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RAPID DETERMINATION OF *p*-HYDROXYLATED METHAMPHETAMINE METABOLITES BY COLUMN LIQUID CHROMATOGRAPHY—ELECTROCHEMISTRY

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SUMMARY

A method for electrochemical determination of *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine in human urine was investigated by column liquid chromatography. A rapid and simple extraction of these substances from urine was performed using a solid-phase extraction column. The detection limit for quantitation was approximately 50 pg for the metabolites. The simplicity and sensitivity of this method allows for analysis of these metabolites in the fields of toxicology and forensic medicine.

INTRODUCTION

Methamphetamine abuse has become a serious problem, and has led to thorough studies of its metabolism, distribution and excretion. Methamphetamine is metabolized along two pathways, either by hydroxylation of the aromatic ring or by demethylation of the side-chain. It has been reported that the main metabolites in man are the unchanged drug and *p*-hydroxymethamphetamine and that minor ones include amphetamine and *p*-hydroxyamphetamine [1].

Many procedures for determination of methamphetamine and amphetamine have been developed for qualitative and quantitative purposes. They include colour tests [2], UV spectrophotometry [3], immunological methods [4], gas chromatography (GC) [5, 6], gas chromatography—mass spectrometry (GC—MS) [7, 8] and column liquid chromatography (LC) [9, 10]. Various methods for analysis of the metabolites of methamphetamine and amphetamine also have been reported, including thin-layer chromatography

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using radiolabelled methamphetamine [1, 11], GC—MS [12], GC—electron-capture detection [13] and GC—nitrogen—phosphorus detection [14]. However, there have been few communications on the determination of the metabolites using LC [15, 16].

The objective of this study was the determination of *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine.

EXPERIMENTAL

Reagents

Methamphetamine was obtained from Dainippon Pharmaceutical (Osaka, Japan). *p*-Hydroxymethamphetamine was kindly supplied by Dr. S. Takahashi (Izumo, Japan) and *p*-hydroxyamphetamine by Dr. K. Ishikawa (Tokyo, Japan). Reagent-grade methyl alcohol, ethyl acetate, trifluoroacetic anhydride (TFA), potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), acetic acid, ethylenediaminetetraacetic acid (EDTA), 1 *M* hydrochloric acid, and special-reagent-grade acetonitrile, tetrahydrofuran (THF), perchloric acid (PCA) were purchased from Nakarai Chemicals (Kyoto, Japan).

Apparatus and conditions

The LC instrumentation used was assembled from commercially available components (IRICA Instruments, Kyoto, Japan), and included an LC pump (Chromatic P-321) equipped with a universal injector (U-350), an amperometric detector (E-308) and a data processor (7000 A). A Cosmosil ($5C_{18}$) packed column, average particle size 5 μm, 150 × 4.6 mm I.D. (Nakarai Chemicals) was used. The recorder was set at 32 nA full scale. The detector potential was 850 mV versus the Ag/AgCl reference electrode. The buffer for chromatography contained the following components: 0.1 *M* KH_2PO_4–K_2HPO_4 (pH 6.0), 2% acetonitrile, 0.2% THF and 0.005% EDTA. The flow-rate was set at 1.0 ml/min.

The GC analysis was performed with a Shimadzu GC-9A equipped with a flame-ionization detector and a digital integrator (Shimadzu, Chromatopac C-R2A, Kyoto, Japan). The column packing for GC was 2% OV-17 on Chromosorb WAW-DMCS (60—80 mesh) in a glass column (2.1 m × 3.2 mm I.D.). The running conditions were: column temperature, 150°C; detector temperature, 190°C; carrier gas (nitrogen) flow-rate, 60 ml/min.

Extraction procedure

The human urine sample containing methamphetamine and its metabolites was appropriately diluted with double-distilled water to 2.0 ml, and then passed through a solid-phase extraction column, Bond-Elut C_{18}® (Analytichem International, Harbor City, CA, U.S.A.), which was prewashed according to the manufacturer's directions. The Bond-Elut column was washed with 4.0 ml of double-distilled water, with 20 ml of 30% methyl alcohol and with 4.0 ml of acetonitrile. After washing, the substances were eluted three times with 0.5 ml of acetonitrile—1 *M* hydrochloric acid (90:10).

For the LC analysis of the *p*-hydroxylated metabolites, the eluate was added

with 100 μl of acetic acid and evaporated under a stream of nitrogen. The residue was dissolved in 100 μl of 0.1 *M* PCA, and 5-μl aliquots were injected into the LC column.

