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Short communication

Determination of amphetamine in human urine by dansyl derivatization and high-performance liquid chromatography with fluorescence detection

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Abstract

A simple procedure for the determination of amphetamine in urine with minimal sample preparation is described. This method involves direct addition of human urine to an acetone–dansyl chloride solution for simultaneous deproteinization and fluorescence derivatization. The derivatized amphetamine is then measured by HPLC with fluorescence detection. It eliminates the extraction procedures often required by other HPLC or GC methods. The effects of pH, temperature and reaction time on the derivatization reaction were investigated. The stability of amphetamine–dansyl chloride in different storage conditions was examined. The detection limit and linearity associated with this assay are discussed.

Keywords: Amphetamine

1. Introduction

The abuse of amphetamine and related compounds is a serious problem in Taiwan. The development of a rapid, sensitive, and accurate analytical method for the determination of amphetamine is an important task.

Many analytical methods including gas chromatography–mass spectrometry (GC–MS), headspace GC, and GC have been developed for the determination of amphetamine-related compounds in urine and plasma samples [1–3]. These methods have excellent sensitivity and selectivity; however, they often require some sample preparation procedures since neither urine nor plasma samples can be introduced into GC directly. Several steps are involved in the sample preparation for GC, such as deproteinization, extraction of amphetamine by organic solvent, sample transfer, and pre-concentration.

In practice, a simple test (radio-immunoassay, RIA, or enzyme-immunoassay, EIA) is often used to screen urine samples prior to GC–MS analysis [4]. However, the specificity of RIA or EIA testing is poor and false results are frequently caused by common-cold medicines containing ephedrine or phenylpropanolamine [5]. On the other hand, for HPLC analysis, biological fluid samples can be analyzed with less sample preparation. Ion-pairing extraction combined with solid-phase chiral derivation has been successfully used for the enantiomeric analysis of amphetamine in plasma [6]. This method achieved an excellent separation for the analysis of

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enantiomeric amphetamine composition for pharmacokinetic studies. However, the on-line solid-phase extraction and derivatization column has a short lifetime which makes it unsuitable for routine analysis. Determination of amphetamine by solid-phase extraction and HPLC with sodium 1,2-naphthoquinone 4-sulphonate derivatization has been reported [7]. Solid-phase extraction combined with ion-pairing reagents was used for the analysis of amphetamines in biological fluids [8]. Hayakawa et al. [9] reported a method in which amphetamine was extracted by organic solvent, reacted with dansyl chloride, and then analyzed by HPLC with chemiluminescence detection. In this reported method, diethyl ether was used to extract amphetamine and related compounds from urine and then derivatized by dansyl chloride. These reported methods required sample preparation procedures and chemiluminescence detection.

This paper describes a HPLC method for the determination of amphetamines in urine. It includes the addition of dansyl chloride–acetone for deproteinization and derivatization, centrifugation for protein removal, and HPLC-fluorescence detection of amphetamine–dansyl chloride (AP–DanCl).

2. Experimental

2.1. Chemicals

Purified water (>18 MΩ) from a NANOPure water purification system (Barnstead, Dubuque, IA, USA) and HPLC grade acetonitrile and acetone (Labscan, Dublin, Ireland) were used throughout. Sodium hydroxide, ephedrine, phenylpropanolamine [(±)-norephedrine hydrochloride], and amphetamine sulfate were purchased from Sigma (St. Louis, MO, USA). Dansyl chloride was obtained from Aldrich (Milwaukee, WI, USA). Sodium bicarbonate was from Nakarai Chemical (Tokyo, Japan).

2.2. 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, Taipei, Taiwan) and a personal computer were used for data acquisition and processing. A Vercopak Inertsil 5-ODS-80A column, (3.2× 250 mm, 5 μm, Vercotech, Taipei, Taiwan) with an on-line filter (TFE exchange membrane, 82102, IRICA, Kyoto, Japan) was used for separation. Acetonitrile–water (70:30, v/v) was used as a mobile phase. The mixture was filtered through a 0.45-μm membrane (FP-450, Gelman Science, MI, USA) and purged with helium gas for 30 min before use. The HPLC flow-rate was 0.5 ml/min.

2.3. Derivatization of urine samples

To an Eppendorf centrifuge tube, 50 μl of urine sample (spiked with amphetamine or not) were added to 20 μl of 0.1 *M* sodium bicarbonate–NaOH buffer (pH 9.0) and 50 μl of 0.1 m*M* dansyl chloride in acetone. This mixture was centrifuged at 10 000 *g* for 1 h. The clear supernatant was transferred to another Eppendorf centrifuge tube wrapped with aluminum foil and incubated at 45°C in a water bath for 1 h. An aliquot of the solution (20 μl) was injected into the HPLC system for analysis. The effects of pH, temperature and reaction time on the derivation reaction will be discussed in the following paragraphs.

2.4. Standard solutions

Standard solutions (0.01 *M*) of each amine (amphetamine, ephedrine, and phenylpropanolamine) in HPLC-grade water were prepared. These stock solutions were then further diluted to yield the appropriate working solutions. All solutions were stored at 4°C in the dark.

