM, ISTD, CO, OC, and HC, respectively. The recovery for the internal standard was 99.6%.

CONCLUSION

High speed methods have been developed and validated for the determination of COC, and selected metabolites, and five common opiates in human plasma. The methods utilize a new monolithic silica column technology, efficient solid phase extraction procedures, and fast and sensitive gradient reversed phase HPLC analyses with UV detection. The methods are suitable for use in routine determinations of the selected drugs of abuse in human plasma.

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Received May 22, 2002
Accepted June 30, 2002
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