For the GC analysis of methamphetamine, 100 μl of acetic acid were added to the eluate, and this was evaporated under a stream of nitrogen. The residue was dissolved in 100 μl of ethyl acetate, after which 200 μl of TFA were added to it. The sample was incubated at 56°C for 30 min. After the reaction, the sample was again evaporated to dryness under a stream of nitrogen. Then, it was dissolved in 100 μl of ethyl acetate, and 1-μl aliquots were injected into the GC system.

RESULTS AND DISCUSSION

In electrochemical detection, the relationship between applied voltage and sensitivity was examined by changing the applied voltage from +600 to +900 mV versus the reference electrode. *p*-Hydroxymethamphetamine and *p*-hydroxyamphetamine, 1 ng of each, were used as samples. The results are shown in Fig. 1. Both metabolites were initiated to respond at an applied voltage of +650 mV and the response of the two became almost constant in the range between +800 and +900 mV. However, since neither methamphetamine nor amphetamine has a hydroxy group in the aromatic ring, these substances

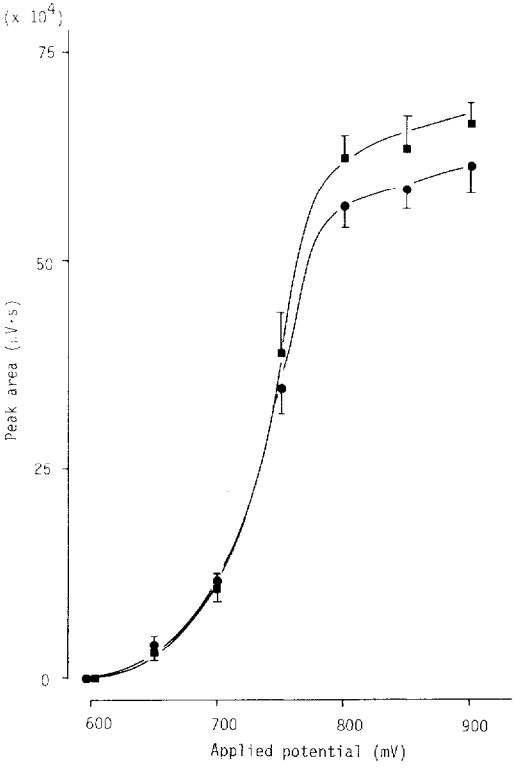

Fig. 1. Relationship between applied voltage and sensitivity of *p*-hydroxymethamphetamine (■) and *p*-hydroxyamphetamine (●). Of authentic substances 1 ng was injected. Each point represents the mean of six experiments and each bar represents the standard deviation.

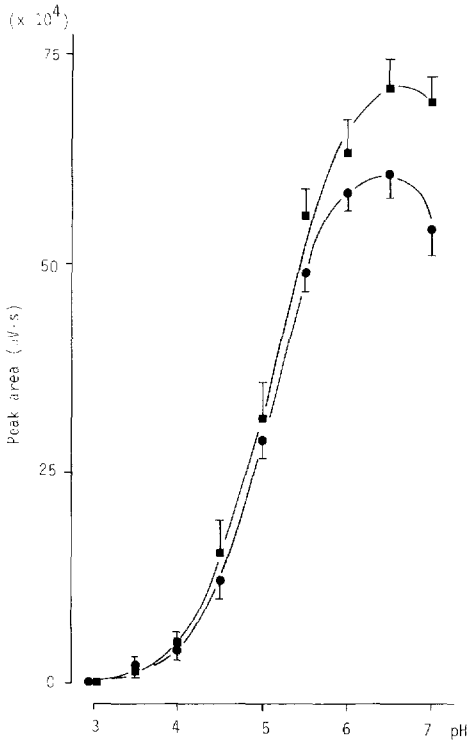

Fig. 2. Relationship between buffer pH and sensitivity of *p*-hydroxymethamphetamine (■) and *p*-hydroxyamphetamine (●). Of authentic substances 1 ng was injected. Each point represents the mean of six experiments and each bar represents the standard deviation.

could not be detected electrochemically. Based on these results, the applied voltage was set at +850 mV versus the Ag/AgCl reference electrode, and at this voltage there was no significant background noise.

