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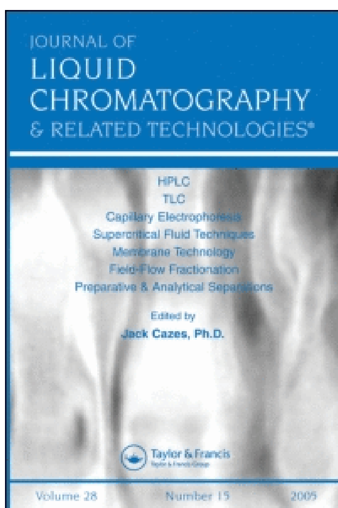

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Determination of Free-Form Amphetamine in Rat's Brain by *In Vivo* Microdialysis and Liquid Chromatography with Fluorescence Detection

M. S. Fuh[a]; T. K. Wang[a]; W. H. T. Pan[b]
[a] Department of Chemistry, Soochow University, Taipei, Taiwan [b] Department of Pharmacology, National Yang-Ming University, Taipei, Taiwan

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DETERMINATION OF FREE-FORM AMPHETAMINE IN RAT'S BRAIN BY *IN VIVO* MICRODIALYSIS AND LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

M. S. Fuh,[1*] T. K. Wang,[1] W. H. T. Pan[2]

[1]Department of Chemistry
Soochow University
Taipei, Taiwan

[2]Department of Pharmacology
National Yang-Ming University
Taipei, Taiwan

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ABSTRACT

The determination of free amphetamine in rat's brain by *in vivo* microdialysis technique and HPLC/Fluorescence derivatization method is described. In vivo microdialysis was used to sample the extracellular amphetamine and its contents were analyzed by HPLC/Fluorescence derivatization assay. The effects of pH, temperature and reaction time on the derivatization were examined. The detection limit, linearity and precision associated with this assay were also evaluated. The stability of amphetamine-dansyl chloride in different storage conditions was determined. A single 3.0 mg/kg i.p. amphetamine administration in rats resulted in a maximum concentration of 654 ± 201 μM in the medial prefrontal cortex.

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By using a non-linear curve fitting of two-compartment open model, the first-order rate constant for the appearance of amhetamine (k_1) was 0.0280 and the first-order rate constant for the disappearance of amhetamine (k_2) was 0.0281. These results document the utility of our method in pharmacokinetics studies.

INTRODUCTION

The number of illegal amphetamine users has increased tremendously in Taiwan. This has signaled many scientists in Taiwan to explore the euphoria mechanism of amphetamine in the human brain. Although, this research topic has been studied for many decades in other parts of world, it still is not clear. Understanding the pharmacokinetics of amphetamine in each key nuclei (e.g. medial prefrontal cortex; mPFC, nucleus accumbens, and striatum) is of interest to all scientists. As we know, only the free form of amphetamine, not the binding form, can have the pharmacological actions in the brain. Therefore, more importantly, monitoring of free amphetamine concentrations in the extracellular space is a preferable method for this study.

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In vivo microdialysis has become a widely accepted sampling device for monitoring extracellular free drug concentrations in rat's brain.[1] Firstly, this method avoids exposure of the brain tissue to the perfusion medium and, therefore, minimizes tissue damage. The dialysis membrane, with a molecular weight cut-off range from 5,000 to 50,000 daltons based on the necessity of the study, eliminates the need of sample clean-up procedures before analytical quantitation. Additionally, samples will not be subjected to further metabolization after collection, since, the analytes can be separated from enzymes by the dialysis membrane.

Finally, the dialysis membrane prevents us to collect the binding form of amphetamine, only the free form. Therefore, using in vivo microdialysis as a sampling technique is a preferred technique to study the pharmacokinetics of the drug in various brain regions in rats.