3. Results and discussion

Acetone was used as a solvent for dissolving dansyl chloride as well as for deproteinization. Different compositions of acetone–water mixtures (100:0, 75:25, and 50:50) were evaluated as solvents for the effectiveness of removing proteins in urine

and for the derivatization reaction. The experimental results indicated that dansyl chloride in 100% acetone is the most effective one.

In a previous report, derivatization was performed at 45°C for 45 min [9]. However, there was no discussion on optimal conditions. We therefore examined the effects of pH, temperature, and reaction time to find the most effective conditions for the derivatization of amphetamines in urine. Urine samples from three volunteers were collected, mixed and stored in a freezer (−20°C) prior to use. The yields of AP–DanCl under different reaction conditions were evaluated by HPLC analysis. The results are shown in Fig. 1A–C. There was little temperature effect on the yield over the range examined. For the ranges of pH and temperature examined, we found the most effective reaction conditions for the urine samples to be at pH 8.5 and a temperature of 45°C. In addition, the results show that there was an increase on the yield of AP–DanCl as the reaction time increased during the first hour and much less effect was observed after the initial 1 h. For practical purposes, a 1-h reaction time was used for the examination of linearity and detection limit for the rest of this study.

Fig. 2 shows typical chromatograms of spiked water, blank urine, and spiked urine samples. The separation was accomplished in less than 12 min. Ephedrine–DanCl co-eluted with a residual compound from the urine. However, the compound of interest, AP–DanCl, was separated from urine residuals and eluted at 10.3 min. These results indicated that this method can differentiate amphetamine and related compounds by retention time.

A calibration curve for amphetamine-spiked urine samples was constructed. The linearity was good from 0.05 to 10 μM ($r^2 = 0.999$). The detection limit was 0.048 μM based on a signal-to-noise ratio of 3. In this study, 20 μl of urine sample was injected to prolong the lifetime of the column. The lifetime of the LC column was for about 300 injections of urine samples.

The intra-day precision of this test method was evaluated by replicated analysis of spiked-urine samples. The intra-day precision showed a coefficient of variation (C.V.) of 1.82% to 6.89%. The inter-day variation was similarly evaluated on several days up to 2 weeks. The inter-day C.V.s varied from 1.76% to 11.81%. Details of the precision study are summarized in Table 1.

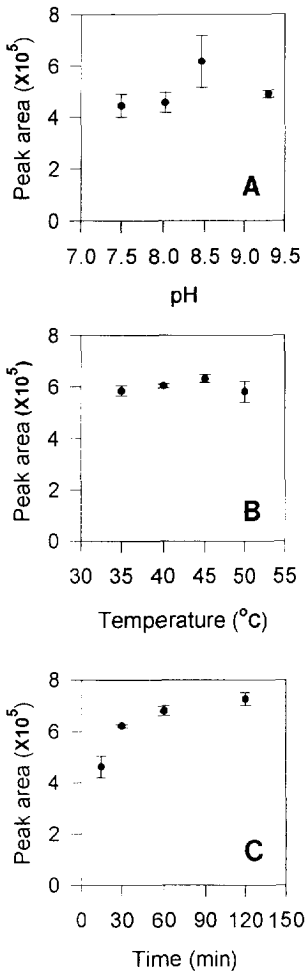

Fig. 1. Results of amphetamine sulfate (2 mg/l) spiked urine sample and dansyl chloride derivatized at (A) 45°C, 60 min, different pH, (B) pH 8.5, 60 min, different temperature, and (C) 45°C, pH 8.5, different reaction time. Data are presented as mean±S.E.M. of six measurements.

The stability of AP–DanCl in urine was evaluated to examine the feasibility of using an autosampler for this assay. The derivatized amphetamine-spiked urine samples were stored in a refrigerator (4°C) or room temperature (26°C) and then injected into the HPLC every 40 min for 20 h. The 0-min sample served as a control. The difference between the results for each of the 30 samples and the control was evaluated by a 1-tailed Student's t-test. $P < 0.05$

