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

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Abstract

An ion-pair liquid chromatography–electrospray mass spectrometry (LC–ESI-MS) method with in vivo microdialysis for the determination of free-form amphetamine in rat brain has been developed. A microdialysis probe was surgically implanted into the striatum of the rat and artificial cerebrospinal fluid (aCSF) was used as the perfusion medium. Samples were collected and then analyzed off-line by LC–ESI-MS. A reversed phase C_{18} column was employed for LC separation. Trifluoroacetic acid (TFA) was added in the mobile phase (acetonitrile–water, 10:90, v/v) as an ion-pair reagent. The ion-pair process disguises the protonated amphetamine cations from the ESI-MS electric field as neutral molecules. Post-column addition of volatile organic acid was utilized to minimize TFA signal suppression effect on ESI-MS detection. More than six-fold enhancement of ESI-MS response was achieved by the post-column addition of propionic acid. Good linearity (0.01–1.00 μg/ml, $r^2 = 0.99$) and detection limit (0.002 μg/ml) were determined. Good precision and accuracy were obtained. The applicability of this newly developed method was demonstrated by continuous monitoring of amphetamine concentrations in rat brain after a single 3.0 mg/kg i.p. administration.

Keywords: Microdialysis; Amphetamine; Amines

1. Introduction

The number of illegal amphetamine users has increased dramatically in Taiwan. Understanding the pharmacokinetics of amphetamine in the key nuclei (e.g. medial prefrontal cortex, mPFC) is of interest to all pharmacologists. This research topic has been investigated for many years; however, it still is not fully understood. As we know, only the free-form amphetamine can have the pharmacological actions in the brain. This is due to the fact that free-form amphetamine can pass through capillary endothelial membrane, reach the target sites, bind to its receptors and finally have the central stimulating effect [1]. However, the protein-bound amphetamine molecules are too large to permeate the membrane. Therefore, monitoring free-form amphetamine in the extracellular space is a preferable approach for this study.

Microdialysis has become a widely accepted sampling device for monitoring drug concentrations in rat brain and blood [2,3]. This method avoids exposure of the brain tissue to the perfusion medium and therefore minimizes tissue damage. The dialysis membrane, with a molecular mass cut-off ranging from 5000 to 50,000 based on the necessity of this study, eliminates the need of sample clean-up procedures before analytical quantitation. In addition, samples will not be subjected to further metabolism after collection since the analyte can be separated from enzymes by dialysis membrane. Thus, in vivo microdialysis technique is a good tool to sample the free-form amphetamine in biological fluids.

Several gas chromatography (GC) and GC–mass spectrometry (MS) methods have been developed for the analysis of amphetamine in human urine and plasma [4–6]. For these GC and GC–MS methods, an extraction procedure is needed to transfer amphetamine into an organic solvent for GC or GC–MS analysis. However, the relatively small amount of microdialysis sample makes it difficult to apply extraction procedure to convert amphetamine into an

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organic solvent for GC or GC–MS analysis. HPLC is an alternative for the determination of amphetamine in biological fluid. Amphetamine was measured directly or after derivatization [7–12]. Recently, capillary electrophoresis has been utilized to analyze amphetamine in urine and serum samples [13–16]. liquid chromatography–electrospray mass spectrometry (LC–ESI-MS) has emerged as a sensitive analytical technique for the determination of various compounds [17–21]. The aim of this investigation is to develop a LC–ESI-MS method to determine amphetamine in artificial cerebrospinal fluid (aSCF) and to incorporate with microdialysis technique to examine the pharmacokinetics of free-form amphetamine in rat brain.

2. Experiment

2.1. Chemicals

Purified water (>18 MΩ) from a NANOPure water purification system (Barnstead, Dubuque, IA, USA) and HPLC-grade acetonitrile (Malinckrodt Baker, Paris, KY, USA) were used throughout. Sodium hydroxide, sodium chloride and amphetamine sulfate were purchased from Sigma (St. Louis, MO, USA). Sodium bicarbonate, magnesium chloride, potassium chloride, calcium chloride, ascorbic acid, formic acid, acetic acid and glucose were purchased from Nakarai Chemical (Tokyo, Japan). Propionic acid and trifluoroacetic acid were purchased from Riedel-de Haen, Germany. Chloral hydrate was obtained from Veterans General Hospital, Taipei, Taiwan.

2.2. 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 i.p. chloral hydrate. Then, the rat was placed on a stereotaxic apparatus (Koff models 1430 and 1460). Anesthesia was maintained with hourly 0.1 ml i.p. injection 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 coordinates used, from bregma, were +3.1 AP, +0.8 ML, and −0.2 V below the skull.

