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Sensitive and specific high-performance liquid chromatographic assay with ultraviolet detection for the determination of cocaine and its metabolites in rat plasma

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Abstract

A sensitive, specific and precise HPLC–UV assay was developed to quantitate cocaine (COC) and its metabolites benzoylecgonine (BE), norcocaine (NC) and cocaethylene (CE) in rat plasma. After adding 50 μl of the internal standard solution (bupivacaine, 8 μg/ml) and 500 μl of Sørensen's buffer (pH 6) to 100 μl of rat plasma sample, the mixture was extracted with 10 ml of chloroform. The organic layer was transferred to a clean test tube and was evaporated under nitrogen. The residue was reconstituted in 100 μl of mobile phase and 35 μl was injected onto the HPLC column. The mobile phase consisted of methanol–acetonitrile–50 m*M* monobasic ammonium phosphate (5:7:63, v/v/v) and was maintained at a flow-rate of 0.4 ml/min. Separation of COC and its metabolites was achieved using a Supelcosil ABZ+plus deactivated reversed-phase column (250×2.1 mm I.D., 5 μm). Calibration curves were linear over the range of 25–5000 ng/ml for COC and its three metabolites. The absolute extraction efficiencies for BE, COC, NC, CE and bupivacaine were 56.6%, 78.6%, 61.1%, 76.4% and 67.0%, respectively. COC and its metabolites were stable in mobile phase for 24 h at room temperature and in rat plasma for 2 weeks at −20°C. The limits of detection for BE, COC, NC and CE were 20, 24, 15 and 12.9 ng/ml, respectively. These values correspond to 0.70, 0.84, 0.525 and 0.452 ng of the according compound being injected on column. The within-day coefficient of variation for the four compounds ranged from 3.0% to 9.9% while the between-day precision varied from 3.6% to 14%. This method was used to analyze rat plasma samples after administration of COC alone and in combination with alcohol. The pharmacokinetic profiles of COC and its metabolites in these rats are also described.

Keywords: Cocaine; Benzoylecgonine; Norcocaine; Cocaethylene

1. Introduction

The prevalence of concurrent abuse of cocaine (COC) and alcohol is very high. It is estimated that 60–80% of COC abusers consume alcohol simultaneously [1]. Due to COC–alcohol interactions, COC metabolism is altered significantly [2–5]. Cocaethylene (CE) is a unique metabolite found in human plasma, urine, and body tissue samples and in experimental animals after combined COC and alcohol administration [6,7]. The importance of this metabolite became evident when it was found to exert central nervous system (CNS) and cardiovascular activities similar to those of COC [8–11]. Ben-

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zoylecgonine (BE) and ecgonine methyl ester are the two major metabolites formed after COC administration [12]. Norcocaine (NC) is a pharmacologically active metabolite of COC formed by N-demethylation via hepatic microsomal oxidative metabolism and can be converted to N-hydroxynorcocaine, a potential hepatotoxin [13–15]. Since changes in COC pharmacokinetics and metabolic profile alter the resulting pharmacological and toxicological effects, studying the factors that cause these changes is of great clinical importance. Therefore, a simple yet selective and sensitive analytical method to quantitate COC and its metabolites simultaneously in biological fluids is needed for such studies.