In the past, several gas chromatography (GC) and GC/mass spectrometry (GC/MS) methods have been developed for the analysis of amphetamine in human urine and plasma.[2,3,4,5] In their methods, samples usually were of a large amount for pre-analysis preparation. For example, an extraction procedure is needed to transfer amphetamine into an organic solvent for GC or GC/MS analysis. Derivatization of amphetamine is always required to avoid decomposition of amphetamine in the GC injection port with high temperature and to enhance the signal to noise ratio by moving the molecular ion from low

100 to over mid 300. However, a relatively small amount of microdialysis sample (about 5 μL to 20 μL) make it difficult to apply extraction and derivatization procedure.

HPLC is an alternative method for the detemination of amphetamine in biological fluids. However, several other HPLC methods have been developed for the measurement of amphetamine in urine and plasma. Amphetamine was quantitated directly by UV,[6,7] photodiode array,[8] chemiluminescence or fluorescence detection.[9,10] However, all these methods are not suitable for small sample sizes of microdialysate samples.

Until recently, a gradient elution HPLC with fluorescence detection method has been developed for the analysis of microdialysate samples.[11] The relative long analysis time of gradient elution makes it less desirable for routine analysis, however. Also, no real application example was provided in their report.

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This paper describes a simple isocratic elution HPLC method for the detemination of amphetamine in microdialysate samples. It includes addition of dansyl chloride for flourescence derivatization and HPLC fluorescence detection of amphetamine-dansyl chloride (AP-DanCl). More importantly, we apply this method to determine the concentration of free amphetamine in rat's medial prefrontal cortex (mPFC) after 3.0 mg/kg i.p. administration.

EXPERIMENTAL

Chemicals and Reagents

Purified water (> 18 MΩ from a NANOPure water purification system (Barnstead Corp., Dubuque, IA, USA) and HPLC grade acetonitrile and acetone (Labscan Limited, Dublin, Ireland) were used throughout. Sodium hydroxide, and amphetamine sulfate were purchased from Sigma (St. Louis, MO, USA). Dansyl chloride was obtained from Aldrich Corp. (Milwaukee, WI, USA). Sodium bicarbonate, sodium chloride, magnesium chloride, potassium chloride, calcium chloride, ascorbic acid, and glucose were purchased from Nakarai Chemical (Tokyo, Japan).

Pentobarbital sodium was obtained from Veterans General Hospital, Taipei, Taiwan. Lidocaine was obtained from the Department of Health, R.O.C.

Microdialysis

The design of a microdialysis method for conscious rats is different from that for anesthetized rats due to increased mobility of conscious rats after amphetamine administration. A piece of dialysis membrane tubing (220 μm i.d.; 6,000 dalton cut-off; Spectrum Medical Industries Inc., Houston, TX USA) was sealed at one end, then the two pieces of fused silica tubing (100 μm o.d., 40 μm i.d.; Polymicro Technologies, Inc., Phoenix, AZ, USA) were placed side by side and the ends of the inlet and outlet tubes were separated by 4 mm. Polyamide sealing resin (Alltech Associates Inc., Deerfield, IL, USA) was used to seal both ends of the dialysis membrane and to coat the excess portion of membrane. The probes were allowed to dry for 12 hours after being sealed by polyamide resin prior to implantation. The inlet and outlet fused silica tubes were protected by an elastic tubing (Baxter Scientific Products, Charlotte, NC, USA). The inlet was connected to a home-made single channel fluid swivel to allow the rat free movement without entanglement with the tubes.[12] The fluid swivel was then connected to a 500 μL syringe via PE-10 tubing. The perfusion syringe containing artificial cerebrospinal fluid (ACSF), was mounted on a microliter syringe pump (Model 22, Harvard Apparatus, S. Natick, MA, USA) at a flow rate of 1.19 μL/min. 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 mixture was adjusted to a pH of 7.2 to 7.4 with 0.1 M sodium hydroxide.