2.3. Microdialysis

After insertion, the microdialysis probe 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 mM magnesium chloride, 2.65 mM potassium chloride, 1.2 ml calcium chloride, 0.25 mM ascorbic acid, and 10 mM glucose. The solution of aCSF was adjusted to a pH of 7.2–7.4 with 0.1 M sodium hydroxide. After 2 h of stabilization, the rat received a single bolus amphetamine injection (3.0 mg/kg i.p.). The dialysis samples were collected in a 200 μl eppendroff tube at 20 min intervals for 6 h. The collected samples were wrapped with aluminum foil and stored in a 0 °C refrigerator prior to analysis.

2.4. HPLC system

An HP1100 LC system which consisted of a quaternary pump, an on-line degaser, an autosampler and a UV-Vis detector (Hewlett-Packard Co., Palo Alto, CA, USA) was used. A SymmetryShield RP18 column (2.1 mm × 150 mm, 5 μm, Waters, Milford, MA, USA) with a SymmetryShield guard column was used for LC separation. A mixture of acetonitrile–water (70:30, v/v) with 0.05% trifluoroacetic acid was used as a mobile phase. The flow rate was set at 200 μl/min. The mixture was filtered through a 0.45 μm membrane (FP-450, Gelman Science, MI, USA) and sparged with helium gas for 30 min prior to use. The injection volume was 15 μl. Post-column addition of propionic acid (25 μl/min) was achieved by a polyether ether ketone (PEEK) tee (Upchurch Scientific, Oak Harbor, WA, USA) and a syringe pump (Harvard Apparatus, Holliston, MA, USA).

2.5. Mass spectrometry

An HP-5988B mass spectrometer with a HP-59987A electrospray interface (Hewlett-Packard, Palo Alto, CA, USA) was used. An 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 solvent from the electrospray chamber and compressed N_2 gas (80 psi; 1 psi = 6894.76 Pa) was used for nebulization. The cylinder electrode in the electrospray chamber was set at −6000 V. The end plate and capillary entrance voltage were set at −3500 and −4000 V. The voltage of skimmer 1, lens 1, skimmer 2, lens 2 and lens 3 were set at 31.0, −1.6, 9.6, 10.8 and −76 V, respectively. The mass spectrometer was tuned with the procedures provided by Hewlett-Packard [21]. The tuning mixture consisting of valine (*m*/*z* 118), tri-tyrosine (*m*/*z* 508) and hexatyrosin (*m*/*z* 997) was obtained from the same company. The collision-induced dissociation (CID) voltage was set at 75 V. The mass spectrometer was operated at positive ion mode and mass spectra were collected in scan mode (*m*/*z* 50–500 in 0.5 s). Nine scans were averaged with a step size 0.1 over the range. For quantitative measurement of amphetamine, selective ion monitoring (SIM) was employed.

2.6. Standard solution

For the examinations of chromatography elution condition, linearity, and detection limit associated with this

method, 10 μg/ml of amphetamine sulfate 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 microdialysate sample, standard solutions were prepared from the solution that was used to inject into rats.

3. Results and discussion

3.1. Electrospray mass spectra

Three major ions ($[M + H]^+$, $[M - NH_2 + H]^+$ and $[C_7H_7]^+$) were detected in the positive mode (capillary exit voltage of 75 V). ESI is a soft ionization technique and CID has been used to promote molecular fragmentation. The effect of CID voltage on the detection of amphetamine was examined. The ESI-MS signal was optimized during continuous infusion of a solution of amphetamine dissolved in aCSF. The intensity of $[M + H]^+$ increased as the CID energy increased from 25 to 75 V and decreased significantly when it passed 75 V. Thus, CID voltage was set at 75 V for the rest of this study. For quantitative analysis, SIM mode ($[M + H]^+$, *m/z* 136) was employed.

3.2. LC separation

The interaction between the silanol group and protonated amphetamine molecule would cause severe tailing effect in LC separation. Ion-pair chromatography is a good approach to minimize this effect. Trifluoroacetic acid (TFA) has been successfully used as an ion-pair reagent for the separation of biochemicals [22,23]. We first examined the effect of TFA on LC separation of amphetamine. As expected, the large excess of TFA is to form [TFA–amphetamine] pair; thus, the peak tailing was reduced and the retention was extended. Furthermore, the ESI-MS intensity of amphetamine diminished as the concentration of TFA increased. Thus, a minimum amount of TFA to achieve adequate separation of amphetamine was added in mobile phase. Fig. 1 shows the typical LC chromatograms. Amphetamine was eluted at approximately 6.6 min. In addition, some intrinsic components in the microdialysis carrier were measured; however, none of them has interfered with amphetamine determination.