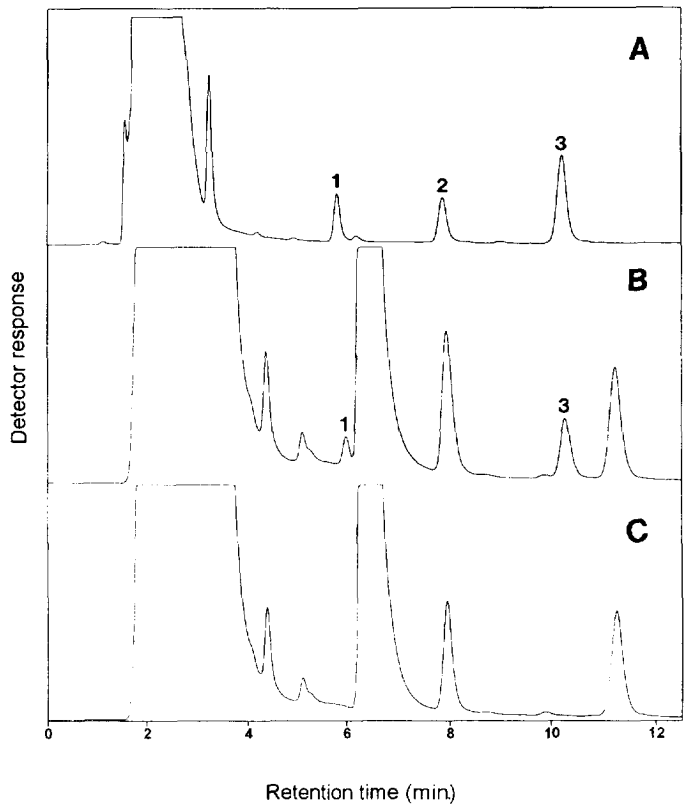

Fig. 2. Typical chromatograms of samples of (A) spiked water sample, (B) spiked urine sample, and (C) urine blank; both the spiked urine and the spiked water sample contain 10 μM of amphetamine sulfate, ephedrine, and phenylpropanolamine. Peaks: 1 = phenylpropanolamine, 2 = ephedrine, 3 = amphetamine.

was taken to indicate a statistical significance. There was no significant decrease in response after 20 h of storage in the refrigerator or at room temperature.

In order to evaluate the effect of the sample-storage condition on this assay, amphetamine-spiked urine samples were stored at room temperature (26°C), in the refrigerator (4°C), and in the freezer (−20°C). Prior to analysis, samples were removed and prepared by the procedures described in previous paragraphs and measured by HPLC. The results are shown in Fig. 3. For the samples stored at room temperature, there was a decrease in recovery of about 5% during the first 24 h of storage and the pressure of HPLC increased substantially after injection of the samples. This increase might be caused by the precipitation of residuals during storage. For the refrigerated samples, there was a tendency to decrease the recovery by about 10% (calculated by the mean value) after 5 days of storage. Moreover, there was approximately 95% recovery (calculated by the mean value) observed after 5 days of storage in the freezer. The results were examined by 1-tailed Student's t-test and $P<0.05$ was taken to indicate a statistical significance. The t-test results indicated that urine samples can be stored in a freezer or a refrigerator for several days prior to analysis without affecting its concentration.

In summary, we have developed a simple, rapid, and sensitive assay to determine amphetamines in urine by HPLC with fluorescence detection. The sample extraction procedures commonly required in other reported methods were eliminated. Deproteinization and derivatization were performed simultaneously. Baseline separation was achieved by a simple isocratic elution in less than 12 min. Although this method is proven to be able to differentiate amphet-

Table 1
Inter-day and intra-day precision of amphetamines in urine

Added concentration (μM)	Intra-day[a]	Inter-day[b]
	Measured concentration (μM)	Measured concentration (μM)
5.0		
Mean	5.01	4.95
S.D.	0.192	0.087
C.V.%	3.83	1.76
1.0		
Mean	1.03	1.03
S.D.	0.071	0.037
C.V.%	6.89	3.59
0.5		
Mean	0.44	0.47
S.D.	0.008	0.024
C.V.%	1.82	5.11
0.1		
Mean	0.12	0.11
S.D.	0.004	0.013
C.V.%	3.33	11.81

[a] Mean and S.D. represent four different urine samples for each concentration.
[b] Inter-day reproducibility was determined from six different runs over a 2-week period.

amines and related compounds by retention time, HPLC–MS or GC–MS analysis is needed for additional confirmation. Currently, we are evaluating the use of a narrowbore column to determine amphetamines in microdialysate and the results will be reported elsewhere.

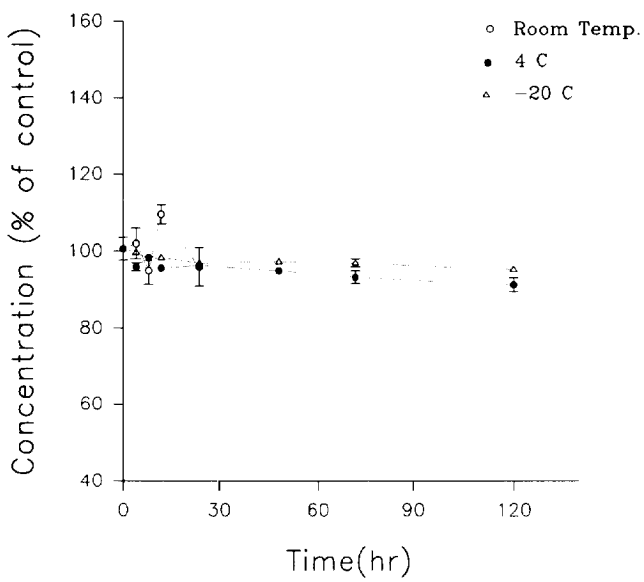

Fig. 3. Recovery of amphetamines added to urine samples which were stored at different temperatures. Amphetamine-spiked urine samples (1 μM) were used for this study. Values in percentages are presented as mean±S.E.M. of three measurements.

Acknowledgments

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