

## Simultaneous Determination of Methamphetamine and Its Metabolites in the Urine Samples of Abusers by High Performance Liquid Chromatography with Chemiluminescence Detection

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A HPLC determination method for methamphetamine (MA) and its metabolites in the urine samples of abusers has been developed. MA, amphetamine (AP), norephedrine (NE), *p*-hydroxymethamphetamine (pOHMA), *p*-hydroxyamphetamine (pOHAP) and an internal standard, namely  $\beta$ -phenylethylamine (PEA) were derivatized with dansyl chloride. They were separated on a reversed phase column with gradient elution using an acetonitrile/tetrahydrofuran/imidazole buffer mobile phase and chemiluminescences determined using bis(2,4,6-trichlorophenyl)-oxalate/hydrogen peroxide as post column reagents. The lower determination limits were as low as  $1 \times 10^{-14}$ — $3 \times 10^{-14}$  mol. AP, NE, pOHAP and PEA were derivatized with naphthalene-2,3-dicarboxaldehyde, and were separated on a reversed phase column using an acetonitrile/imidazole buffer mobile phase and chemiluminescences determined. The lower determination limits were  $3 \times 10^{-16}$ — $1.5 \times 10^{-15}$  mol. Enzymatic hydrolysis of glucuronides of pOHMA (pOHMAG) and pOHAP (pOHAPG) allowed them to be determined as pOHMA and pOHAP, respectively. After adjusting the pH of the urine samples to 10.5 and adding PEA, all metabolites except glucuronides were extracted quantitatively into chloroform-isopropanol (3:1). Utilizing the two methods, MA and all metabolites were determined in urine samples of MA abusers. The tendency, in order of decreasing concentration was: [MA] > [A] > [pOHMAG] > [pOHMA] > [NE] > [pOHAPG] > [pOHAP]. Although ephedrine (EP) was detected in several samples, it was not considered to be a metabolite of MA but rather a component derived from cough medicine.

**Keywords** methamphetamine metabolite; human urine; HPLC; chemiluminescence detection; glucuronide

In recent decades, methamphetamine (MA) abuse has become a serious problem in Japan, and several medicines, from which MA or related compounds are biotransformed, are also being imported or used as street drugs.<sup>1)</sup> Methods such as gas chromatography and gas chromatography/mass spectrometry are used routinely in police laboratories to determine MA in urine samples. In some of these laboratories, amphetamine (AP) is determined simultaneously. To increase the reliability of the evidence, the analytical results must indicate that MA has been administered and excreted into the urine. This can be facilitated if the simultaneous determination of MA and as many as possible of its metabolites can be made.

A great deal of effort has gone into studying the metabolism, distribution and excretion of MA using several animal models.<sup>2)</sup> In the human body, AP, norephedrine (NE), *p*-hydroxymethamphetamine (pOHMA), *p*-hydroxyamphetamine (pOHAP) and *p*-hydroxynorephedrine (pOHNE) were identified as metabolites after oral MA administration.<sup>3)</sup> In the urine of abusers, AP, ephedrine (EP), NE, pOHMA and pOHAP have been determined.<sup>4)</sup> It has also been reported that both pOHMA and pOHAP are further metabolized to their corresponding glucuronides.<sup>5)</sup>

There are several highly sensitive high performance liquid chromatographic (HPLC) methods by which these metabolites can be determined.<sup>5,6)</sup> We developed a means to determine MA by chemiluminescence using dansyl (DNS) chloride or naphthalene-2,3-dicarboxaldehyde (NDA) as a fluorescent labeling reagent.<sup>7)</sup> After extraction from human urine and derivatization with DNS chloride, derivatives of MA and AP were separated on a reversed phase column

and chemiluminescences determined. Detection limits were as low as  $1 \times 10^{-10}$  M in urine, several orders better than that of gas chromatographic or gas chromatography/mass spectrometric methods.<sup>8,9)</sup> HPLC with chemiluminescence detection might thus be very appropriate for the desired purpose.