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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 hour light/dark cycle room with free access to food and water. On the experimental day, animals were first anesthetized with 50 mg/kg i.p. sodium pentobarbital and placed on a stereotaxic apparatus (Koff models 1430 &1460). A local anesthetic (6% lidocaine) was used to relieve pain. A stainless steel guide cannula (C313GP, Plastics One, Inc.) was then lowered to 4 mm above the ventral surface of the medial prefrontal cortex (mPFC). The coordinates used, from bregma, were +3.1 AP, +0.8 ML, and -0.2 V below the skull. The guide cannula was secured to the skull with three mounting screws and dental cement. Intramuscular penicillin (60,000 I.U.) was administered immediately following surgery, then, the dialysis probe was inserted through the guide cannula.

Thereafter, the rat was placed in a behavioral box for at least an 18 hour recovery period prior to amphetamine administration (3.0 mg/kg i.p.). After the recovery period, the blank dialysate was assayed for ensuring no interference of amphetamine detection. After intraperitoneal injection of

amphetamine, dialysis samples were collected for 120 min. at 20 min. intervals. The dialysis fluid was collected in a 200 μL eppendroff tube wrapped with aluminum foil and stored in a -20°C refrigerator prior to analysis.

HPLC System

The HPLC system consisted of a model 750 piston pump from GL Science (Tokyo, Japan), a Waters 470 scanning fluorescence detector (Bedford, MA, USA), and a Rheodyne 7125 injector with a 20 μL injection loop. The excitation was set at 343 nm and the fluorescence was monitored at 500 nm. A SISC chromatography data system (Scientific Information Service Corp., Taipei, Taiwan) was used for data acquisition and processing. A Vercopak Inertsil 5-ODS-80A column, (3.2 x 250 mm, 5 μm, Vercotech Corp., Taipei, Taiwan) with an on-line filter (TFE exchange membrane, 82102, IRICA Instrument Inc., Kyoto, Japan) was used for separation. A mixture of 70 % acetonitrile and 30% water (v/v) was used as a mobile phase for chromatography analysis. The mixture was filtered through a 0.45 μm membrane (FP-450, Gelman Science, Michigan, USA) and sparged with helium gas for 30 minutes before use. The HPLC flow rate was 0.5 mL/min.

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Standard Solution

For the examination of chromatography elution conditions, linearity, and detection limit associated with this method, 1 mM of amphetamine in ACSF was prepared and stored at 4°C in the dark. This stock solution was prepared weekly, but the working solutions were diluted with ACSF to appropriate concentration daily. For interday and intraday studies, the stock and working solutions were prepared daily.

For the quantitative analysis of microdialysate samples, standard solutions were prepared from the same solution that was used to inject the rats.

Derivatization of Samples

Into an eppendorf centrifuge tube, 15 μL of microdialysate was added to 8 μL of 0.1 M sodium bicarbonate/NaOH buffer (pH 9.0) and 15 μL of 0.1 mM dansyl chloride in acetone. The eppendorf tube was wrapped with aluminum foil, then the mixture was incubated at 45°C in a water bath for 1 hour. An aliquot of the solution (20 μL) was injected into the HPLC system for analysis.

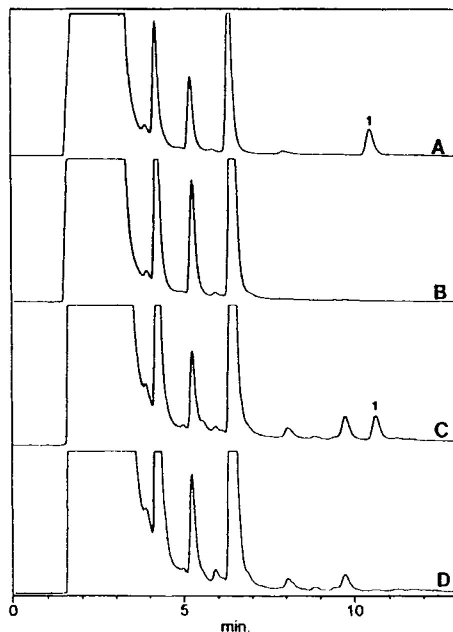

Figure 1. Typical chromatograms of a spiked amphetamine sample in ACSF (A), an ACSF only (B), a real microdialysis sample (C), and a microdialysis sample before amphetamine administration (D). Peak 1= amphetamine

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RESULTS AND DISCUSSION

Figure 1 shows the typical chromatograms of ACSF blank, amphetamine spiked ACSF, and mice brain microdialysate samples. There are many substances in the ACSF and brain microdialysate, which reacted with the dansyl chloride; however, none of them interfered with AP-DanCl. AP-DanCl was eluted at 10.6 minutes, with the separation completed in less than 12 minutes.

