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Determination of methamphetamine, amphetamine and piperidine in human urine by high-performance liquid chromatography with chemiluminescence detection

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ABSTRACT

A high-performance liquid chromatographic method for the determination of trace levels of methamphetamine, amphetamine and piperidine in human urine is reported. The three compounds, extracted into diethyl ether from alkaline urine, were derivatized with dansyl chloride, then separated on a reversed-phase column and detected by chemiluminescence after reaction with bis(2,4,6-trichlorophenyl) oxalate and hydrogen peroxide. The corresponding peaks obtained from human urine were identified as the dansyl derivatives by mass spectrometry. Methamphetamine levels as low as $2 \cdot 10^{-10}$ *M* in urine were determined. The sensitivity of the method is higher than that of Simon's reagent test and gas chromatography.

INTRODUCTION

The abuse of methamphetamine (MA) is a serious drug problem in Japan. Administration of MA has usually been proved by the detection of MA in urine, because a large fraction of the MA administered is excreted into urine as MA itself. Many methods have been developed for the determination of MA and related compounds, including colour tests, UV spectrometry, immunological methods, thin-layer chromatography, gas chromatography (GC), GC mass spectrometry (MS) and high-performance liquid chromatography (HPLC). In these methods, Simon's reagent test has commonly been used as a simple and selective colour test[1], and both

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GC[2–4] and GC–MS[5] have been used as highly sensitive methods. HPLC with UV detection has not been widely used in the determination of MA in biological fluids because of its low sensitivity[6–8]. However, both fluorescence and electrochemical detection have been used for the determination of MA and its hydroxylates by HPLC[9–11].

The reliability of the detection of MA in suspected human urine is obtained by satisfying the following two requirements. The first is that MA must be detected by more than two different and sensitive methods; a sensitive method comparable to GC or GC–MS is necessary. Second, the method should indicate that MA has not been added artificially but excreted into the suspected human urine; the simultaneous determination of MA, its metabolites and, if possible, some urinary compounds is necessary.

We have previously reported the determination method of MA and related compounds by HPLC with chemiluminescence detection[12]. The sensitivity of chemiluminescence detection for primary or secondary amines was much higher than fluorescence detection by using dansyl chloride[13] or naphthalenedialdehyde[12] as derivatizing reagents. The high sensitivity of the method with dansylation satisfies the first of the requirements. The interesting fact was that chromatograms of suspected human urine samples usually showed three major peaks that had the same retention times as dansyl-MA, dansyl-amphetamine (A) and dansyl-piperidine (P). A is a metabolite of MA[14] and P is a compound usually contained in human urine[15]. If the three peaks are identified as dansyl-MA, -A and -P, the second requirement described above is satisfied.

The purposes of this work were to identify dansyl-MA, -A and -P in the corresponding peaks from suspected human urine and to demonstrate several potential advantages of the present method.

EXPERIMENTAL

Chemicals

MA (hydrochloride), A (sulphate) and P were purchased from Dainippon Pharmaceutical (Osaka, Japan), Takeda Pharmaceutical (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. All other chemicals were of analytical-reagent grade or better and used without further purification.

HPLC

The pre-treatment procedures for human urine were as follows. To 2 ml of urine in a tube, 0.2 ml of 10% sodium hydroxide solution and 2.0 ml of diethyl ether were added and the mixture was shaken vigorously for 2 min. After centrifugation (1000 *g*, 10 min), the ether layer was collected. The extraction with diethyl ether was repeated again and the two extracts were combined. If the concentration of MA was not high enough, the ether extract (4 ml) was dried in a stream of nitrogen after the addition of a drop of diethyl ether containing 0.1 *M* acetic acid.

For dansylation, to 0.1 ml of the ether extract (4 ml) or to the evaporated residue, 1.0 ml (1.1 ml for the latter) of 10 m*M* carbonate buffer (pH 9.0) and 0.9 ml of acetone containing 1.0 m*M* dansyl chloride were added successively, and the mixture was incubated at 45°C for 1 h. An aliquot of the solution was injected into the HPLC system.