We here describe a HPLC method which simultaneously determines MA and its metabolites in urine samples.

### Materials and Methods

**Chemicals and Solutions** MA hydrochloride and EP hydrochloride were obtained from Dainippon Pharmaceutical (Osaka, Japan). NE hydrochloride and AP sulfate were obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and Takeda Pharmaceutical (Osaka, Japan), respectively.  $\beta$ -Phenylethylamine (PEA) hydrochloride was purchased from Nacalai Tesque (Kyoto, Japan). Other MA related compounds used in this work were provided by the Forensic Science Laboratory, Ishikawa Prefectural Police Headquarters.

DNS chloride and NDA were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Molecular Probes (Eugene, U.S.A.), respectively. Bis(2,4,6-trichlorophenyl)oxalate (TCPO) and 30% hydrogen peroxide were obtained from Tokyo Kasei Kogyo and Kanto Chemicals (Tokyo, Japan), respectively.  $\beta$ -Glucuronidase (EC 3.2.1.31, 200 U/ml) was purchased from Behringer Mannheim (Mannheim, Germany). All other chemicals used were of analytical grade.

Standard solutions of MA and related compounds were prepared by dissolving appropriate amounts of chemicals in water and then diluting them. Solutions used in the pretreatment of urine were the following: acetate buffer (0.2 M, pH 4.5) was prepared by dissolving acetic acid and sodium acetate in water. Borate buffer (0.5 M, pH 10.5) was prepared by dissolving boric acid and sodium hydroxide in water.  $\beta$ -Glucuronidase solution (20 U/ml) was prepared by diluting ten times the above  $\beta$ -glucuronidase solution with water.

Fluorescent labeling solutions were the following: carbonate buffer (0.02 M, pH 9.0) was prepared by dissolving sodium bicarbonate and sodium hydroxide in water, and borate buffer (0.05 M, pH 9.0) was prepared by

TABLE I. Urine Samples of MA Abusers

Urine		User			Administration		Sampling time of urine (h)
No.	pH	Sex <sup>a)</sup>	Age	Type <sup>b)</sup>	Dose (g)	Route <sup>c)</sup>	
H1-49	6	M	53	B	0.03	V	11
H2-06	6	M	59	R	0.06	V	5
H2-30	6	M	34	B	0.05	V/H	22
H2-33	6	F	17	B	Unknown	V/H	39
H2-36	6	M	44	B	0.06	V	32
H2-45	6.5	M	55	B	0.03	V	11
H2-74	6.5	M	41	B	0.03	V	33
H3-33	6.5	M	44	R	0.03	V	33
H3-36	6	M	45	R	0.05	V	0.2
H3-40	6.5	M	35	R	0.25	V	11
H3-51	7.5	F	56	B	0.03	V	19
H3-58	6.5	M	43	R	0.03	V	30

a) M, male; F, female. b) B, first time user; R, regular user. c) V, intravenous; H, inhaled. User type, dose, administration route and sampling time were drawn from individual confessions.

dissolving boric acid and sodium hydroxide in water. DNS chloride ( $2.0 \times 10^{-3}$  M), NDA ( $4.0 \times 10^{-3}$  M), and sodium cyanide (0.02 M) solutions were prepared by dissolving each in acetone, methanol and water, respectively.

Urine samples used in this report are listed in Table I with informations drawn from confessions. In all urine samples, MA was identified by thin layer chromatography/Simon's reagent spot test, gas chromatographic and gas chromatography/mass spectrometric analysis at the Forensic Science Laboratory, Ishikawa Prefectural Police Headquarters. Samples were stored in a freezer ( $-80^\circ\text{C}$ ). Just before use, they were shaken in a water bath ( $30^\circ\text{C}$ ) for 5 min to assure they were completely dissolved.

**Urine Sample Pretreatment** Hydrolysis of glucuronides was done as follows: to 2.0 ml of urine, 0.3 ml of acetate buffer (pH 4.5) and 0.2 ml of  $\beta$ -glucuronidase solution (20 U/ml) were added and the mixture was incubated at  $37^\circ\text{C}$  for 4 h. When urine was not treated with  $\beta$ -glucuronidase, 0.2 ml of water was added to the sample.

