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Determination of free-form of cocaine in rat brain by liquid chromatography–electrospray mass spectrometry with in vivo microdialysis

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Abstract

A rapid liquid chromatography–electrospray mass spectrometry (LC–ES-MS) method with in vivo microdialysis for the determination of free-form of cocaine (COC) in rat brain has been developed. A C_{18} column and a gradient elution were employed for the separation. The $[M+H]^+$ ($m/z=304$) and a fragmented ion ($m/z=182$) were detected using positive ion mode detection. Selective ion monitoring was utilized for quantitative measurement. The linearity of this assay was good ranging from 0.01 to 1.0 μM ($r^2=0.999$). The inter- and intra-day precisions showed relative standard deviations ranging from 1.0% to 3.3% and 1.0% to 3.6%, respectively. In addition, the detection of one COC metabolite, benzoylecgonine (BE), by this assay was also investigated. The linearity, precision, and detection limit associated with this method for BE were determined. The application of this newly developed method was demonstrated by examining the pharmacokinetics of COC in rat brain.

Keywords: Cocaine

1. Introduction

Cocaine (COC) has been used for medical purposes for a long time. However, the abuse of COC has become a serious problem in recent years. COC can easily penetrate the blood–brain barrier and block the reuptake process of cathecholamine neurons. Therefore, COC can enhance dopamine and norepinephrine neurotransmission and generate a euphoria effect. Monitoring the COC concentration and its metabolite in the extracellular space is an important step in understanding the psychological effect of COC. In the past, the effects of COC and its metabolites on the extracellular environment and its pharmacokinetics in the rat brain have been widely studied [1–3]. Two major metabolites of COC are benzoylecgonine (BE) and ecgonine methyl ester [4,5]. Since these two metabolites have not been shown to significantly affect dopamine reuptake, they have not received extensive studies in the past [4,6].

Several analytical methods utilizing gas chromatography (GC), liquid chromatography (LC), GC–mass spectrometry (MS) and LC–MS have been

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developed to evaluate the concentration of COC and its metabolites in biological fluid and in human hair [7–12]. However, derivatization prior to analysis is often needed for GC and GC–MS determination. LC with UV detection has been utilized to determine COC in rat brain; however, a method with better sensitivity is often needed for pharmacokinetic study. LC–electrospray (ES) MS has emerged as a sensitive and accurate analytical technique [13–17]. ES generates ions under atmospheric pressure and at relatively low temperature which minimizes thermal decomposition of labile compound. In addition, an aqueous sample can be analyzed with little or no sample preparation.

In vivo microdialysis has become a popular sampling technique for the study of drug distribution in the rat brain [18,19]. In biological fluids, cocaine exists as protein binding form and free form. There are two important characteristics for the free-form cocaine. First, it can easily transport in and out through cellular membranes. Secondly, it can bind onto their own binding sites (re-uptake transporters of dopaminergic nerves) and produce their drug's effect. The protein binding form cannot penetrate through the membrane into the microdialysis probe; therefore, only the free-form cocaine was sampled.

This paper describes an LC–ES-MS method for the determination of COC and BE in artificial cerebrospinal fluid (aCSF). The effects of modifier and flow-rate of mobile phase on the sensitivity of ES-MS will be presented. The linearity, detection limit and precision associated with this newly developed method will be discussed. In addition, this newly developed LC–ES-MS method with microdialysis was utilized to examine the concentration of COC and pharmacokinetics in rat brain.

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile (Malinckrodt Baker, Paris, KY, USA) and HPLC-grade water (Labscan, Dublin, Ireland) were used throughout the experiment. Sodium hydrogencarbonate, sodium chloride, magnesium chloride, potassium chloride, calcium chloride, ascorbic acid, glucose and acetic acid were purchased from Nacalai Tesque (Kyoto, Japan). Cocaine and sodium hydroxide were from Sigma (St. Louis, MO, USA). Benzoylecgonine was obtained from Radian International (Austin, TX, USA). Chloral hydrate was from Riedel-de Haen (Seelze, Germany).

