Simultaneous analysis of cocaine and its metabolites in rat liver microsome s_9 fraction by liquid chromatography-mass spectrometry

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Abstract: A method utilizing liquid chromatography-mass spectrometry for the simultaneous analysis of cocaine and its in-vitro metabolites in rat liver micro some S_9 fraction is described. Cocaine was incubated with rat liver micro some S_9 fraction firstly, and then the incubation sample mixture was analyzed by liquid chromatography-mass spectrometry in full-scan mode. The main metabolites of cocaine were identified from the determination of molecular ions and the results of tandem mass spectrometry analysis. There were two metabolites were identified in the rat liver S_9 fractions referring to the literature reports.

Keywords: liquid chromatography-mass spectrometry; liver microsome; cocaine; metabolism.

INTRODUCTION

Liquid chromatography-mass spectrometry (LC-MS) has been widely applied in the analysis of drugs and their metabolites (Xiang *et al*, 2009). The approach has shown higher sensitivity and superior selectivity for the analysis of thermo-labile, highly polar and non-volatile compounds compared to other methods such as high-performance liquid chromatography (HPLC), gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The utilizing of electro spray ion trap mass spectrometry (ESI-ITMS) technique in LC-MS can acquire rich structural information of the analyses (Duan *et al*, 2013). Because drug metabolites often keep the core structure of parent drug after biotrans formation, the fragmentation of parent drug obtained by multi-stage tandem mass spectrometric techniques (MS^n) can be used for the identification of metabolites. Consequently, LC-ESI-ITMS has also been widely using in the metabolism analysis of many drugs (Cai *et al*, 2002).

As an alkaloid being contained in the leaves of *Erythroxylon coca* Lam. or *Erythroxylon novogranatense* (Morris) Hieronymus of Erythroxylaceae plants, cocaine is being used as a local anaesthetic. It also shows stimulating action on the central nervous system, when it is used continuously, psychic dependence on its use appears, resulting in its chronic toxicosis. Cocaine is being abused worldwide and is the second most widespread illicit drug in western countries at present (Nadia *et al*, 2012). Many analytical strategies have been developed for the determination of cocaine in plants, pharmaceutical samples and biomaterials, including GC (Wang *et al*, 2010), GC-MS (Marchei *et al*, 2008), HPLC (Sun *et al*, 2002) and LC-MS (Jagerdeo *et al*, 2008).

In the present study, a specific and sensitive method applying LC-MS was developed and utilized for the simultaneous analysis of cocaine and two metabolites in rat liver S_9 fraction after *in vitro* metabolism study.

MATERIAL AND METHODS

Chemicals and reagent

Chromatography grade methanol, acetonitrile and analytical grade acetic acid were purchased from Shield Co., Ltd (Tianjin, China). Ammonium acetate was purchased from Dima Technology Inc. (USA). Cocaine was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). β-Nicotinamide-adenine dinucleotide phosphate reduced form (β-NADPH) was obtained from iPhase Pharma Services (Beijing, China). Rat liver S_9 fraction was prepared from male Sprague Dawley (S.D.) rats (about 8 weeks old) that were purchased from Laboratory Animal Services Center, Liaoning University of Traditional Chinese Medicine. The protein concentration of rat liver S_9 fraction was determined by Bradford method and the concentration of micro some was determined by Omura method.

In vitro incubation and sample extraction

The procedure of *in vitro* incubation of sample has been described in details by Zeng (2006) and it was applied in our work as following: stock solution of cocaine was prepared in water at a concentration of 50.0μg/mL. 20μL of stock solutions were added to 200μL SD rat liver micro some S_9 fraction solution containing 2.0mg of micro somal protein. The mixture was shaken 5 min for equilibration in a water bath at 37°C and then the incubation was initiated by adding NADPH in the mixture. The final concentrations of test compound, NADPH and micro somal protein were 5.0μg/mL, 1.0 mmol/L and 1.0mg/mL, respectively. The reaction was quenched by adding 150μL of acetonitrile after incubation for 120min. The samples were then vortex-mixed, placed

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in ice for 5 min and centrifuged at 14000 rpm for 20 min. The supernatant was pooled together and evaporated to dry at 37°C under nitrogen stream. The residue was reconstituted in 50µL of aconitrile, centrifuged at 14000 rpm for 20min. An aliquot of 10µL of the supernatant was used for LC-MS^n analysis.

LC/MS^n analysis

Finnigan Surveyor liquid chromatography (San Jose, CA, USA) equipped with a Waters X-terra MS-C_8 reversed-phase column (100 × 2.1mm, 3.5µm) was used for the LC separation. The mobile phase consisted of phase A (water, 10 mM ammonium acetate, adjusted to pH 4.0 with formic acid) and B (methanol). The gradient program started with 5% B and held for 1 min, then increased to 90% B within 5min, and held for 5 min. The flow rate was 0.2mL/min. The effluent in the first 1 min was diverted to waste to minimize the contamination of the ion source.