The HPLC system was as described previously report[12] except that a Jasco (Hachioji, Tokyo, Japan) 880-PU post-column pump and a Soma (Hinode, Tokyo, Japan) S-3400 chemiluminescence monitor (spiral flow cell, 100 μl) were used.

The mobile phase was acetonitrile–water (7:3, v/v) containing 1 m*M* imidazole, with a pH of 7.0 adjusted with nitric acid. The chemilumigenic reagent solution was acetonitrile solution containing 0.5 m*M* bis(2,4,6-trichlorophenyl)oxalate (TCPO) and 0.15 *M* hydrogen peroxide. Other operating conditions were as in the previous study[12].

Mass spectrometry

The HPLC fractionation for the three peaks used the same system as described above except that the loop volume of the injector was 100 μl and a fraction collector was attached just after the analytical column through a switching valve. Acetonitrile–water (7:3, v/v) was used as the mobile phase. After pretreatment (extraction and derivatization) of suspected human urine as described above, an aliquot (100 μl) of the sample solution was injected into the HPLC system. The fractions of the three peaks corresponding to dansyl-MA, -A and -P were collected. The injection of urine was repeated five times.

Electron impact (EI) mass spectra were obtained using a Shimadzu QP-1000 instrument. The ionization voltage and current were 200 eV and 60 μA, respectively.

Simon's reagent test

To 5.0 ml of urine in a tube, 0.5 ml of 10% sodium hydroxide solution and 5.0 ml of diethyl ether were added and the mixture was shaken vigorously for 2 min. After centrifugation (1000 *g*, 10 min), the ether layer was dehydrated with about 1 g of sodium sulphate and decanted. After addition of several drops of acetic acid, the solution was dried in a stream of nitrogen. An aliquot of the precipitate was dissolved in several drops of methanol and spotted on a paper. At the same point of the paper, 20% sodium carbonate, acetaldehyde–ethanol (1:1, v/v) and 1% sodium nitroprusside were spotted successively. If a positive purple-blue coloration was observed, the sample was applied to thin-layer chromatography and the spot of the same R_F value as MA was checked by the same coloration test.

GC

The same residue of the ether extracts obtained for Simon's reagent test was used for GC. Volumes of 200 μl each of ethyl acetate and trifluoroacetic anhydride were added to the residue and the mixture was incubated at 55°C for 1 h. After drying under a flow of nitrogen, the residue was dissolved in ethyl acetate containing diphenylmethane as an internal standard.

The conditions for GC were as follows: apparatus, Shimadzu GC-14A; column, Chromosorb W coated with 2% OV-17 (1.1 m × 2.6 mm I.D.); carrier gas, helium (30 ml/min); detector, flame ionization; column temperature, 130°C; injection port temperature, 160°C; and injection volume, 1 μl. Trifluoroacetyl (TFA) derivatives of A and MA and the internal standard eluted at 2.1, 3.7 and 4.5 min, respectively. TFA-P was eluted too fast to be separated from the large peak of the solvent front.

RESULTS AND DISCUSSION

Typical chromatograms of suspected and control human urine samples

Typical chromatograms of a standard solution and suspected and control human urine are shown in Fig. 1. Peaks of dansyl-A, -P and -MA were observed at 11.9, 12.7 and 18.8 min, respectively, in the standard chromatogram (A). Three peaks (labelled I, II and III from the earliest) were found in the chromatogram of the suspected human urine (B) at the same retention times as the three standards. The concentrations of MA and P were calculated to be $1.6 \cdot 10^{-3}$ and $2.9 \cdot 10^{-2}$ *M*, respectively, in the urine, assuming that peaks I and II are dansyl-A and -MA, respectively. In the chromatogram of the control human urine (C), which showed peak II, no interfering peaks were observed at the retention times of peaks I and II. Further, all other suspected human urine samples tested showed the same three peaks and all control human urine samples showed only peak II.