2.2. HPLC system

A HP1100 LC system which consisted of a quaternary pump, an on-line degaser, an autosampler and a UV-visible detector (Hewlett-Packard, Palo Alto, CA, USA) was used. A LiChroCART RP-18e column (Purospher, 125×3 mm, 5 μm, Merck, Germany) with a LiChroCART 4-4 on-line guard column was used for separation. The mobile phase was acetonitrile containing 0.05% acetic acid (solution A) and 0.05% acetic acid (pH 3.55, solution B). The flow-rate was 0.5 ml/min. and the injection volume was 10 μl. The following gradient conditions were used: 0 min 15% of solution A and 85% of solution B and held for 4 min and a linear gradient from 15% A to 65% A within 6 min. After completing the chromatographic elution, the mobile phase was programmed to its initial condition within 2 min while an 8 min re-conditioning time was set before the next injection.

2.3. Mass spectrometry

A HP-5988B mass spectrometer with a HP-59987A electrospray interface (Hewlett-Packard) was used. HP Chemstation (G1034C, version C.03.00) was utilized for system control, data acquisition and data analysis. Heated N_2 gas (350°C, 12.5 l/min) was used to evaporate the solvent from the electrospray chamber and compressed N_2 gas (80 p.s.i.) was used for nebulization (1 p.s.i.=6894.76 Pa). The cylinder electrode in the electrospray chamber was set at −6000 V. The end plate and capillary entrance voltages were set at −3500 V and −4000 V, respectively. The voltages of skimmer 1, lens 1, skimmer 2, lens 2 and lens 3 were set at 31.0 V, −1.6 V, 9.6 V, 10.8 V and −76 V, respectively. The mass spectrometer was tuned with the procedures provided by Hewlett-Packard [20]. The tuning mixture consisting of valine (m/z=118), trityrosine (m/z=508) and hexatyrosin (m/z=997)

was obtained from the same company. The collision-induced dissociation (CID) voltage was set at 125 V. The mass spectrometer was operated in the positive ion mode and mass spectra collected in scan mode were obtained by scanning from 50 to 800 in 0.5 s. Nine scans were averaged with a step size of 0.1 over the range.

2.4. Standard solution

For the examinations of chromatography elution conditions, linearity, and detection limit associated with this method, 1 m*M* of COC in aCSF was prepared and stored at 4°C in the dark. This stock solution was prepared weekly and the working solutions were diluted with aCSF to appropriate concentration daily. For inter- and intra-day studies, the stock and working solution were prepared daily. For the quantitative analysis of microdialysis samples, standard solutions were prepared from the solution that was used to inject into rats.

2.5. Animals and surgeries

Adult male Sprague–Dawley rats weighing 250±20 g on arrival were supplied by the Animal Center of National Yang-Ming University (Taipei, Taiwan). They were housed in a 12 h light–dark cycle room with free access to food and water. On the experimental day, a rat was first anesthetized with 400 mg/kg intraperitoneal (i.p.) chloral hydrate and then placed on a stereotaxic apparatus (Koff Models 1430 and 1460). Anesthesia was maintained with hourly 0.1 ml i.p. injections of 200 mg/ml chloral hydrate. Body temperature was maintained throughout the experiment with a 37°C heating pad. Thereafter, a laboratory-made microdialysis probe (active length 4 mm) was lowered into the medial prefrontal cortex (mPFC) of the rat. The coordinate used, from bregma, were +3.1 AP, +0.8 ML, and −0.2 V below the skull.

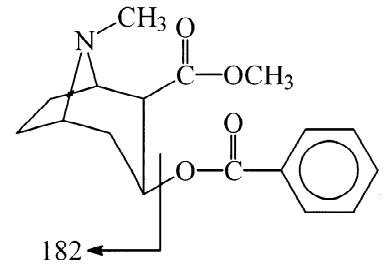
2.6. Microdialysis

After insertion of the dialysis probe, it was perfused with aCSF using a microliter syringe pump (Model 22, Harvard Apparatus, S. Natick, MA, USA) at a flow-rate of 1.19 μl/min. The aCSF is composed of 0.13 *M* sodium chloride, 0.98 m*M* magnesium chloride, 2.65 m*M* potassium chloride, 1.2 ml calcium chloride, 0.25 m*M* ascorbic acid, and 10 m*M* glucose. The solution of aCSF was adjusted to pH 7.2 to 7.4 with 0.1 *M* sodium hydroxide. After 2 h of stabilization, the rat received a single bolus COC injection (30 mg/kg i.p.). The dialysis samples were collected in a 200-μl Eppendorf tube at 20 min intervals for 160 min. The collected samples were wrapped with aluminum foil and stored at −20°C in a refrigerator prior to LC–ES-MS analysis.