ESI-MS analysis was performed on a LXQ ion trap mass spectrometer (Thermo Fisher, USA). Positive ion mode was used for the determination of cocaine and its metabolites. The MS parameters were optimized by direct infusion of 5.0µg/mL cocaine in acetonitrile at the flow rate of 10.0µL/min. Capillary temp was 350°C. The flow rates of sheath gas, aux gas and sweep gas were 30.00, 8.00 and 2.00, respectively. The voltages of source, capillary and tube lens were 5.00kv, 1.00v and 5.00v, respectively.

RESULTS

Optimization of LC-MS conditions

Methanol and water with 0.1% acetic acid and 10mM ammonium acetate were selected as the mobile phases for the LC-ESI-ITMS analysis of cocaine according to the reference (Johansen *et al*., 2007; Jiang *et al*., 2005). Solvent gradient-elution program was established by comparing the peak resolution of cocaine obtained from different gradient-elution modes. The $[M+H]^+$ ion of cocaine at m/z 304 was chosen as parent ion for the fragmentation in MS/MS mode. The base daughter ion was m/z 182 in MS/MS spectrum. Under the optimized gradient-elution conditions, the retention times of cocaine was 8.30 min (fig. 1).

Method validation

LC-MS/MS was used for the quantitative analysis of cocaine in rat liver tissue sample. The base fragment ion at m/z 182 was chosen for the quantitative analysis ion, and the MS/MS spectrum of cocaine was shown in fig. 2B. Selectivity of the method was tested by comparing the chromatogram of blank sample extract of rat liver S_9 fraction with the corresponding spiked extract of cocaine. The obtained result showed that there was no background interference to the analysis of the compound under the optimal LC-MS/MS conditions. Calibration linearity was investigated by analyzing the spiked S_9 fraction extract standards with different concentrations for cocaine. The calibration curve was obtained by plotting the peak-area against the spiked concentrations of cocaine. Good linearity was obtained in the ranges of 0.025µg/mL to 5.0 µg/mL for cocaine with r^2=0.9999 and the linearity equation was Y=169851X-1846.67. In order to estimate the limit of detection (LOD), spiked samples at different concentrations were analyzed. The LODs developed in the present work was calculated as 0.006µg/mL for cocaine on the basis of the chromatographic peak for which the signal-to-noise ratio was 3 (S/N=3). The limits of quantitative (LOQ) was calculated as 0.018µg/mL based on signal to noise ratio of chromatographic peak equal 10 (S/N=10). The recovery test was also processed with spiking 0.18µg/mL and 1.8µg/mL of cocaine solution in rat liver micro some S_9 fraction and the mean recovery was 88.3% (n = 5).

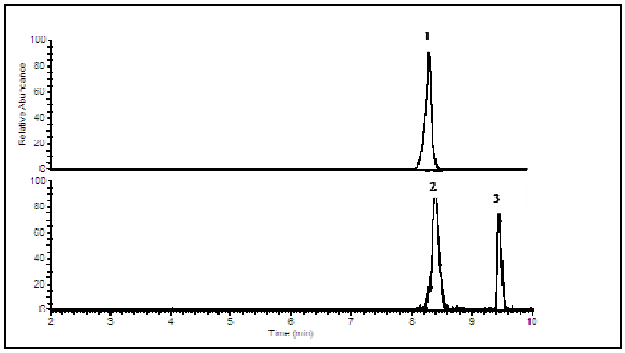

Fig. 1: LC-MS chromatograms of cocaine and its metabolites under the optimized gradient-elution conditions Peaks identification: 1. Cocaine; 2. nor cocaine; 3. benzoylecgonine.

In vitro metabolism study

An *in vitro* metabolism experiment of cocaine was conducted with rat liver micro some S_9 fraction. Metabolic stability was determined at the incubation time of 120 min based on the comparison of peak intensities of cocaine at the incubation time of 120min to that at 0 min (Cai *et al* 2000). The metabolic rate was 83.4%, indicating that cocaine could be easily metabolized in the rat liver S_9 fraction, which agreed with the results reported previously (Sara *et al*, 2011). Therefore, not only the detection of cocaine is important, attention should also be paid on the examination of metabolites in the case of drug poisoning.