Peak identification by MS

In previous work, imidazole buffer was used as a catalyst to enhance the generation of chemiluminescence in the TCPO–hydrogen peroxide system[16]. However, the buffer was removed from the mobile phase in the fractionation experiment in order to reduce its interference in MS analysis. The retention times of the three dansyl derivatives were not changed by the removal of imidazole buffer, although their peak heights in chemiluminescence detection became smaller. The three standard peaks (dansyl-A, -P and -MA) and the corresponding three peaks (I, II and III) fractionated from suspected human urine (No. 40) were characterized by MS as in Table I. All characteristic fragment peaks in each standard fraction were observed in the corresponding fractions from the urine with similar relative intensities. The possible

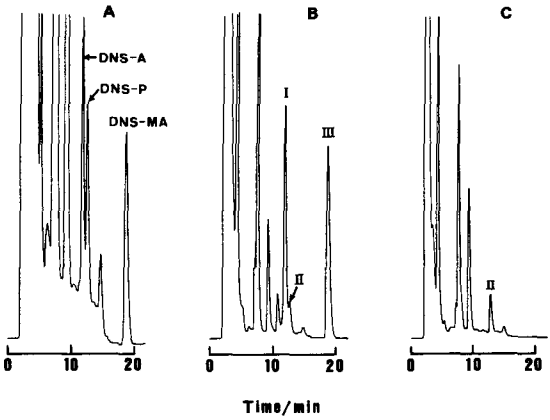


Fig. 1. Typical chromatograms of suspected human urine and control human urine. Analytical column, Inertsil ODS-2; detector, Soma S-3400 with range 64; mobile phase, acetonitrile–water (7:3, v/v) containing 1 m*M* imidazole (pH adjusted at 7.0 with nitric acid); chemiluminescence reagent solution, acetonitrile containing 0.5 m*M* TCPO and 0.15 *M* hydrogen peroxide. (A) Standard solution of dansyl (DNS)-MA, -A and -P (each $1.0 \cdot 10^{-6}$ *M*; attenuation of integrator, 5); (B) suspected human urine diluted 100-fold (attenuation 4 before 16 min and 8 thereafter); (C) control human urine (attenuation 9). For other conditions, see text.

TABLE I

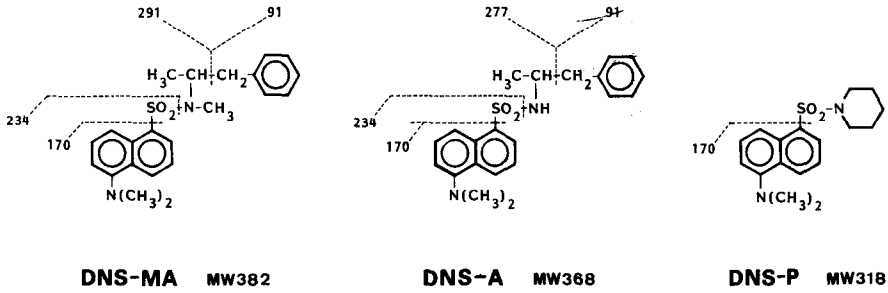
m/z VALUES OF CHARACTERISTIC MASS FRAGMENTS

Standard		*Suspected human urine*	
Peak	*m/z (relative intensity)*	*Peak*	*m/z (relative intensity)*
Dansyl-A	91(11), 154(10), 170(100), 234(15), 277(16), 368(23)	I	91(15), 154(43), 170(100), 234(10), 277(10), 368(11)
Dansyl-P	170(100), 318(22)	II	170(100), 318(20)
Dansyl-MA	91(8), 154(10), 170(100), 234(14), 291(38), 382(8)	III	91(11), 154(9), 170(100), 234(16), 291(43), 382(8)

degradations of the three compounds are shown in Fig. 2. These results suggested that peaks I, II and III were mainly dansyl-A, -P and -MA, respectively.