3.3. Post-column modification

The disadvantage of the addition of TFA to the elution solvent was that the ESI-MS signal was greatly suppressed. This is because ion-pair process disguises the protonated amphetamine cations from the ESI-MS electric field as neutral molecules [23,24]. Post-column addition of organic acid has been successfully utilized to minimize the signal suppression from ion-pair formation. The effects of ESI-MS detection by the addition of various organic acids (formic acid, acetic acid and propionic acid) were evaluated and the results are summarized in Fig. 2. The enhancement increased in the order of formic, acetic and propionic acid. This might be attributed to the fact that the less volatile organic acid (e.g. propionic acid) would displace strong [TFA–amphetamine] ion-pair and form weak [organic acid–amphetamine] ion-pair and subsequently generate protonated amphetamine ion and then detected by MS. Furthermore, the ESI-MS response increased as the flow rate of post-column addition of propionic acid increased from zero to 25 μl/min.; however, the extent of signal improvement decreased as the flow rate went beyond 25 μl/min. More than six-fold enhancement of ESI-MS response was achieved by the post-column addition of propionic acid. The post-column addition of propionic acid was set at 25 μl/min for the rest of this study.

3.4. Linearity and detection limit

The linearity of this newly developed assay was evaluated by a series of amphetamine (in aCSF) solution. A linear calibration graph was constructed using least-squares regression of quantities versus peak area. Good linearity ($r^2 = 0.999$) was determined from 0.01 to 1.0 μg/ml. The detection limit based on a signal-to-noise ratio of 3 was 0.002 μg/ml (11 nM). It is approximately equal to the value (5 nM) of the previously published fluorescence derivatization–HPLC method [12]. However, much less sample preparation was required in this newly developed ion-pair LC–ESI-MS method.

3.5. Precision and accuracy

The precision of the method was evaluated by the replicate analyses of aCSF containing amphetamine at all concentrations utilized for constructing calibration curves as shown in Table 1. 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 intra- and inter-day precisions showed relative standard deviations (R.S.D.s) ranging from 1.0 to 5.5% and 1.0 to 7.4%, respectively. The accuracy of the method was expressed by (mean measured concentration/spiked concentration) × 100%. Acceptable accuracy ranging from 94 to 105% was determined.

3.6. Analysis of microdialysis sample

The application of this newly developed ion-pair LC–ESI-MS method was demonstrated by evaluating free-form amphetamine in the mPFC region of rat brain. Amphetamine can be readily detected in the extracellular fluid after i.p. administration. A typical chromatogram of rat brain microdialysate after amphetamine was administered is shown in Fig. 1c. In SIM detection (*m/z* 136), there was no endogenous interference component observed in