The effects of pH, reaction temperature and reaction time on fluorescence derivatization reaction were evaluated by the method described in a previous report.[11] We found the most effective reaction condition for ACSF samples to be at pH 8.75 and a temperature of 45°C. For practical purposes, the fluorescence derivatization reaction condition was set at 45°C, pH 8.75 and 1 hour of reaction time throughout the remainder of this study.

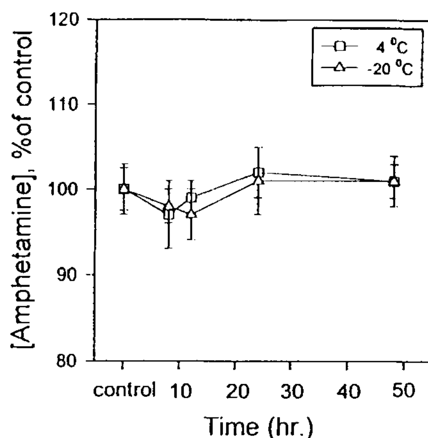

Figure 2. Stability of the spiked amphetamine samples in ACSF (0.1μM) versus time, which were stored at either 4°C or -20°C. The calculated concentration of a fresh prepared solution was used as 100%. Values are represented as the percentile changes of control (mean ± S.E.M.; n=3).

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The effect of sample storage conditions on this assay was then evaluated. Amphetamine spiked ACSF samples (0.1μM) were stored in a freezer (-20°C) and a refrigerator (4°C). The samples were prepared by the procedures described in previous paragraphs and measured by HPLC for 24 hours. The results as shown in Figure 2 were examined by a one-tailed student-T test and $P<0.05$ was taken to indicate statistical significance. There was no significant decrease in response after 24 hours of storage in the refrigerator or freezer. The measurements of amphetamine in microdialysate for the remainder of this study were done within 24 hours after collection.

The stability of AP-DanCl in ACSF was evaluated and the results are summarized in Figure 3. The derivatized amphetamine spiked ACSF samples (0.1 μM) were stored either in a refrigerator at 4°C or at room temperature (about 26°C). Samples were withdrawn from both solutions hourly during a ten hour period by injection into HPLC for measurement to insure the stability of amphetamine. The 0 hour sample served as a control. The difference between the results of each of the 10 samples and the control was examined by a one-tail student-T test. $P<0.05$ was taken to indicate statistical significant. There was no significant decrease in response after storage in the refrigerator or freezer.

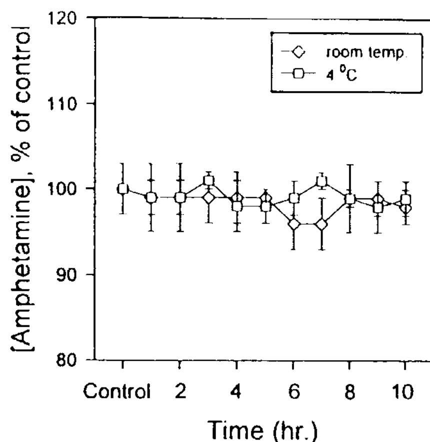

Figure 3. Stability of dansyl chloride derivatized amphetamine samples (0.1μM) versus time, which were stored at either room temperature (26°C), or 4°C. Values are represented as the percentile changes of control (mean ± S.E.M.; n=3).