Purification and enrichment

Sample purification is necessary in the simultaneous determination of MA, A and P in order to remove interfering compounds such as peptides and proteins which react with dansyl chloride. For the purification of MA or A in urine, extraction with organic solvents[17,18], extraction with an ion-pair reagent[19,20], headspace purification[4] and column treatment[6,21] have been reported. We reported previously that MA was easily extracted into diethyl ether from urine by the addition of sodium hydroxide and that there was no interfering peak in the determination of dansyl-MA by HPLC with chemiluminescence detection[12]. Therefore, the optimum conditions for diethyl ether extraction from alkaline urine were examined in this work for the purification of the three compounds. The percentage extraction of the three compounds increased with increase in the concentration of sodium hydroxide, and both MA and A were extracted quantitatively at pH > 9. This result was in agreement with the fact that the pK_a values of alkylamines are generally over 9. The extraction of P was lower than those of MA and A at every pH, and was as low as 50% at concentrations of sodium hydroxide over 10%. The high pK_a of piperidine (11.1) might be considered as a reason. Although the low extraction efficiency may decrease the accuracy, the determination of P is not as important as those of MA and A, as the purpose of the



DNS-MA MW382 **DNS-A** MW368 **DNS-P** MW318

Fig. 2. Possible fragments of dansyl derivatives identified by electron impact MS. MW = Molecular weight.

TABLE II

EFFECT OF ACETIC ACID ON RECOVERIES OF METHAMPHETAMINE (MA) AND PIPERIDINE (P) IN DIETHYL ETHER BY ENRICHMENT WITH A STREAM OF NITROGEN

Each value is the mean ± standard deviation (%) (n = 4).

Addition	*Recovery (%)*			
	MA		*P*	
	$5 \cdot 10^{-8}$ *M*	$5 \cdot 10^{-10}$ *M*	$5 \cdot 10^{-8}$ *M*	$5 \cdot 10^{-10}$ *M*
None	100 ± 3.0	55 ± 5.4	73 ± 1.7	59 ± 3.9
Acetic acid	97 ± 3.9	92 ± 6.2	100 ± 2.2	100 ± 14.1

detection of P was to show that the sample solution tested was urine. Hence a concentration of sodium hydroxide of 10% was adopted in subsequent experiments.

The recoveries of both MA and A were over 99% at concentrations of $5.0 \cdot 10^{-7}$ *M*, which are almost the same as those in the previous study[12]. Linear calibration graphs were also observed in the range $2 \cdot 10^{-8}$–$2 \cdot 10^{-5}$ *M* in urine with a correlation coefficient of 0.996 for MA and 0.994 for A. If 0.1 ml of the diethyl ether extracts was subjected to dansylation, both MA and A at levels as low as $8 \cdot 10^{-9}$ *M* could be detected in urine as the detection limits (signal-to-noise ratio = 3) were $4 \cdot 10^{-15}$ mol[12]. Although the sensitivity of the present method is comparable to that of GC–MS, enrichment is necessary in order to determine trace levels of MA and A accurately. The use of a stream of nitrogen is a well known and easy enrichment technique. However, the recoveries of MA and P from diethyl ether alone decreased at lower concentrations because of the higher volatility of the compounds whose amino groups were unprotonated. To decrease the loss, a small amount of acetic acid was added to the diethyl ether. Without the addition of a drop of diethyl ether containing 0.1 *M* acetic acid, the recoveries of MA at $5 \cdot 10^{-10}$ *M* and of P at both $5 \cdot 10^{-8}$ and $5 \cdot 10^{-10}$ *M* were low. However, the addition of a drop of diethyl ether containing 0.1 *M* acetic acid increased the recoveries of MA and P to 92% and 100%, respectively, as shown in Table II. Using the enrichment technique, MA at levels as low as $2 \cdot 10^{-10}$ *M* was detected in aqueous solution. A linear calibration graph for MA with a correlation efficient of 0.988 was observed in the range $5 \cdot 10^{-10}$–$1 \cdot 10^{-7}$ *M*. This technique was effective for the determination of MA at concentrations less than $1 \cdot 10^{-7}$ *M* in urine.