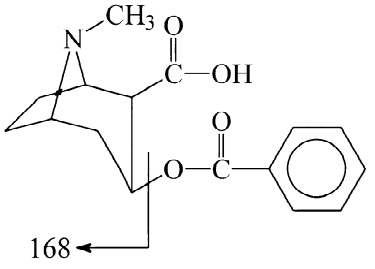
3. Result and discussion

The separation of COC and BE was performed using a C_{18} reversed-phase column with gradient elution. Acetic acid was added to the mobile phase to improve the separation results. Various amounts of acetic acid (0.01%, 0.05% and 0.1%, v/v) were added to the mobile phase to examine the effect of LC separation. For BE and COC, the retention factor increased as the amount of acetic acid increased from 0.01% to 0.05%. The symmetries of both BE and COC peaks deteriorated greatly when the amount of acetic acid increased from 0.05% to 0.1%. This might be attributed to the protonation of the weak basic tertiary amines in both COC and BE when the amount of acid in the mobile phase was increased. The interaction between the silanol group and protonated amines caused the band broadening. Therefore, we added 0.05% of acetic acid to the mobile phase for LC separation.

ES is a soft ionization technique with little fragmentation of molecules; however, CID has been used to promote molecular fragmentation [21,22]. CID occurs in the space between the capillary exit and the skimmer in the electrospray source. The ions accelerate and collide with the drying gas molecules and fragmented ions are generated when the difference between the voltage applied to capillary exit and that applied to the skimmer is sufficient [23]. This work investigated the effect of CID voltage on the fragmentation of COC and BE. The structures of COC, BE and proposed fragmented ions are shown in Fig. 1. For COC, two major ions (m/z=M+H and 182) were determined. The ion intensities of $[M+H]^+$ ions increased greatly when the voltage increased



Cocaine (mol. wt. = 303)



Benzoylecgonine (mol. wt. = 289)

Fig. 1. Molecular structures of cocaine and benzoylecgonine.

from 25 to 100 V. When the voltage was increased from 100 to 175 V, the abundance of fragmented ion (m/z=182) increased while the abundance of $[M+H]^+$ diminished. However, the intensities of both ions decreased significantly when the voltage was higher than 175 V. For BE, three major ions (m/z= M+Na, M+H and 168) were also detected. A similar effect of CID voltage on the fragmentation of the BE molecule was observed. In this study, CID voltage was set at 125 V and the mass spectra of COC and BE are shown in Fig. 2.

The addition of acetic acid to the mobile phase also has a profound effect on the detection sensitivity of ES-MS. For BE, the intensities of $[M+H]^+$ and fragmented ion (m/z=168) increased as the amount of acetic acid increased from 0 to 0.1%. This might be attributed to the higher degree of protonation of the BE molecule at higher concentration of acid in mobile phase. Surprisingly, the reverse trend was observed in COC. In order to achieve good LC separation and adequate ES-MS sensitivity, 0.05% of acetic acid was added to the mobile phase for this study.

The LC–ES-MS chromatogram of BE and COC in aCSF solution is shown in Fig. 3a. BE and COC were eluted at approximately 4.8 and 8.3 min, respectively. Although some substances were detected in the microdialysate of rat brain, none of them interfered with COC or BE. Quantitative results were obtained in the selective ion monitoring (SIM) mode. The linearity of this newly developed method was evaluated by analyzing a series of COC and BE standards. Good linearities (r^2=0.999) for COC and BE were determined from 0.01 to 1.0 μ*M* and 0.35 to 35 μ*M*, respectively. The detection limits based on a signal-to-noise ratio of 3 were about 5 n*M* for COC and 35 n*M* for BE. Compared with previously published reports, the detection limit of this newly developed method is approximately 100 times better [24,25].

The inter- and intra-day precisions of this method were evaluated by replicated analysis of COC and BE spiked samples. Calibration standards were prepared and analyzed each day. A total of three series of samples were analyzed over a week-long period and each sample was measured in triplicate. The results of precision study are summarized in Tables 1 and 2. For COC, the intra- and inter-day precisions showed relative standard deviations (RSDs) ranging from 3.6 to 1.0% and 3.3 to 1.0%, respectively. Acceptable accuracy ranging from 98.0 to 120.0% was obtained. For BE, a small increase in both intra- and inter-day precisions was observed; nevertheless, comparative accuracy was detected.