MA and its metabolites (except glucuronides) were extracted into chloroform-isopropanol (3:1) according to the previous report<sup>10)</sup> with some modifications. To the urine, 50  $\mu\text{l}$  of  $1.0 \times 10^{-3}$  M PEA hydrochloride solution (the internal standard), 5.0 ml of 10% sodium carbonate, and 2.0 ml of borate buffer (pH 10.5) were added successively. After the addition of 2.0 ml of chloroform-isopropanol (3:1), the mixture was shaken vigorously and centrifuged at  $3000 \times g$  for 10 min. Then, the organic phase was transferred. This procedure was repeated two more times. All the organic phases were combined, divided into two equal aliquots and then dried under a stream of nitrogen at  $37^\circ\text{C}$ . The resultant residues were used in the following fluorescent labeling step.

**Fluorescent Labeling** Both DNS chloride and NDA were used as outlined in our previous report.<sup>7)</sup> One ml of carbonate buffer (pH 9.0) and 1.0 ml of DNS solution were added successively to one of the test tubes containing dried residues. After mixing, the test tube was placed in the dark and incubated at  $45^\circ\text{C}$  for 1 h. The resultant solution which contained DNS derivatives was diluted adequately with acetonitrile-water (1:1) and kept in an ice bath until injection. To the other test tube, 0.8 ml of borate buffer (pH 9.0)-acetonitrile (3:4), 0.4 ml of acetonitrile, 0.1 ml of sodium cyanide solution and 0.1 ml of NDA solution were added, successively. After mixing, the test tube was placed in the dark and allowed to settle at room temperature for 2 h. This resulted in the formation of N-substituted 1-cyanobenz[*f*]isoindole (CBI) derivatives. The resultant solution was diluted adequately (more than 10 times) with acetonitrile-water (1:1).

**HPLC and Its Conditions** The HPLC system consisted of two Shimadzu (Kyoto, Japan) LC-6A pumps, a Rheodyne (Cotati, CA, U.S.A.) 7125 injector (20  $\mu\text{l}$  loop), a Nacalai Tesque (Kyoto, Japan) guard column (Cosmosil 5C<sub>18</sub>, 4.6 mm i.d.  $\times$  50 mm), a GL Sciences (Tokyo, Japan) analytical column (Inertsil ODS-2, 4.6 mm i.d.  $\times$  250 mm), a Shimadzu CTO-6A column oven ( $40^\circ\text{C}$ ), a Shimadzu SCL-6A system controller, a mixing joint (Y-type, Teflon), a Sanuki (Tokyo, Japan) DMX-2000 dual-head short-stroke pump, a Soma (Tokyo, Japan) S-3400 luminomonitor (70  $\mu\text{l}$  spiral cell) and a Shimadzu C-R3A integrator.

The separation of DNS derivatives was carried out by a gradient elution with the following two solutions: solution I,  $1.0 \times 10^{-3}$  M imidazole buffer

(the pH adjusted to 7.0 with nitric acid)-tetrahydrofuran (THF) (5:1) and solution II, acetonitrile-THF (5:1). The percent of solution II in the mobile phase increased from 30% (0 min) to 50% (55 min) with two phases. The gradient profile is shown in Fig. 1(A). Total flow rate was 1.0 ml/min. The mobile phase for CBI derivatives consisted of acetonitrile- $1.0 \times 10^{-3}$  M imidazole buffer (pH 7.0)-THF (51:47:2), and the flow rate was 1.0 ml/min. The post-column chemiluminescence reagent solution was acetonitrile solution containing  $5.0 \times 10^{-4}$  M TCPO and  $1.5 \times 10^{-1}$  M hydrogen peroxide. The flow rate was 1.0 ml/min.