The enrichment technique was applied to suspected human urine (No. 9) in which MA was not detected either by Simon's reagent test nor by GC. The peak of dansyl-MA was not large enough to be quantified without any enrichment, as shown in Fig. 3A. The peak size was increased about 40-fold by the enrichment, although a small peak was observed near the peak of dansyl-MA, as shown in Fig. 3B. The concentration of MA in the urine was calculated to be $1.3 \cdot 10^{-8}$ *M*. However, the peak of dansyl-A could not be detected in the same chromatogram because the level of A was usually much lower than that of MA, as shown in Table III.

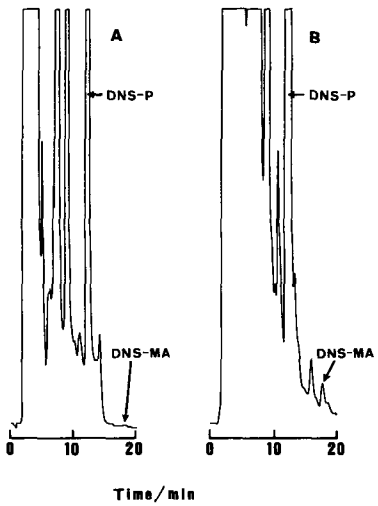


Fig. 3. Chromatograms of suspected human urine (No. 9) (A) without and (B) with enrichment by a stream of nitrogen. Range of the detector, 8; attenuation of the integrator, 6. For other conditions, see Fig. 1.

Comparison with other methods

Both Simon's reagent test and GC are popular detection methods for MA. Therefore, analytical results for the three compounds in several suspected human urine samples obtained by Simon's reagent test, GC and the present HPLC method are compared in Table III. Simon's reagent test, with a detection limit of $6 \cdot 10^{-8}$–$7 \cdot 10^{-7}$ mol, detected MA but did not detect A as the reagent is sensitive to only secondary amines. GC with a flame ionization detector determined TFA derivatives of both MA and A simultaneously and had the same sensitivity for MA as Simon's reagent test. The sensitivity to these compounds of GC with an electron-capture detector might be

TABLE III

COMPARISON OF RESULTS FOR MA, A AND P IN SUSPECTED HUMAN URINE SAMPLES OBTAINED BY THE PRESENT HPLC METHOD, SIMON'S REAGENT TEST AND GC

Urine No.	*MA (mol/l)*			*A (mol/l)*		*P (mol/l)*	
	Simon's reagent	*GC*	*HPLC*	*GC*	*HPLC*	*GC*	*HPLC*
40	D[a]	$1.2 \cdot 10^{-3}$	$1.3 \cdot 10^{-3}$	$2.6 \cdot 10^{-5}$	$3.5 \cdot 10^{-5}$	ND[a]	$7.3 \cdot 10^{-5}$
44	D	$1.9 \cdot 10^{-4}$	$1.7 \cdot 10^{-4}$	$5.0 \cdot 10^{-6}$	$3.0 \cdot 10^{-6}$	ND	$2.8 \cdot 10^{-5}$
41	D	$1.0 \cdot 10^{-4}$	$9.9 \cdot 10^{-5}$	$6.7 \cdot 10^{-6}$	$7.5 \cdot 10^{-6}$	ND	$1.4 \cdot 10^{-5}$
42	D	$6.7 \cdot 10^{-5}$	$8.3 \cdot 10^{-5}$	$3.8 \cdot 10^{-6}$	$3.3 \cdot 10^{-6}$	ND	$2.8 \cdot 10^{-5}$
43	ND	ND	$4.2 \cdot 10^{-8}$	ND	ND	ND	$2.4 \cdot 10^{-6}$
39	ND	ND	$3.6 \cdot 10^{-8}$	ND	ND	ND	$2.8 \cdot 10^{-5}$

[a] D = Detected; ND = not detected.

increased by a factor of about 100–1000. The retention of TFA-P was too weak to be determined under the conditions used. In contrast to these two methods, the present method determined MA, A and P simultaneously, and its sensitivity was much higher than those of the other two methods.