The application of this newly developed LC–ES-MS method was demonstrated by evaluating COC concentration in the mPFC region of rat brain. The mPFC is a terminal region of the mesocortical dopaminergic pathway. This region has been recognized to correlate to depression, cocaine addiction and psychosis. Therefore, study of cocaine pharmacokinetics in the mPFC is very important for understanding its effect in the brain. The LC–ES-MS chromatogram of rat brain microdialysate after COC was administered is shown in Fig. 3c. No BE is detected in microdialysate after COC injection. About 80–90% of cocaine was hydrolyzed to ecgonine methyl ester (EME) by esterase enzyme in plasma [26]. In addition, the hydrolysis of cocaine to BE is a non-enzymatic process [27]. Then, it most likely takes place in the brain. Therefore, monitoring

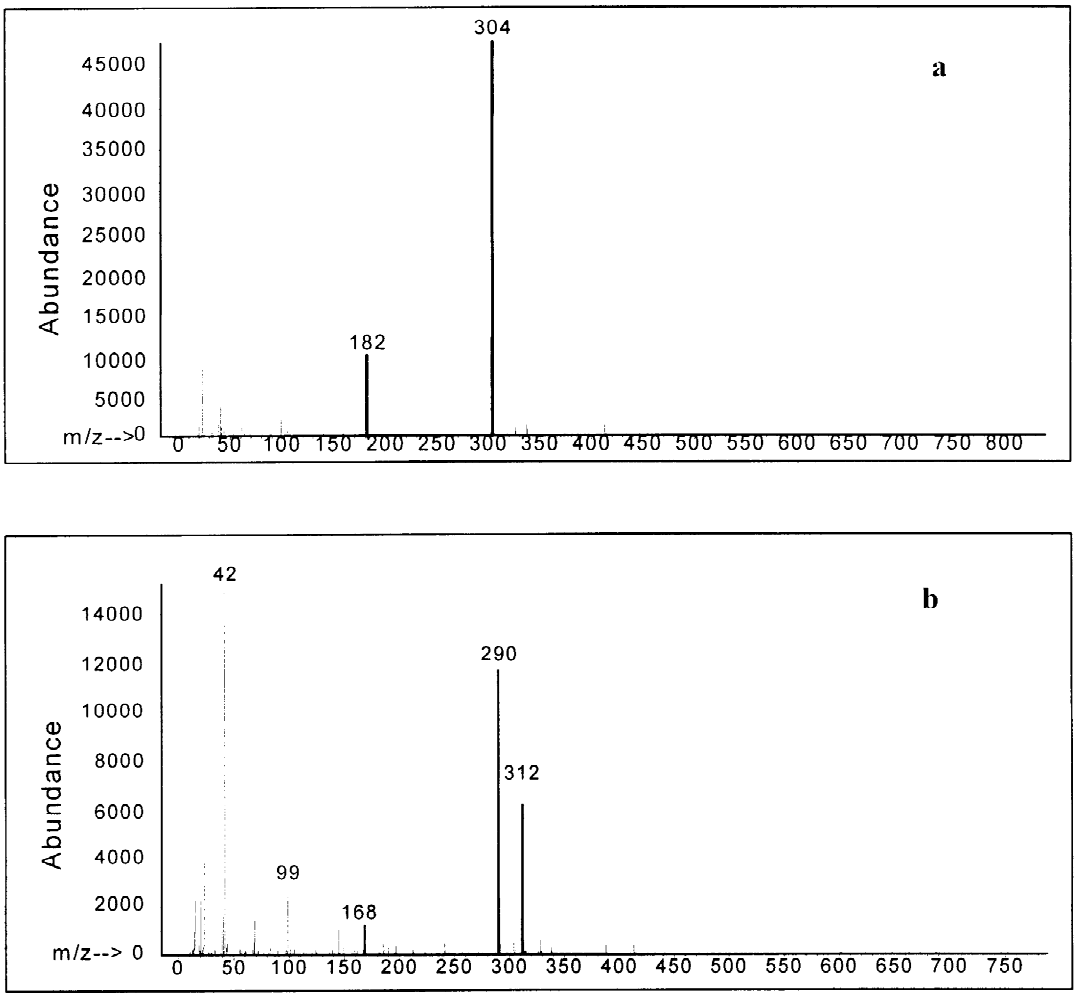


Fig. 2. Positive electrospray ionization (CID voltage: 125 V) mass spectra of cocaine and benzoylecgonine. (a) Cocaine, (b) benzoylecgonine.