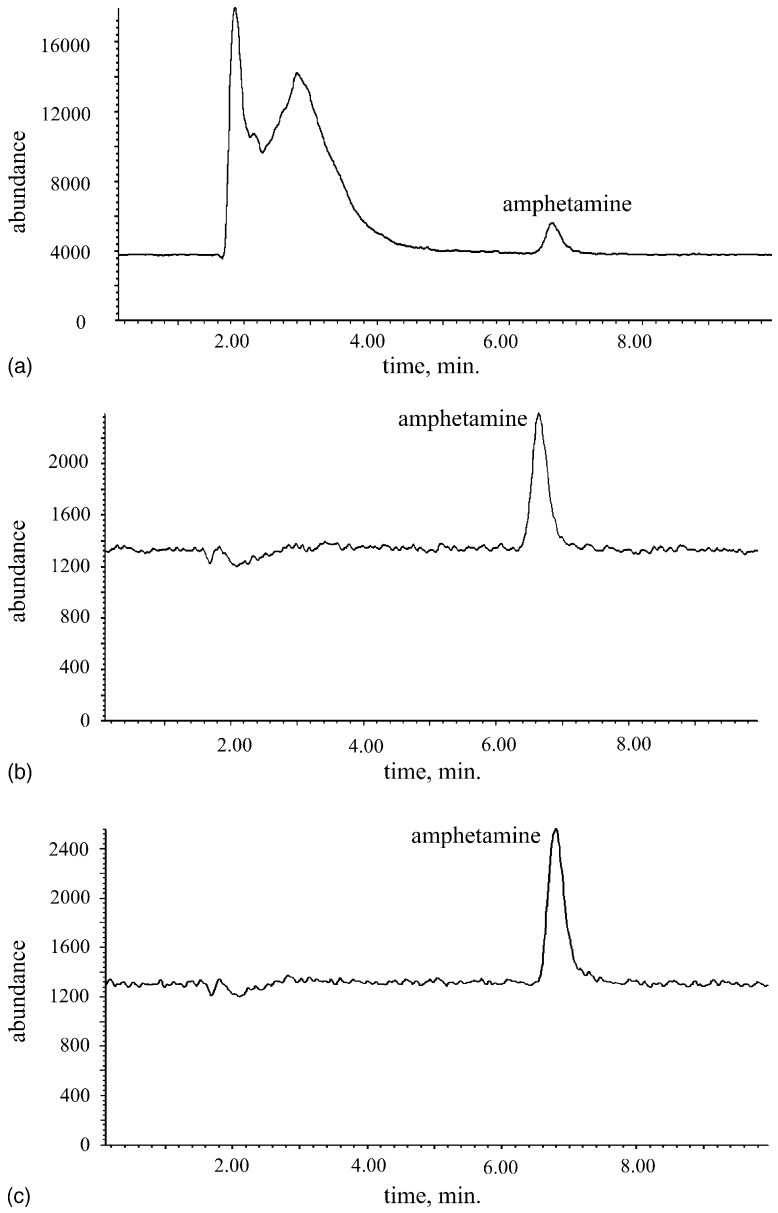

Fig. 1. (a) Reconstructed ion chromatogram of amphetamine (0.05 μg/ml) added aCSF. (b) Extracted ion chromatogram (*m*/*z* 136) of amphetamine (0.05 μg/ml) added aCSF. (c) Extracted ion chromatogram (*m*/*z* 136) of rat brain microdialysate sample after amphetamine administration.

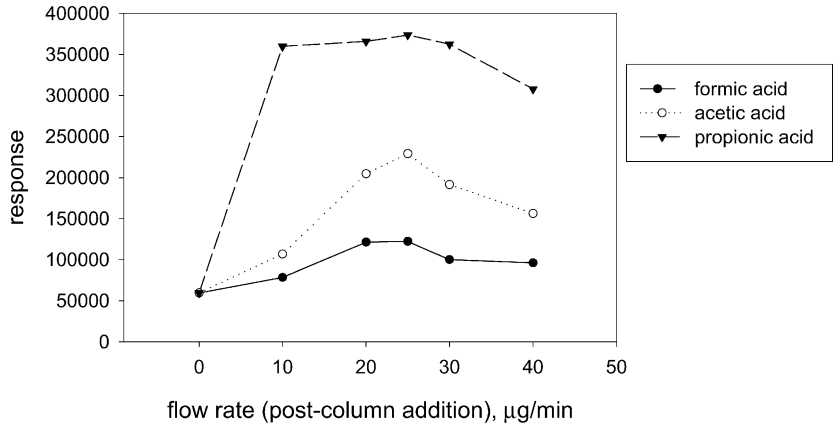

Fig. 2. Effects of post-column addition of various organic acids.

Table 1
Precision and accuracy study

Concentration (μg/ml)	Intra-day[a] measured concentration (μg/ml)		Accuracy (%)	Inter-day[a] measured concentration (μg/ml)	Accuracy (%)
1.000	Mean	1.015	101.5	0.998	99.8
	S.D.	0.013		0.011	
	R.S.D. (%)	1.3		1.1	
0.500	Mean	0.499	99.8	0.504	100.8
	S.D.	0.005		0.005	
	R.S.D. (%)	1.0		1.0	
0.100	Mean	0.102	100.2	0.105	105.0
	S.D.	0.002		0.003	
	R.S.D. (%)	2.0		2.8	
0.050	Mean	0.051	102.0	0.052	104.0
	S.D.	0.001		0.002	
	R.S.D. (%)	2.0		3.8	
0.010	Mean	0.0094	94.0	0.0095	95.0
	S.D.	0.0005		0.0007	
	R.S.D. (%)	5.5		7.4	

[a] $n = 3$.

rat's brain microdialysate. Profile of extracellular concentrations of amphetamine in the mPFC versus time ($n = 6$) after a single 3.0 mg/kg intraperitoneal administration. The time course of amphetamine increase and decrease is quite rapid. Amphetamine reached a maximum concentration of 0.103 ± 0.023 μg/ml during 20–40 min collection interval, which is no significant different as compared with the previous published value [12].

4. Conclusion

In conclusion, we have developed a simple and sensitive ion-pair LC–ESI-MS method for the determination of free-form amphetamine in rat brain. This method is sensitive enough for the continuous monitoring of amphetamine concentrations in microdialysis samples from rat brain. No sample pre-treatment or derivatization is required. Post-column addition of propionic acid at the flow rate of 25 μl/min resulted in more than six-fold improvement of signal. The method has been shown to be useful for the analysis of different polar/ionic compounds.

Acknowledgements

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