The concentrations of MA determined by the present method were very close to those given by GC. The differences in the concentrations of A between the two methods seemed to be larger than those of MA, possibly became the concentrations of A were much lower than MA in all samples. The concentrations of P in six suspected human urine samples were detected in the range $2.4 \cdot 10^{-6}$–$7.3 \cdot 10^{-5}$ *M*. The level was comparable to the normal value ($1.46 \cdot 10^{-5}$ *M*). These results indicate the high reliability of the present method for the simultaneous determination of MA, A and P in human urine. The precision of the method might be improved by the use of an internal standard. The previous study[12] suggested that the dansyl derivatives of both phenylbutylamine and N-methylphenethylamine eluted between dansyl-P and -MA, and dansyl-N-isopropylbenzylamine was eluted after dansyl-MA. These amines are considered to be suitable internal standards for addition to urine.

REFERENCES

1 F. Feigl, *Spot Tests in Organic Analysis*, Elsevier, Amsterdam, 1956, p. 260.
2 G. Belvedere, S. Caccia, A. Frigerio and A. Jori, *J. Chromatogr.*, 84 (1973) 353.
3 M. Terada, S. Yoshimura, T. Yamamoto, T. Yoshida and Y. Kuroiwa, *Eisei Kagaku*, 29 (1983) 143.
4 H. Tsuchihashi, K. Nakajima, H. Ono, A. Matsushita and M. Nishikawa, *Eisei Kagaku*, 34 (1988) 146.
5 T. Yamamoto, M. Terada, S. Yoshimura, T. Sato, H. Kitagawa, T. Yoshida, H. Aoki and Y. Kuroiwa, *Eisei Kagaku*, 27 (1981) 331.
6 N. Takayama, H. Kobayashi and A. Tsuji, *Eisei Kagaku*, 30 (1984) 14.
7 K. Slais, M. W. F. Nielen, U. A. Th. Brinkman and R. W. Frei, *J. Chromatogr.*, 393 (1987) 57.
8 Y. Hosoi, *Eisei Kagaku*, 33 (1987) 170.
9 B. M. Farrell and T. M. Jefferies, *J. Chromatogr.*, 272 (1983) 111.
10 K. Ishikawa, J. Martinez and J. McGauch, *J. Chromatogr.*, 306 (1984) 394.
11 K. Shimosato, M. Tomita and I. Ijiri, *J. Chromatogr.*, 377 (1986) 279.
12 K. Hayakawa, K. Hasegawa, N. Imaizumi, O. S. Wong and M. Miyazaki, *J. Chromatogr.*, 464 (1989) 343.
13 S. Kobayashi, K. Imai, *Anal. Chem.*, 52 (1980) 424.
14 T. Baba and H. Yoshimura, *Forensic Toxicol. News*, 6 (1988) 7.
15 Y. Okano, T. Kadota, M. Naka, J. Nagata, S. Ijima, A. Matsuda, H. Iwamura, T. Hitoshi, K. Takahama and T. Miyata, *J. Pharmacobio-Dyn.*, 8 (1985) 487.
16 N. Imaizumi, K. Hayakawa and M. Miyazaki, *Analyst (London)*, 114 (1989) 161.
17 Y. Yamamoto, A. Fukui and K. Kondo, *Jpn. J. Legal Med.*, 33 (1979) 105.
18 T. Mitsui and Y. Fujimura, *Bunseki Kagaku*, 64 (1988) T27.
19 S. Motomizu, M. Iwachi and K. Toei, *Bunseki*, 64 (1980) 234.
20 M. Syoyama and T. Sano, *Eisei Kagaku*, 31 (1985) 410.
21 I. Une, M. Yashiki, J. Yamauchi and T. Kojima, *Jpn. J. Legal Med.*, 37 (1983) 63.