The pH of the buffer was investigated to attain the maximal sensitivity of the metabolites. Of each of the metabolites 1 ng was injected into the liquid chromatograph at +850 mV applied voltage. Fig. 2 shows the results of this investigation. Both metabolites were initiated to respond at a pH of 3.5 and the sensitivity was increased progressively up to pH 6.5, followed by a decline at pH 7.0. Although the maximal sensitivity of the metabolites was obtained by using the pH 6.5 buffer, under this condition the cell current meter of the detector indicated a high current value of the basal level. Therefore the pH of the buffer was adjusted to 6.0 for chromatography of the *p*-hydroxylated methamphetamine metabolites.

The detection limit of the metabolites was evaluated on the basis of calibration curves. The calibration curves of these substances were obtained by plotting the peak area against the amounts injected. Good linearity in these two metabolites was achieved within ranges of 1—5 ng and 50—1000 pg. The coefficient of correlation (r) for *p*-hydroxymethamphetamine was 0.9918 in the range 1—5 ng and 0.9961 in a range 25—1000 pg. For *p*-hydroxyamphetamine, it was 0.9946 on the large scale and 0.9965 on the small scale. The detection limit for the metabolites was approx. 50 pg.

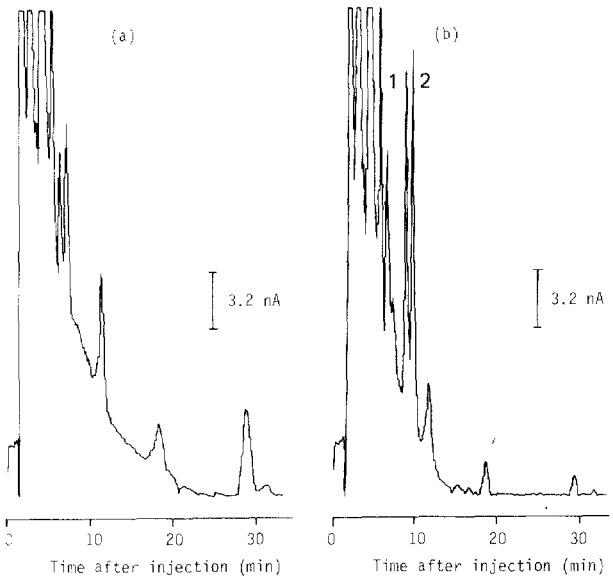

Fig. 3. Chromatograms of the urine blank (a) and the urine containing the metabolites (b). Peaks 1 and 2 represent the recovery from 1 ml of urine spiked with 40 ng of the metabolites and correspond to *p*-hydroxyamphetamine and *p*-hydroxymethamphetamine, respectively.

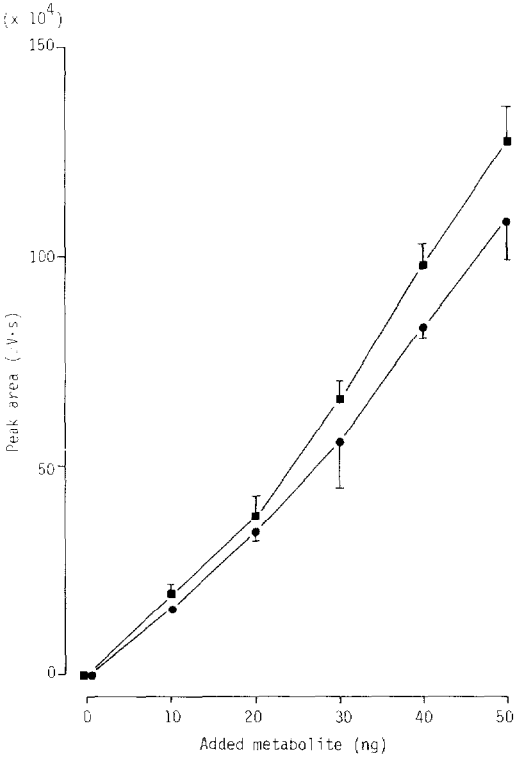

Fig. 4. Recovery curves of exogenously added *p*-hydroxymethamphetamine (■) and *p*-hydroxyamphetamine (●). Indicated amounts of the metabolites were added to 1 ml of urine and extracted as described in the text. Each point represents the mean of six experiments and each bar represents the standard deviation.