Several assays have been reported for the determination of BE, COC, NC and CE. Immunoassays lack sufficient selectivity when COC metabolites are present, and confirmation with chromatographic assay is often required [16]. Gas chromatography (GC), mass spectrometry (MS) and GC–MS are very specific and sensitive [17–21] but are expensive and not readily available compared to high-performance liquid chromatography (HPLC). Moreover, derivatization of some analytes are often needed to produce relatively intense high-molecular-mass ions before running GC–MS assays [17,18,22]. Other techniques such as atomic continuous-flow [23], potentiometry [24] and calorimetry [25] have also been described. HPLC is a relatively simple and inexpensive technique and its reversed-phase version is especially suitable for analyses of biological fluids such as plasma samples. It has rapid sample throughput and permits automated fraction collection of column effluent and is thus applicable to experiments with radioactive labeling. Assays for quantitating COC and its metabolites in plasma require sample preparation procedures that will not affect the stability of these compounds and a suitable chromatographic condition to separate them in one run within a reasonable period of time. Recently, a sensitive and specific GC–MS method was developed for the simultaneous measurement of COC, CE and seven other related compounds in biological fluids [17,18]. This assay involved one solid-phase extraction (SPE) procedure and the limits of detection were ~1 ng/ml for BE, COC and ecgonine methyl ester and 3–6 ng/ml for the other analytes. On the other hand, most of the reported HPLC techniques cannot be applied to determine the polar BE and the non-polar COC, NC, and CE simultaneously [26–29]. Another recently published HPLC method described the quantitation of benzoylnorecgonine, BE, COC, NC, CE and norcocaethylene in rat plasma and urine samples [30]. However, it required two successive liquid–liquid extractions and two separate injections onto two different columns. The primary objective of this research was to develop a simple, sensitive, accurate and precise HPLC–UV method for the determination of BE, COC, NC and CE in rat plasma microsamples (100 μl). This assay was used to determine plasma concentrations of COC and its metabolites in rats after treatment with COC alone and in combination with alcohol.

2. Experimental

2.1. Chemicals and reagents

BE, COC, NC and CE were obtained from Research Biochemicals (Natick, MA, USA). Bupivacaine hydrochloride was purchased from Sigma (St. Louis, MO, USA). Acetonitrile was supplied by Burdick and Jackson (Muskegon, MI, USA). Chloroform, methanol, ethanol, monobasic ammonium phosphate, monopotassium phosphate and disodium phosphate were products of J.T. Baker (Phillipsburg, NJ, USA). All chemicals were of analytical reagent grade and all solvents were of HPLC grade. All aqueous solutions were prepared with Milli-Q water (CDMF01205 Milli-Q water purification system, Millipore, Bedford, MA, USA).

2.2. Preparation of standards and reagents

The working solution (8 μg/ml) of the internal standard was prepared by dilution with water from an 80 μg/ml bupivacaine stock solution. The pH 6 Sørensen's buffer [31] was prepared by mixing stock solutions A (1/15 *M* monopotassium phosphate) and B (1/15 *M* disodium phosphate) at 87.7:12.3 ratio. Stock solutions of 500 μg/ml for BE and COC and 50 μg/ml for NC and CE were prepared in methanol. Nine standard solutions with each containing

COC and its three metabolites were prepared by serial dilution with methanol. The standard solutions contained concentrations of 25, 50, 100, 200, 400, 600, 1000, 2000 and 5000 ng/ml of COC and its metabolites. To test the possibility of COC degradation in methanol, we diluted COC stock solution (500 μg/ml in methanol, stored at −20°C for 8 months) to 1000 ng/ml by mobile phase. We injected the diluted solution onto the HPLC system and found that degradation of COC in methanolic solution stored at −20°C for 8 months was less than 0.2%.

2.3. The HPLC–UV system and conditions

An HPLC system equipped with a variable-wavelength detector and an autoinjector was used in our study (Model LC-10, Shimadzu Scientific Instrument, Columbia, MD, USA). Separation of BE, COC, NC, CE and bupivacaine was accomplished on a 250×2.1 mm I.D., 5 μm Supelcosil ABZ+plus deactivated reversed-phase column (Supelco, Bellefonte, PA, USA). Mobile phase, consisted of 120 ml of methanol, 180 ml of acetonitrile and 1500 ml of 50 m*M* monobasic ammonium phosphate (pH 4.5), ran through the column at a flow-rate of 0.4 ml/min. The typical column pressure under this condition was, on average, about 160 atm (1 atm=101325 Pa). The column effluent was monitored at 235 nm and peak heights were calculated with an electronic integrator (CR501 Chromatopac, Shimadzu Scientific Instrument).