DISCUSSION

Because full-scan mass spectra were acquired during the quantitative analysis for metabolic stability, potential metabolites of drugs could be simultaneously detected without the need for sample reanalysis (Lu *et al*, 1995). Extracted ion peaks of molecular ions of the predicted

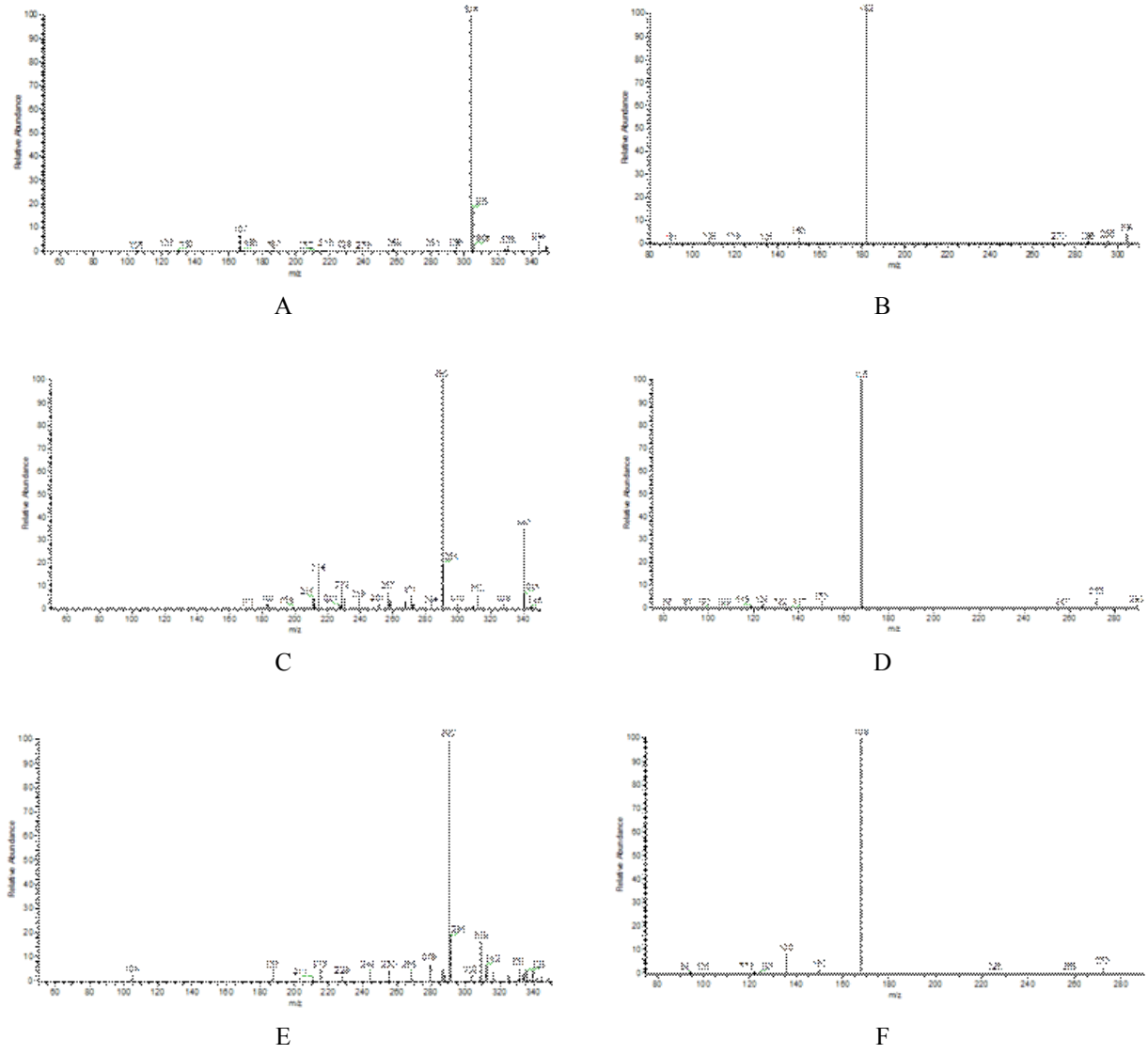

Fig. 2: MS and MS/MS spectrums of cocaine and its metabolites

(A) MS spectrum of cocaine; (B) MS/MS spectrum of cocaine; (C) MS spectrum of Nor cocaine; (D) MS/MS spectrum of nor cocaine; (E) MS spectrum of benzoylecgonine; (F) MS/MS spectrum of benzoylecgonine