BE concentration in the brain is more important to understand the metabolism route of cocaine in the brain. Only when concentration of cocaine is higher than 1000 ng/ml, was it hydrolyzed directly to BE [26]. Therefore, the concentration of BE in the mPFC after 30 mg/kg i.p. was lower than our quantitative limit (0.35 μM) of BE. The change of extracellular COC concentration in the mPFC over time (n=5) is shown in Fig. 4. COC concentration quickly increased in the mPFC after 30 mg/kg COC i.p. administration. It reached a maximum concentration of 0.8±0.05 μM during the 20–40 min collection interval after drug administration. A non-linear curve-fitting computer program, Minsq, written by MicroMath Scientific Software (Salt Lake City, UT, USA) was used to fit an equation consisting of the difference of two first-order kinetic processes for the appearance and disappearance of COC in the brain to experimental COC data:

$$\mathrm{COC} = A \cdot [\exp(-k_1 t) - \exp(-k_2 t)],$$

where $A = [k_1/(k_1 + k_2)]C_0$

Three parameters fit into the data: A, k_1, and k_2. A is a concentration and absorption efficiency factor, k_1 is the first-order rate constant for the appearance of

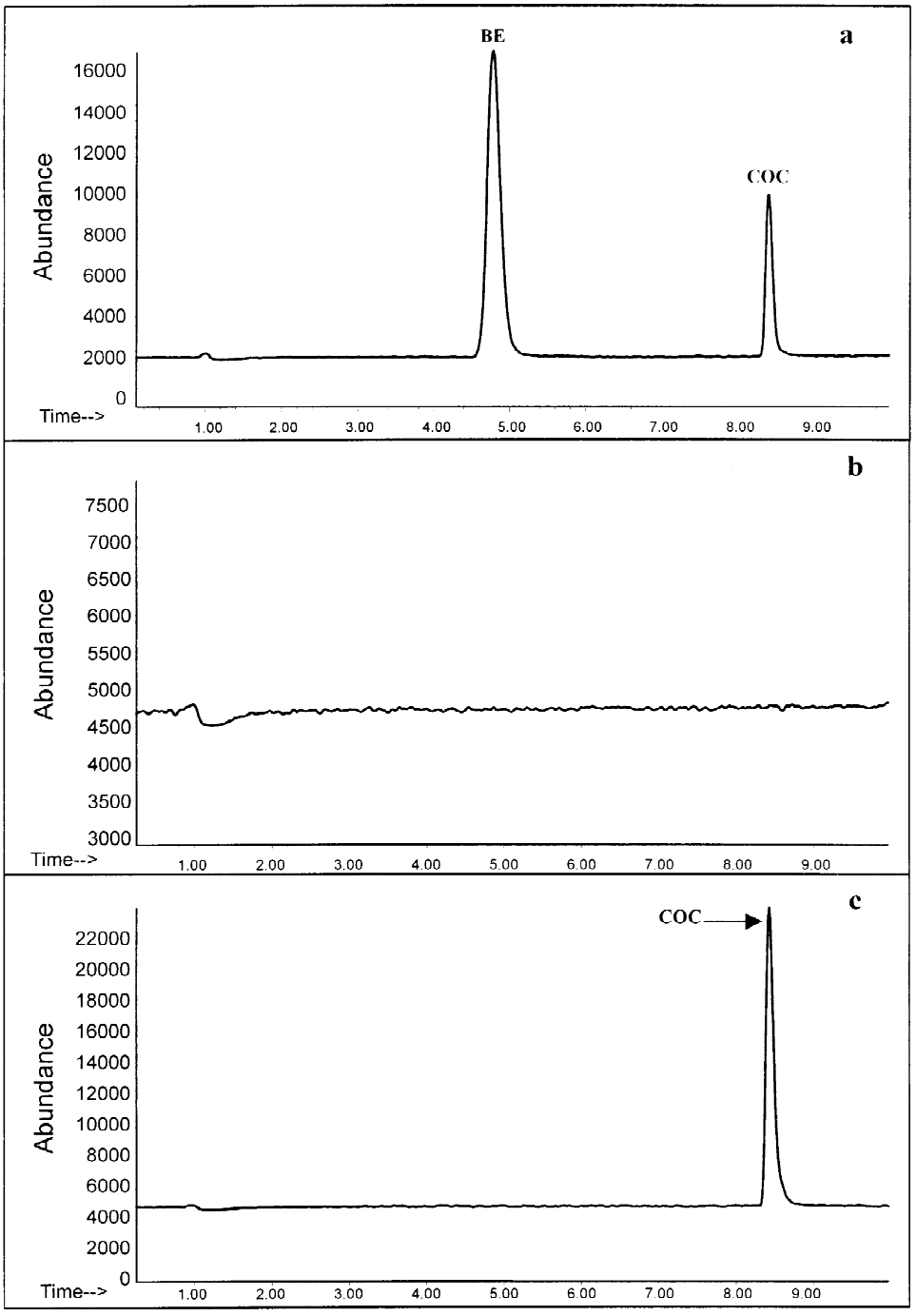


Fig. 3. Reconstructed ion chromatogram [m/z=290 (289.70 to 290.70) and 304 (303.70 to 304.70)]. (a) Standard (BE=5 μ*M*, COC=0.5 μ*M*), (b) rat brain microdialysate before COC injection, (c) rat brain microdialysate after COC administration.

Table 1
Intra- and inter-day precision and accuracy of cocaine in aCSF

Added concentration (μM)		Intra-day		Inter-day	
		Found concentration (μM)	Accuracy (%)	Found concentration (μM)	Accuracy (%)
0.01	Mean	0.01	100.0	0.01	100.0
	SD	0.0001		0.0001	
	RSD (%)	1.0		1.0	