2.4. Calibration curves

Aliquots from each of the standard solutions (100 μl) were transferred to 13 ml clean centrifuge tubes (Kontes of California, Hayward, CA, USA) and were dried under nitrogen with an analytical evaporator (Organomation Associates, Berlin, MA, USA). The residue in each tube was reconstituted in 100 μl of rat plasma. Then 50 μl of the bupivacaine working solution and 500 μl of the pH 6 Sørensen's buffer were subsequently added. Samples were extracted with 10 ml of chloroform by shaking for 10 min in a mechanical shaker (Eberbach Corporation, Ann Arbor, MI, USA) and centrifuging for another 10 min at 2000 rpm (873 *g*) with an IEC EXD Centrifuge (International Equipment Company, Needham HTS, MA, USA). The chloroformic layer was transferred to a clean tube and then dried under nitrogen in the analytical evaporator. The dried residue in each tube was reconstituted in 100 μl mobile phase and 35 μl was injected onto the HPLC. Analyte peak height/internal standard peak height for BE, COC, NC and CE were plotted against the according concentrations and linear regression was performed.

2.5. Selectivity

The selectivity of the assay for the analysis of COC and its metabolites was evaluated by chromatographing a group of over-the-counter medications and some controlled substances including their major metabolites under the same conditions of our assay. These drugs included ascorbic acid, morphine, oxymorphone, noroxymorphone, norhydromorphone, ethylmorphine, codeine, norcodeine, oxycodone, hydrocodone, nalorphine, procaine, acetaminophen, caffeine, lidocaine, benzoylnorecgonine, ketamine, acepromazine, salicylic acid, benzoic acid, thebaine, cocaine propyl ester, benzocaine, tetracaine and pentobarbital.

2.6. Stability of COC and its metabolites in mobile phase and in frozen plasma

Stability of COC and its metabolites in mobile phase (at room temperature for 24 h) was determined by preparing 100, 600 and 2000 ng/ml solutions of COC and its metabolites in 100 μl of mobile phase and adding 100 μl of internal standard solution. After mixing for 30 s, samples were injected immediately onto the HPLC and once again 24 h later. For each compound, the concentrations measured at time zero and after 24 h were compared using paired *t*-tests to determine whether there were significant changes in concentration over time. To study the stability of COC and its metabolites in frozen rat plasma, blank rat plasma was spiked with COC and its metabolites to prepare samples containing 100, 600 and 2000 ng/ml of each compound. These

plasma samples were then frozen at −20°C and analyzed two weeks later.

2.7. Absolute extraction efficiency

The absolute extraction efficiencies of COC, it metabolites and bupivacaine in rat plasma were determined at concentrations of 100, 600 and 2000 ng/ml. Two sets of standards were prepared: one in mobile phase and the other in rat plasma. The standard solutions in the mobile phase were injected directly onto the HPLC while the plasma standards underwent the entire extraction procedures before injection. The absolute peak heights for each compound in the nonextracted and extracted samples were compared to determine the absolute extraction efficiency for each compound.

2.8. Precision and accuracy

The within-day precision of the assay was determined by the analysis of six replicates of rat plasma samples containing 100, 600 and 2000 ng/ml of each compound on the same day. The between-day precision was evaluated at the same three concentrations by the analysis of six sets of standards on six different days within two months. The coefficient of variation (C.V.) of the measured concentration and the percent of nominal concentration were used to determine the precision and accuracy of the assay. All samples for this study were freshly prepared on the day of analysis.

2.9. Pharmacokinetic studies

Male Wistar rats weighing 300–350 g (Simonsen Labs., Gilroy, CA, USA) were maintained on a 12-h light/dark cycle with Purina chow pellets and water ad libitum for at least seven days before experiments. Rats were kept three/cage in a temperature and humidity controlled environment. Eight rats were randomly chosen and were treated with either COC (30 mg/kg, i.p.) alone or in combination with 5 g/kg of alcohol (per os, 20 min before COC administration) in a balanced cross-over design. Blood samples were drawn through the femoral artery cannula at 0, 2, 5, 10, 15, 30, 60, 90, 120, 180 min after COC administration. Vacutainers for sample collection (3 ml, Becton Dickinson VACUTAINER Systems, Rutherford, NJ, USA) were pretreated with 2 drops of heparin (1000 U/ml) and 3 drops of saturated sodium fluoride solutions. They were then evaporated to dryness at 100°C. Heparin and sodium fluoride can help reduce blood clotting and minimize the degradation of COC, NC and CE by plasma carboxylesterases [32,33]. Plasma samples were obtained by centrifugation for 5 min using a TRIAC centrifuge (Clay Adams, Division of Becton Dickinson and Company, Rutherford, NJ, USA). Samples were kept in 13 ml centrifuge tubes at −20°C until analysis. Rat plasma samples obtained after COC and COC plus alcohol administrations were treated utilizing the same procedures as described above. Concentrations of unknown samples were calculated from the calibration curves for COC and its metabolites. The average concentration–time profiles for COC and its metabolites obtained from eight rats were plotted on semi-logarithmic paper for pharmacokinetic evaluations.