## Results and Discussion

**Fluorescent Labeling and HPLC Conditions** As described above, our previous reports<sup>7-9)</sup> suggested the possibility of the sensitive determination of not only MA, AP, EP but also NE, pOHMA, pOHAP and pOHNE as DNS or CBI derivatives. Therefore, at the beginning of this work, we tested DNS derivatization of NE, pOHMA, pOHAP and pOHNE under the same conditions as in the earlier reports. With the addition of DNS chloride, all test solutions showed strong chemiluminescence; their intensities increased and became constant after 1 h. Also, in earlier reports the mixture of acetonitrile- $1.0 \times 10^{-3}$  M imidazole buffer (pH 7.0) (7:3) was used as a mobile phase for the separation of DNS derivatives of MA and AP on a reversed phase column. Although all DNS derivatives tested in this work were eluted within 20 min using the same mobile phase, urine samples produced several interfering peaks, especially around early eluting analyte peaks. Great improvement was obtained by the gradient elution with acetonitrile-imidazole buffer and by the addition of THF to the mobile phase. As shown in Fig. 1, all DNS derivatives were separately eluted within 50 min. Figure 1(A) shows a typical chromatogram of DNS derivatives of MA, AP, EP, pOHMA, pOHAP and PEA. When PEA was used as an internal standard, calibration curves of MA, AP and EP were linear down to  $1 \times 10^{-14}$  mol by either peak height or peak area method. Calibration curves of pOHMA and pOHAP were linear down to  $3 \times 10^{-14}$  mol. Figure 1(B) shows a chromatogram of  $\beta$ -glucuronidase treated urine sample from a MA abuser after DNS derivatization, and peaks of MA, AP, pOHMA, pOHAP and PEA (internal standard) are seen at the same retention times as those in Fig. 1(A). A small NE peak also eluted just before the large pOHMA peak, although the resolution of the NE and

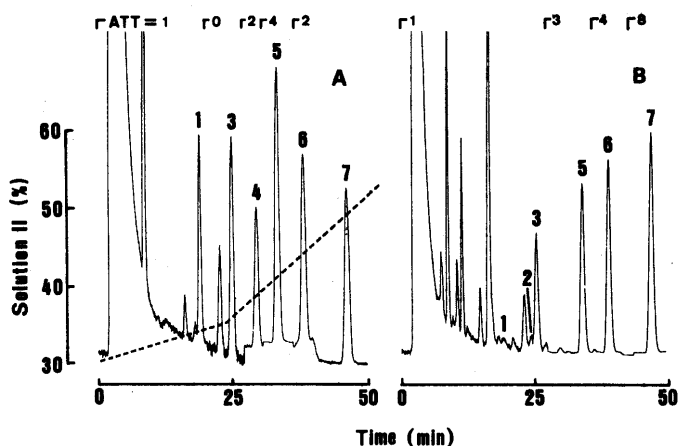


Fig. 1. Chromatograms of (A) Standard Mixture and (B)  $\beta$ -Glucuronidase Treated Urine Sample of a MA Abuser after DNS Derivatization

Peaks: 1, pOHAP; 2, NE; 3, pOHMA; 4, EP; 5, PEA; 6, AP; 7, MA. Injection amount of (A): each  $1.0 \times 10^{-13}$  mol except PEA ( $5.0 \times 10^{-13}$  mol). Urine sample of (B): H3-40.

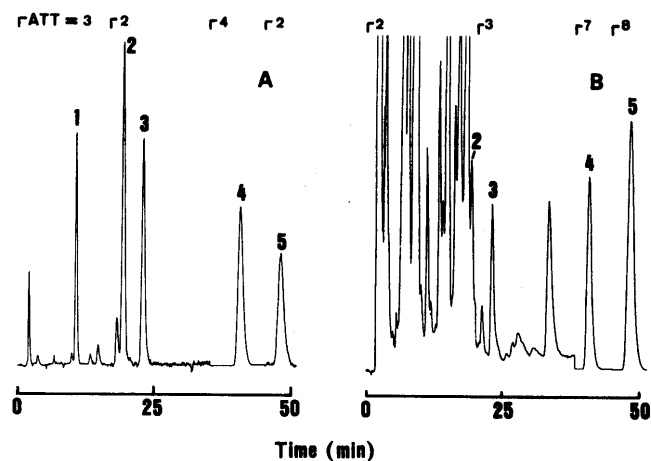


Fig. 2. Chromatograms of (A) Standard Mixture and (B)  $\beta$ -Glucuronidase Treated Urine Sample of a MA Abuser after CBI Derivatization