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Solutions of amphetamine spiked ACSF samples (5.0, 1.0, 0.5, 0.1, 0.05, 0.01 μM) were prepared, then each solution was derivatized throughout the procedure as described in a previous section. Each standard was analyzed six times. A calibration curve for amphetamine spiked ACSF samples was constructed and the linearity was good from 5 to 0.01 μM (r^2=0.999). The detection limit was 0.005 μM based on a signal-to-noise ratio of 3.

The analytical precision of this method was evaluated by replicated analysis of amphetamine spiked ACSF samples. A new calibration standard set was prepared and analyzed each day. A total of six series of samples were analyzed over a two week period and each sample was determined in triplicate. The results of interday and intraday studies are summarized in Table 1. The intraday precision showed a coefficient of variation (CV) ranging from 1.8% to 8.3%. The interday variation was similarly evaluated on several days and the CV varied from 0.2% to 10.0%.

The time course of amphetamine concentration (n=5) in the extracellular fluid in mPFC of rats which received 3 mg/kg amphetamine i.p. is shown in Figure 4. Amphetamine reached a maximum concentration of 654 ± 201μM during the 20-40 min collection interval. The time course of amphetamine change was rapid.

Table 1

Interday and Intraday Precision of Amphetamine in ACFS

Added Conc.		Intraday[1] Measured Conc. μM	Interday[2] Measured Conc. μM
5.0 μM	mean	4.99	4.98
	S.D.	0.089	0.010
	CV	1.8%	0.2%
1.0 μM	mean	1.01	1.04
	S.D.	0.040	0.030
	CV	4.0%	2.9%
0.5 μM	mean	0.54	0.51
	S.D.	0.014	0.028
	C.V.	2.6%	5.6%
0.1 μM	mean	0.09	0.10
	S.D.	0.007	0.006
	C.V.	7.8%	6.0%
0.05 μM	mean	0.046	0.049
	S.D.	0.004	0.005
	C.V.	8.7%	10.2%

[1]Mean and S.D. represent four different ACSF samples for each concentration.
[2]Interday reproducibility was determined from six different runs over a two week period.

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A non-linear curve-fitting computer program, Minsq, written by MicroMath Scientific Software (Salt Lake City, Utah, U.S.A.) was used to fit an equation consisting of the difference of two first-order kinetic processes for the appearance and disappearance of amphetamine in the extracellular fluid of the mPFC to experimental amphetamine data:

$$\text{Amphetamine} = A\,(\exp(-k_1 t)-\exp(-k_2 t))$$

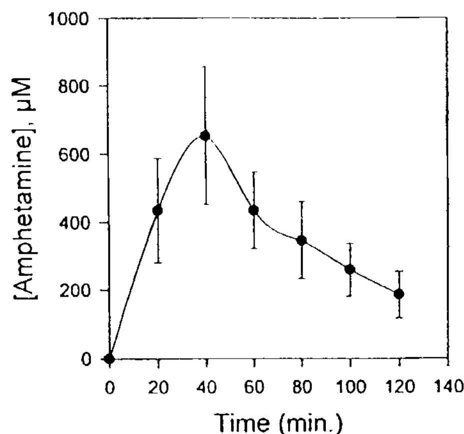

Figure 4. Time course of extracellular amphetamine concentration at the medial frontal cortex in rats (n=5) following a 3.0 mg/kg amphetamine i.p. injection.

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Three parameters fit into the data: A, k_1, and k_2. A is a concentration and adsorption efficiency factor, k_1 is the first-order rate constant for the appearance of amphetamine, and k_2 is the first-order rate constant for the disappearance of amphetamine. Both rate constants have the units of min^{-1}. After this non-linear fit, the k_1 was 0.0280 and k_2 was 0.0281. The 95% non-linear confidence intervals for these rate constants were: $[0.0212 < k_1 < 0.0349]$ and $[0.0211 < k_2 < 0.0350]$.

In conclusion, we have successfully evaluated the concentration of free amphetamine in rat brain by microdiaysis technique and a simple, sensitive and accurate HPLC with fluorescence derivatization assay.

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