metabolites in the incubated samples were examined, followed by the investigation on the full-scan mass spectra. Metabolite was detected if the mass chromatographic peak of an expected $[M+H]^+$ ion was observed in incubated sample but not in the corresponding control sample. Two metabolites were detected both at m/z 290 in the incubated sample of cocaine with different retention time. Interpretation of ESI mass spectra of the detected metabolite indicated that the detected ions represented singly charged molecular ions. The corresponding ion peaks were not observed from the analysis of the control samples, indicating that the detected components in the 120min sample were not resulted from background materials. In order to confirm the metabolite identification, LC-MS^n analysis were performed on the detected ions. The LC-MS^n product ion spectra of metabolites, as shown in fig. 2C, fig. 2D, fig. 2E and fig. 2F, were referenced to the corresponding spectra obtained from the analysis of cocaine standards. fig. 2C shows that the $[M+H]^+$ ion at m/z 290 and retention time with 8.38 min shows a product ion at m/z 168, resulting from the loss of the methyl group, so the metabolite was identified as nor cocaine, which has been identified in the metabolism of cocaine reported in literature (Lu *et al*, 1995). In the same way another metabolite with $[M+H]^+$ ion at m/z 290 and retention time

with 9.46 min shows a product ion at m/z 168 and m/z 136 as shown in fig. 2F, resulting from the loss of the methyl group, so it was identified as benzoylecgonine based on the report in literatures (Johansen *et al*, 2007).

CONCLUSIONS

The described LC-MS method provided simultaneous determination of cocaine as well as its major metabolites in rat liver micro some S_9 fraction. LC-MS^n analysis was used for the identification of the metabolites nor cocaine and benzoylecgonine, the use of LC-MS technique permitted rapid and direct detection of metabolites when the samples were analyzed for the quantification of the parent compound. Because many toxic cases of cocaine poison may be caused by oral intake of plant medicines containing the drugs, the simultaneous detection of the parent alkaloids and their metabolites may be useful for the simulation of actual poison case. Therefore, this method can be applied for the rapid identification of poisonous alkaloids and their metabolites in forensic and clinically toxicological relevant cases.

REFERENCES

Cai ZW, Lee FSC, Wang XR and Yu WJ (2002). A capsule review of recent studies on the application of mass spectrometry in the analysis of Chinese medicinal herbs. *J. Mass Spectrom.,* **37**: 1013-1024.

Cai ZW, Sinhababu AK, Harrelson S (2000). Simultaneous quantitative cassette analysis of drugs and detection of their metabolites by high performance liquid chromatography/ion trap mass spectrometry. Rapid. *Commun. Mass Spectrom.,* **14**: 1637-1643.

Castiglioni S, Bagnati R, Melis M, Panawennage D, Chiarelli P, Fanelli R and Zuccato E (2011). Identification of cocaine and its metabolites in urban wastewater and comparison with the human excretion profile in urine. *Water Res.,* **45**: 5141-5150.

Duan JP, Zeng YT, Zhou T, Zhang Y and Xu F (2013). Study on the Pharmacokinetics of tacrolimus in depression model rats. *Int. J. Pharm.,* **9**(4): 265-270.

Jagerdeo E, Montgomery MA, Lebeau MA and Sibum M (2008) An automated SPE/LC/MS/MS method for the analysis of cocaine and metabolites in whole blood. *J. Chromatogr. B.,* **874**: 15-20.

Jiang HP (2005). Study on determining the cocaine and benzyolecgonine in the urine. *J. Sichuan Univ. (Natural Science Edit ion),* **42**(4): 794-797.

Johansen SS and Bhatia HM (2007). Quantitative analysis of cocaine and its metabolites in whole blood and urine by high-performance liquid chromatography coupled with tandem mass spectrometry. *J. Chromatogr. B.,* **852**: 338-344.

Lu HM, Lu FQ and Tan JY (2005). Toxicological analysis. Policeman Education Press, Beijing, China, pp.479-485.

Marchei E, Colone P, Nastasi GG, Calabr`o C, Pellegrini M, Pacifici R, Zuccaro P and Pichini S (2008). On-site screening and GC-MS analysis of cocaine and heroin metabolites in body-packers urine. *J. Pharma. Biomed. Anal.,* **48**: 383-387.

Nadia DG and Daniela M (2012). Cocaine and its metabolites in the placenta: A systematic review of the literature. *Reprod. Toxicol.,* **33**: 1-14.

Sun L, Song J, He W and Ren JQ (2002). Determination of cocaine in rat's serum by high performance liquid chromatography. *Chin. J. Forensic Med.,* **17**(3): 165-166.

Wang XB, Ye NS, Wang JF and Gu XX (2010). Simultaneous determination of cocaine and its metabolite ecgoninemethylester in human blood using microwave extraction-gas chromatography. *Chin. J. Chromatogr.,* **28**(7): 673-767.

Xiang P, Shen M and Zhuo XY (2009). Application of LC-MS in the analysis of medicine and abused drugs. Shanghai Science and Technology Press, Shanghai, China, pp.182-188.

Zeng S (2006). Drug metabolism. Zhejiang University Education Press, Hangzhou, China, pp247-250.