Peaks: 1, pOHNE; 2, pOHAP; 3, NE; 4, PEA; 5, AP. Injection amount of (A), each  $8.0 \times 10^{-15}$  mol except PEA ( $4.0 \times 10^{-14}$  mol). Urine sample of (B), H3-40.

pOHMA peaks was not complete. The pOHNE peak, whose retention time was 9.5 min, was not detected.

We also reported that CBI derivatives of primary amines are very sensitive to chemiluminescence detection and that their detection limits are lower than those of DNS derivatives.<sup>7)</sup> Thus, NDA was tested as a fluorescent labeling reagent for AP, NE, pOHAP and pOHNE under the same conditions. With the addition of NDA, all test solutions showed strong chemiluminescence, whose intensities increased and became constant after 2 h. The resultant CBI derivatives were all separately eluted within 50 min from a reversed phase column using acetonitrile-imidazole buffer as the mobile phase. Figure 2(A) shows a typical chromatogram of CBI derivatives of AP, NE, pOHAP, pOHNE and PEA. When PEA was used as an internal standard, calibration curves of AP, NE, pOHAP and pOHNE were linear down to  $1.5 \times 10^{-15}$ — $3 \times 10^{-16}$  mol in urine by either peak height or peak area method. Figure 2(B) shows a chromatogram of  $\beta$ -glucuronidase treated urine sample from a MA abuser after CBI derivatization,

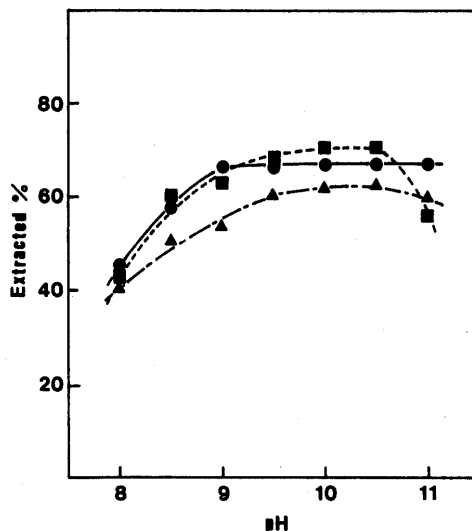


Fig. 3. Effect of Aqueous pH on % of pOHMA, pOHAP and pOHNE Singly Extracted into Chloroform-Isopropanol (3:1)

Symbols: ●, pOHMA; ■, pOHAP; ▲, pOHNE. Experimental conditions, see text.

with peaks of AP, NE and pOHAP at the same retention times as in Fig. 1(A). The pOHNE peak was not observed, however, most likely because of the extremely low level of pOHNE.

The question arises again of which derivatization method is better to provide evidence that MA has been administered and excreted into the urine. MA and pOHMA, which are secondary amines, were detected in Fig. 1(B) but not detected in Fig. 2(B). The EP peak was observed in several urine samples after DNS derivatization as described below. These differences suggest that DNS derivatization is more effective than CBI derivatization for the above purpose.