3. Results and discussions

3.1. Chromatograms and selectivity of the assay

Fig. 1. shows representative chromatograms for an extracted blank rat plasma sample (A), an extracted blank rat plasma sample spiked with COC and its metabolites (B), and a typical chromatogram for a plasma sample obtained from an alcohol pretreated rat 15 min after COC administration (C). These chromatograms show that the peaks for COC and its metabolites were fully separated and the endogenous peaks from rat plasma did not interfere with the separation of the analytes.

The selectivity of the assay was evaluated by examining possible interferences from commonly used over-the-counter drugs, local anesthetics and some drugs of abuse including their major metabolites. Table 1 summarizes the retention times and capacity factors for 29 drugs (including COC and its metabolites) and their column selectivities relative to COC. Results show that lidocaine and benzoylnorecgonine interfere with BE quantitation. However, we found that it was possible to separate BE from lidocaine by decreasing methanol concentration in

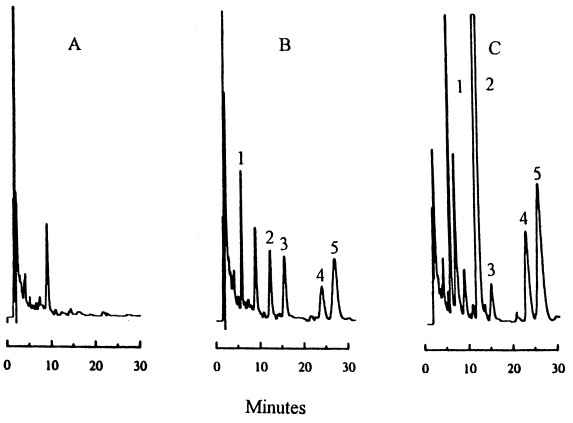

Fig. 1. Representative chromatograms for cocaine and its metabolites under the chromatographic conditions outlined in Section 2.3: (A) blank rat plasma; (B) extracted blank rat plasma spiked with cocaine and its metabolites (100 ng/ml) and bupivacaine (2 μg/ml); (C) extracted plasma sample from a rat 15 min after cocaine (30 mg/kg, i.p.) and 35 min after alcohol (5 g/kg, p.o.) administrations. Peaks: 1=benzoylecgonine; 2=cocaine; 3=norcocaine; 4=cocaethylene; 5=bupivacaine.

the mobile phase. This resulted in longer retention times for COC and its metabolites and a slight reduction in the sensitivity of the assay. Although benzoylnorecgonine has the same retention time as BE in our assay, its effect on BE quantitation in rat and human plasma samples obtained after COC administration is minimal. This is because benzoylnorecgonine was not identified in rats in previous studies after 15 mg/kg or 10 mg/kg of COC i.p. administrations [26,30]. In another report, GC–MS monitoring of benzoylnorecgonine in plasma samples from human subjects after smoking 42 mg of crack cocaine revealed that concentrations of this compound were below the limit of detection (which is ~3–6 ng/ml) of the assay [17]. Also, with our one-step extraction procedure, the absolute extraction efficiency of benzoylnorecgonine from rat plasma spiked with 200, 600 and 2000 ng/ml of this analyte was only 6.5±0.38% (n=3). The peak of thebaine superimposed on that of COC and the peak of cocaine propyl ester partially overlapped with that of CE. All the other tested drugs did not interfere with quantitation of COC, its metabolites, and the internal standard, bupivacaine.