Recovery experiments for *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine were performed with Bond-Elut C_{18} in healthy urine samples to which known amounts of the metabolites were exogenously added. The chromatograms of the urine blank and the urine containing the metabolites are shown in Fig. 3. In the urine with the drugs, *p*-hydroxyamphetamine was detected at 9 min and *p*-hydroxymethamphetamine at 10 min (Fig. 3b). In the urine blank, on the other hand, neither metabolite appeared at the corresponding retention times (Fig. 3a). Fig. 4 shows the recovery curves of the exogenously added metabolites by plotting the peak area against the added amounts. A good correlation between the peak area and the amounts was obtained. The coefficient of correlation of *p*-hydroxymethamphetamine was calculated to be 0.9869 and that of *p*-hydroxyamphetamine was 0.9828. We found, however, that the recovery rate attained was lower when the amounts of metabolites added to urine were smaller. The recovery rates for the metabolites are summarized in Table I. When 100 ng each of *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine were added to 1 ml of urine, recovery rates of 77 ± 7 and 76 ± 4%, respectively, were obtained. When 20 ng each of the metabolites were added, 55 ± 7% of the former and 61 ± 4% of the latter were recovered. Based on these results, it is recommended that recovery curves should be utilized for determination of these metabolites.

TABLE I

RECOVERIES OF EXOGENOUSLY ADDED *p*-HYDROXYMETHAMPHETAMINE AND *p*-HYDROXYAMPHETAMINE FROM URINE SAMPLES

Amount added* (ng)	Recovery (mean ± S.D., $n = 6$) (%)	
	p-Hydroxymethamphetamine	*p*-Hydroxyamphetamine
100	77 ± 7	76 ± 4
50	74 ± 6	77 ± 7
40	72 ± 4	74 ± 2
30	64 ± 5	66 ± 14
20	55 ± 7	61 ± 4
10	57 ± 4	57 ± 4

*Indicated amounts of the metabolites were added to 1 ml of urine and extracted as described in the text.

The urine samples from the methamphetamine addicts were analysed both by our procedure for *p*-hydroxylated metabolites and by GC for methamphetamine. A typical chromatogram of the metabolites from an addict is shown in Fig. 5, and the concentrations of methamphetamine, *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine in the urine samples of eight addicts are summarized in Table II. The concentrations of *p*-hydroxymethamphetamine fluctuated from 7.5 to 90.5 ng/ml of urine while those of methamphetamine varied from 4.5 to 157.5 μg/ml of urine. In addition, the contents of *p*-hydroxyamphetamine were in the range of 0.00- 5.56 ng/ml of urine. These values on the metabolites appear small when compared with the data of Table 6 of Caldwell et al. [1]. The reason for this discrepancy may lie in the fact that

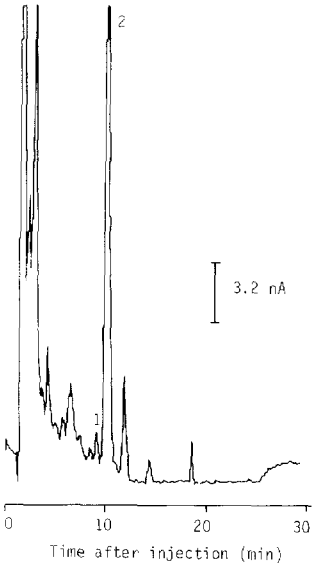

Fig. 5. Typical chromatogram of a urine sample from a methamphetamine addict. Peaks 1 and 2 correspond to *p*-hydroxyamphetamine and *p*-hydroxymethamphetamine, respectively.

TABLE II

CONCENTRATIONS OF METHAMPHETAMINE, *p*-HYDROXYMETHAMPHETAMINE AND *p*-HYDROXYAMPHETAMINE IN THE URINE SAMPLES OF THE ADDICTS

Addict No.	Concentration		
	Methamphetamine (μg/ml)	*p*-Hydroxymethamphetamine (ng/ml)	*p*-Hydroxyamphetamine (ng/ml)
1	4.5	10.0	N.D.*
2	5.0	7.5	Trace
3	15.7	27.5	2.16
4	21.5	12.0	Trace
5	58.0	90.5	5.56
6	63.0	27.5	2.23
7	117.5	57.5	1.46
8	157.5	33.5	Trace

*N.D. = Not detected.

Caldwell et al. [1] quantitated both free and conjugated metabolites together in the first 24-h urine after dosing, while we analysed only the free form of the metabolites in the urine of addicts collected at an arbitrary time.

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