**Extraction of pOHMA, pOHAP and pOHNE** Before the fluorescent labeling, sample clean-up is necessary. MA and AP are known to be extracted quantitatively into diethylether from aqueous solution at alkaline pH. It has been reported that pOHMA and pOHAP can be extracted into chloroform-isopropanol (3:1) from aqueous solution with the addition of sodium carbonate.<sup>10)</sup> Thus simultaneous extraction of MA and all metabolites into chloroform-isopropanol (3:1) may be possible except glucuronides. However, the final pH of urine samples, after hydrolysis with  $\beta$ -glucuronidase at acidic pH, did not appear to be optimum for this extraction. To pinpoint the optimum pH, 0.2 ml of 0.5 M borate buffers at differing pHs were added to 2.0 ml of standard pOHMA, pOHAP and pOHNE solutions (each  $2.0 \times 10^{-4}$  M). Figure 3 shows the percentage singly extracted of each of the three compounds into 4.0 ml of chloroform-isopropanol (3:1) as a function of urinary pH. The maximum percentages, 63–70%, were observed at pHs 10 and 10.5 for all three, at which the percentages of MA, AP, NE and EP extracted into the organic phase were all over 90%. Therefore, in the following experiments, the pH of all urine samples was roughly adjusted to about 10 with sodium carbonate and then borate buffer (pH 10.5) was added. Extracting urine samples three times at pH 10.5, using 2.0 ml of chloroform-isopropanol (3:1) each time, recoveries of pOHMA, pOHAP and pOHNE were more than 95%.

**Enzymatic Hydrolysis of Glucuronides** When urine

samples of MA abusers before and after  $\beta$ -glucuronidase treatment were compared, peak heights of pOHMA and pOHAP increased after treatment, while peak heights of MA, AP and NE remained constant. Figure 4 shows time course measurements of pOHMA and pOHAP in a urine sample of a MA user with  $\beta$ -glucuronidase (2 U/ml of urine). Both peak heights increased and became constant after 4 h. Coefficient of variation (CV) (%) of these peak heights at 4 h were 4.3% and 10.0%, respectively ( $n=3$ ); thereafter, the maximum peak heights did not increase with increase in the amount of  $\beta$ -glucuronidase. The increases were suppressed by the addition of glucuronic acid as an inhibitor of  $\beta$ -glucuronidase. These results suggested that concentrations of pOHMAG and pOHAPG could be calculated as the differences in peak heights of pOHMA and pOHAP with and without  $\beta$ -glucuronidase treatment, respectively.

**Concentrations of MA and Its Metabolites in Urine Samples** Urine samples of twelve MA users were tested by this method and MA and its metabolites determined

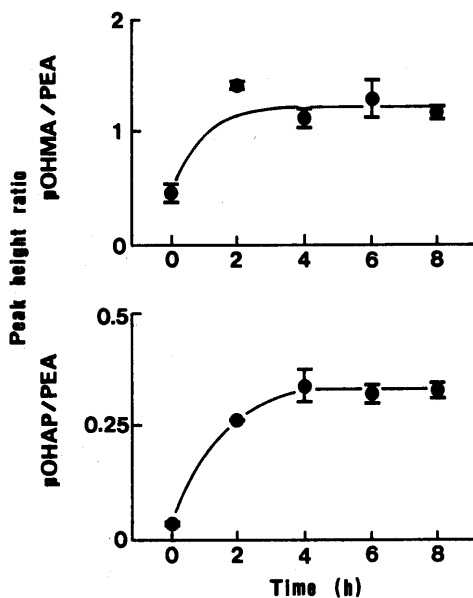


Fig. 4. Time Course Plots of Concentrations of pOHMA and pOHAP in the Urine Sample of a MA Abuser with  $\beta$ -Glucuronidase Added

Urine sample, H3-33. Each symbol and vertical bar represent the mean and S.D. ( $n=3$ ). Experimental conditions, see text.

(Table II). MA, AP, pOHMA, pOHMAG, pOHAP, pOHAPG and EP were determined after DNS derivatization. The lowest concentration of MA was  $9.15 \times 10^{-7}$  M, since the detection limit (cut-off level) by the three screening methods used is around  $1 \times 10^{-6}$  M, while only NE was determined after CBI derivatization because of the incomplete resolution of DNS derivatives of NE and pOHMA as described above. Recently, a HPLC/chemiluminescence detection method has been reported for the simultaneous determination of these metabolites with detection limits as low as  $0.25 \times 10^{-13}$ — $1.33 \times 10^{-13}$  mol on column using 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole as the fluorescent labeling reagent.<sup>11)</sup> However, the determination of NE in urine samples of MA abusers was often impossible due to co-eluting peaks. The fact that NE less than  $1 \times 10^{-7}$  M in urine was determined in Table II suggests the effectiveness of the CBI derivatization/HPLC method in the trace determination of NE.