Table 1
Retention times, capacity factors and column selectivities of cocaine, its metabolites, and some other compounds under the chromatographic conditions described in Section 2.3

Compound	t_R (min)	k'	A
Ascorbic acid	1.642	0.09467	0.1326
Morphine	2.078	0.3853	0.1679
Oxymorphone	2.189	0.4593	0.1768
Noroxymorphone	2.267	0.5113	0.1831
Norhydromorphone	2.425	0.6167	0.1959
Norcodeine	2.914	0.9427	0.2354
Codeine	2.933	0.9553	0.2369
Nalorphine	3.032	1.021	0.2449
Procaine	3.204	1.136	0.2588
Acetaminophen	3.313	1.209	0.2676
Oxycodone	3.550	1.367	0.2868
Hydrocodone	4.015	1.677	0.3243
Caffeine	4.325	1.883	0.3494
Ethylmorphine	4.870	2.247	0.3934
Lidocaine	5.780	2.853	0.4669
Benzoylecgonine	5.888	2.925	0.4756
Benzoylnorecgonine	5.922	2.948	0.4784
Ketamine	6.301	3.201	0.5090
Acepromazine	7.475	3.983	0.6038
Salicylic acid	8.317	4.545	0.6718
Benzoic acid	9.092	5.061	0.7344
Thebaine	12.35	7.233	0.9976
Cocaine	12.38	7.253	1.000
Norcocaine	15.63	9.420	1.263
Cocaethylene	24.07	15.05	1.944
Cocaine propyl ester	25.12	15.75	2.029
Bupivacaine	26.90	16.93	2.173
Benzocaine	32.73	20.82	2.644
Tetracaine	57.27	37.18	4.626
Pentobarbital	62.52	40.68	5.050

t_R, retention time; k', capacity factor; A, column selectivity compared to cocaine (the retention time of the compound divided by that of cocaine).

3.2. Stability of COC and its metabolites in mobile phase and in frozen plasma

COC undergoes hydrolysis via two mechanisms: spontaneous or carboxylesterase catalyzed hydrolysis of the alkyl ester group to yield BE and serum cholinesterase catalyzed hydrolysis of the phenyl ester group to form ecgonine methyl ester. It was reported that alkaline pH and elevated temperature increase COC hydrolysis [33]. The same study also showed that COC was stable in human blood for at least 150 days if the temperature was maintained at

below 4°C and the pH was adjusted to 5 in the presence of either 2% sodium fluoride or organophosphate. Our method involves buffering the rat plasma samples with pH 6 Sørensen's solution so as to optimize the extraction efficiency of COC and its metabolites and minimize their spontaneous chemical hydrolysis. In our routine COC pharmacokinetic studies, rat plasma samples were frozen at most for two weeks before analysis. The extracted samples were reconstituted in mobile phase and automatically injected within 24 h. For these reasons, we investigated the stability of COC and its metabolites in mobile phase for 24 h and in frozen rat plasma (−20°C) for two weeks. Table 2 shows that there were no significant differences ($p>0.05$, paired *t*-test) between the measured mean concentrations at time zero and after 24 h for all compounds. The low pH (4.5) of the phosphate solution in the mobile phase may be the major factor for stabilizing COC and its metabolites for at least 24 h at room temperature (23°C). Mean while, no decomposition of any of these compounds in frozen rat plasma samples was observed within two weeks (Table 2). The results presented here indicate that rat plasma samples containing COC and its metabolites pretreated with sodium fluoride can be stored at −20°C for at least two weeks and that samples reconstituted in mobile phase are stable for at least 24 h.