0.05	Mean	0.06	120.0	0.06	120.0
	SD	0.001		0.002	
	RSD (%)	1.6		3.3	
0.10	Mean	0.11	110.0	0.11	110.0
	SD	0.004		0.003	
	RSD (%)	3.6		2.7	
0.50	Mean	0.52	104.0	0.53	106.0
	SD	0.012		0.016	
	RSD (%)	2.3		3.0	
1.00	Mean	0.98	98.0	0.98	98.0
	SD	0.015		0.001	
	RSD (%)	1.5		0.1	

COC, and k_2 is the first-order rate constant for the disappearance of COC. Both rate constants have the units of min^{-1}. After this non-linear fit, k_1 was 0.0181 ± 0.0003 and k_2 was 0.0263 ± 0.0004. The value obtained for the concentration factor, A, was 5.92 ± 0.54 μM.

In summary, the results of the work described here demonstrate the potential of LC–ES-MS with mi-

Table 2
Intra- and inter-day precision and accuracy of benzoylecgonine in aCSF

Added concentration (μM)		Intra-day		Inter-day	
		Found concentration (μM)	Accuracy (%)	Found concentration (μM)	Accuracy (%)
0.35	Mean	0.37	108.8	0.39	114.7
	SD	0.024		0.018	
	RSD (%)	6.4		4.6	
1.73	Mean	1.87	108.0	1.95	112.7
	SD	0.077		0.090	
	RSD (%)	4.1		4.6	
6.92	Mean	6.98	100.8	6.82	98.5
	SD	0.161		0.145	
	RSD (%)	2.3		2.1	
17.30	Mean	17.72	102.4	17.56	101.5
	SD	0.35		0.36	
	RSD (%)	2.0		2.1	

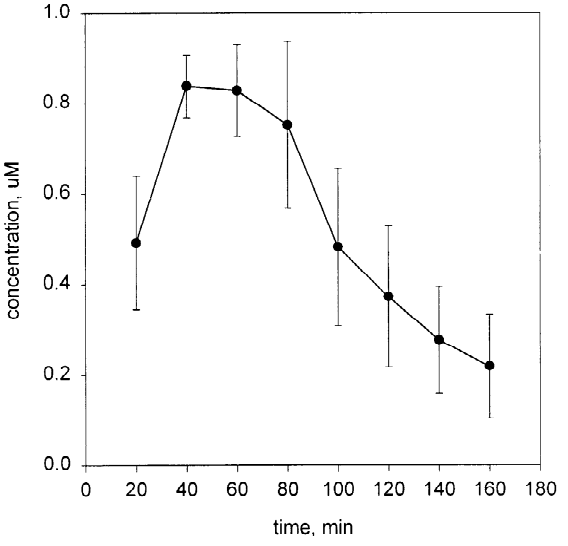


Fig. 4. Time course of COC in rat brain ($n=5$) following a 30 mg/kg i.p. injection.

crodialysis for the determination of COC in rat brain. Linearities of COC and BE ranged from 0.01 to 1.0 μM and 0.35 to 35 μM, respectively. Good precision and accuracy were determined. The newly developed LC–ES-MS method was coupled with microdialysis to measure the concentration of free-form of cocaine in rat brain. In addition, the pharmacokinetics of cocaine in rat brain were evaluated.

Acknowledgements

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