3.3. Absolute extraction efficiency and reproducibility

The mean absolute extraction efficiencies in the concentration range of 100–2000 ng/ml (bupivacaine at 4 μg/ml) for BE, COC, NC, CE and bupivacaine were 56.6, 78.6, 61.1, 76.4 and 67.0%, respectively (Table 3). Extraction efficiencies achieved in our assay were not corrected for any volume loss during aqueous phase aspiration and subsequent organic phase transfer. We would, therefore, expect that the actual extraction efficiencies were much higher. Sukbuntherng et al. [30] reported a two-step extraction procedure for COC, NC and CE plus a third step for BE and benzoylnorecgonine from rat plasma samples. The absolute extraction efficiencies achieved with this method were not reported. Other reports showed that extraction efficiencies for COC and its metabolites from human [17] and rat [29] biological fluids ranged from 36.2±10.2% to 98.5±3.1% utilizing SPE. Lau et al. [26] reported using liquid–liquid extraction method with 100 μl borate buffer (1 *M*) and 1 ml chloro-

Table 2
Stability of cocaine and its metabolites in mobile phase and in frozen rat plasma

Compound	Nominal concentrations (ng/ml)	Concentration found[a] at $t=0$ h (mg/ml) Mean±S.D.	Concentration found[a] at $t=24$ h (ng/ml) Mean±S.D	Concentration found[b] in frozen plasma (ng/ml) Mean±S.D
Benzoylecgonine	100	92±2.3	95±3.7	107±4.8
	600	620±18	625±9.0	660±22
	2000	2000±37	2000±21	2270±272
Cocaine	100	100±5.8	100±5.4	110±3.4
	600	600±27	600±30	630±14
	2000	2010±14	2010±13	2140±191
Norcocaine	100	94±4.4	96±1.6	115±12
	600	610±11	610±12	620±16
	2000	2010±39	1990±13	2040±89
Cocaethylene	100	100±4.9	100±5.1	105±4.4
	600	600±24	600±20	646±11
	2000	2000±9.2	2000±12	2160±148

[a] $n=6$ for each presented data point. Paired *t*-test result showed that there was no significant difference ($p>0.05$) between the measured means at each concentration for all compounds at the two tested times.
[b] $n=6$ for each presented data point. Samples were prepared and stored at −20°C for 2 weeks before analysis.

Table 3
Absolute extraction efficiency, reproducibility, limit of detection and quantitation for cocaine and its metabolites in rat plasma

Compound	Nominal concentrations (ng/ml)	Extraction efficiency (%) (mean±S.D.) (n=6)	Mean slope±S.D.[a] (n=6) (ml/μg)	Limit of detection (n=20) (ng/ml)	Limit of quantitation (n=20) (ng/ml)
Benzoylecgonine	100	59.6±4.1	4.3±0.24[c]	20±1.1[c]	65±3.6[c]
	600	55.5±2.6			
	2000	54.7±4.8	5.6±0.47[d]	1.07±0.090[d]	3.6±0.30[d]
	Grand mean[b]	56.6±2.3			
Cocaine	100	80.9±3.6	3.2±0.16[c]	24±1.2[c]	81±4.1[c]
	600	81.4±3.0			
	2000	73.6±7.9	3.2±0.14[d]	1.88±0.082[d]	6.3±0.27[d]
	Grand mean[b]	78.6±3.1			
Norcocaine	100	64.0±3.6	2.4±0.26[c]	15±1.6[c]	50±5.4[c]
	600	62.0±3.2			
	2000	57.3±8.8	3.1±0.14[d]	1.29±0.087[d]	6.6±0.29[d]
	Grand mean[b]	61.1±3.3			
Cocaethylene	100	76.5±4.3	1.63±0.076[c]	12.9±0.60[c]	43±2.0[c]
	600	79.9±2.7			
	2000	72.7±9.0	1.71±0.099[d]	3.5±0.20[d]	11.7±0.68[d]
	Grand mean[b]	76.4±3.4			
Bupivacaine (I.S.)		67.0±3.1			

[a]Each standard curve covers the concentration range of 25–5000 ng/ml.
[b]S.D. for the grand mean was calculated following the rule of propagation of uncertainty [35].
[c]In plasma.
[d]In mobile phase.

form–ethanol (82.5:17.5, v/v) to achieve 87.0, 81.0 and 89.0% extraction efficiencies for BE, COC and NC from rat plasma samples. He also reported a similar method without ethanol to achieve extraction efficiencies of 86.0, 89.7 and 98.3% for COC, NC and CE, respectively [27]. However, neither of these methods can be used to extract BE, COC, NC and CE simultaneously. High pH can increase extraction efficiencies of alkaloids such as COC and its metabolites [26–28], but it can also increase the spontaneous degradations of COC, NC and CE during extraction since all of these compounds have easily hydrolyzible alkyl ester groups. With our easy, one-step sample preparation procedure using only one extraction solvent, all four compounds were recovered with relatively good efficiencies that were concentration independent.

Calibration curves obtained during a period of two months showed very good linearity for all four compounds within the concentration range of 25–5000 ng/ml. The coefficients of determination for the calibration curves were all greater than 99% and the intercepts were all close to zero (data not shown). Slopes (including standard deviations) of calibration curves for compounds in both extracted plasma and nonextracted mobile phase are presented in Table 3. The C.V.s for the slopes were between 4.4–10.8% indicating very good reproducibility of the assay.

3.4. Limits of detection and quantitation

The limits of detection and quantitation were determined using the method reported previously by Skoog and Leary [34]. We performed 20 blank measurements to calculate absolute peak heights for the noise at the corresponding retention times for each compound and divided them by the grand mean of the bupivacaine absolute peak heights (either in extracted plasma or in mobile phase). The mean±S.D. for these peak-height ratios was calcu-

lated and the S.D. was multiplied by 3 and divided by the slope of the calibration curve to obtain the limit of detection for each compound. The limit of quantitation was calculated in the same way except the noise S.D. was multiplied by 10 instead of 3. Table 3 shows that in plasma, the limits of detection for BE and COC were 20 and 24 ng/ml and those of NC and COC were 15 and 12.9 ng/ml, respectively. The limit of quantitation for the four compounds was between 43 to 81 ng/ml in rat plasma (Table 3). To the best of our knowledge, there are three HPLC methods that reported the limits of detection for COC and its metabolites in plasma samples. Lau et al. [26] and Lau [27] achieved detection limit of 5 ng/ml for cocaine and its metabolites. However, these two methods could not quantitate the polar BE and non-polar COC, NC and CE simultaneously. On the other hand, our assay has much lower limits of detection and quantitation than the method of Virag et al. [29].

3.5. Precision and accuracy

The within-day and between-day precision and accuracy of the assay were evaluated at three different concentrations (100, 600 and 2000 ng/ml). The C.V.s for the within-day analysis ranged from 3.0–9.9% and the measured concentrations were between 90.0–110.0% of the nominal concentrations. The C.V.s for the between-day analysis ranged from 3.6–

Table 4
Analytical precision and accuracy for cocaine and its metabolites in rat plasma determined by the HPLC method

Compound	Nominal concentrations (ng/ml)	Concentrations found (mean±S.D.)	C.V. (%)	% of nominal concentration
Within-day[a]				
Benzoylecgonine	100	90±8.9	9.9	90.0
	600	600±29	4.8	100.0
	2000	1900±85	4.5	95.0
Cocaine	100	92±3.2	3.5	92.0
	600	600±20	3.3	100.0
	2000	1920±88	4.6	96.0
Norcocaine	100	100±8.5	8.5	100.0
	600	630±39	6.2	95.0
	2000	2200±160	7.3	110.0
Cocaethylene	100	90±2.9	3.2	90.0
	600	600±18	3.0	100.0
	2000	1920±63	3.3	96.0
Between-day[b]				
Benzoylecgonine	100	90±11	12	90.0
	600	610±36	5.9	101.7
	2000	2060±88	4.3	103.0
Cocaine	100	100±10	10	100.0
	600	600±30	5.0	100.0
	2000	2030±81	4.0	101.5
Norcocaine	100	100±14	14	100.0
	600	620±55	8.9	103.3
	2000	2100±121	5.8	105.0
Cocaethylene	100	96±6.0	6.3	96.0
	600	610±25	4.1	101.7
	2000	2040±73	3.6	102.0

[a] $n=6$ for each presented data point. Samples were prepared and analyzed on the same day.
[b] $n=6$ for each presented data point. Samples were prepared and anlyzed on six different days within two months.

14% and the measured concentrations were between 90.0–105.0% of the nominal concentrations (Table 4). These results indicate very good precision and accuracy of the assay.

3.6. Pharmacokinetic results

The developed HPLC assay was used to study the pharmacokinetics of COC and its metabolites in the rat after treatment with COC only and in combination with alcohol. The mean±S.E.M. (standard error of the mean) concentration–time profile for COC and it metabolites after COC and alcohol administration to eight rats are presented in Fig. 2. COC was rapidly absorbed from the peritoneal cavity after i.p. administration, reached the maximum concentration within 10 min, underwent a distribution phase and was then eliminated rapidly. The major metabolites BE, NC and CE appeared rapidly after COC and alcohol administrations and reached maximal concentrations within 10 to 30 min. The average plasma peak concentrations for BE, COC, NC and CE were 3100±660, 5100±870, 690±130 and 670±180 ng/ml, respectively. These results indicate that this assay can be used to accurately quantitate plasma concentrations of BE, COC, NC and CE over a period of 3 h after i.p. administration of 30 mg/kg of COC in the rat. Detailed pharmacokinetic analysis of these results will be discussed in a separate forthcoming paper.

4. Conclusions

The developed assay involves a single step liquid–liquid extraction procedure that maintains the stability of COC and its metabolites during the entire sample preparation process. Separation of BE, COC, NC, CE and the internal standard bupivacaine were achieved within 30 min using an isocratic mobile phase. In fact, this method has been used to quantitatively detect COC and its metabolites in rat brain microdialysate without any modifications [5,36]. One significant advantage of using this assay is that only 100 μl of plasma is required for the analysis. Small sample size is critical when the animal species under investigation is relatively small and repeated blood sampling is needed. This will minimize the occurrence of physiological perturbations in the experimental animals and thus increase the accuracy of the results. This assay is sensitive enough to quantitate COC and its metabolites in plasma samples in

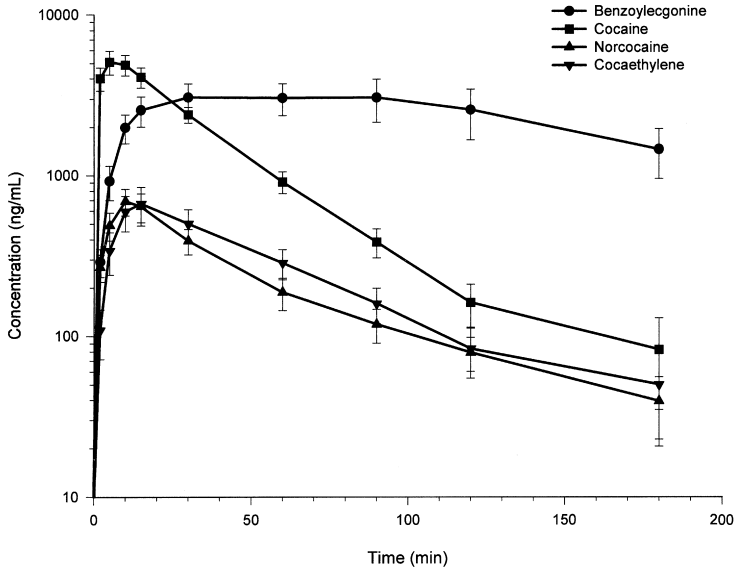

Fig. 2. Plasma concentration–time profile of cocaine and its metabolites in the rat after cocaine (30 mg/kg, i.p.) and alcohol (5 g/kg, p.o.) administrations. The graph represents data (mean±S.E.M.) from eight rats.

the range of concentrations often observed after administration of COC to humans and laboratory animals. The method we described has advantages compared to the previously published reports that utilized HPLC–UV as a means of separation and detection. It is a simple, rapid, sensitive, precise and specific assay suitable for studying the pharmacokinetics of COC and its major UV-detectable metabolites in rat plasma samples. This method will help significantly in exploring the pharmacokinetic and pharmacodynamic interactions between COC and alcohol and in studying COC metabolite pharmacokinetics.

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