Table II shows several interesting features of urinary excretions of MA metabolites. First, EP was detected in only two samples (H2-74 and H3-36), and there was no

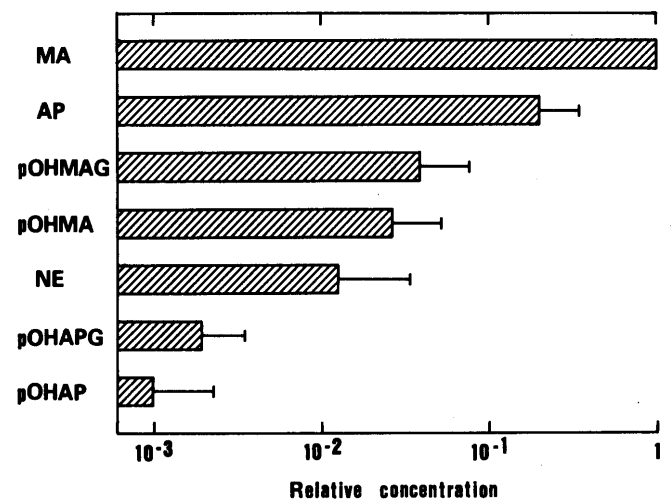


Fig. 5. Relative Concentrations of MA Metabolites in Abuser Urine Samples

Relative concentrations of the six metabolites of MA were calculated for each urine sample in Table II except H2-06, H2-74 and H3-36. Each column and horizontal bar represent the mean and S.D. of the nine samples.

TABLE II. Concentrations of MA and Its Metabolites in Abuser Urine Samples

Urine	Concentration (M)							
	MA	AP	NE	pOHMA	pOHMAG	pOHAP	pOHAPG	EP
H1-49	$1.68 \times 10^{-4}$	$2.32 \times 10^{-5}$	$7.07 \times 10^{-7}$	$2.13 \times 10^{-6}$	$1.02 \times 10^{-6}$	$7.29 \times 10^{-8}$	$4.16 \times 10^{-7}$	ND
H2-06	$9.15 \times 10^{-7}$	$4.84 \times 10^{-7}$	ND	ND	ND	ND	ND	ND
H2-30	$2.12 \times 10^{-4}$	$1.62 \times 10^{-5}$	$4.08 \times 10^{-7}$	$7.42 \times 10^{-7}$	$2.24 \times 10^{-6}$	$9.45 \times 10^{-8}$	$5.65 \times 10^{-8}$	ND
H2-33	$1.95 \times 10^{-4}$	$7.34 \times 10^{-5}$	$3.07 \times 10^{-6}$	$3.53 \times 10^{-6}$	$4.00 \times 10^{-6}$	$4.27 \times 10^{-7}$	$1.63 \times 10^{-7}$	ND
H2-36	$3.26 \times 10^{-5}$	$1.07 \times 10^{-5}$	$2.05 \times 10^{-7}$	$1.08 \times 10^{-6}$	$2.47 \times 10^{-6}$	$1.40 \times 10^{-8}$	$3.16 \times 10^{-8}$	ND
H2-45	$1.49 \times 10^{-4}$	$1.38 \times 10^{-5}$	$8.00 \times 10^{-7}$	$5.15 \times 10^{-6}$	$1.02 \times 10^{-5}$	$1.27 \times 10^{-8}$	$6.22 \times 10^{-7}$	ND
H2-74	$5.75 \times 10^{-6}$	$2.83 \times 10^{-6}$	$5.95 \times 10^{-8}$	ND	ND	ND	ND	$1.15 \times 10^{-6}$
H3-33	$6.92 \times 10^{-4}$	$3.71 \times 10^{-5}$	$1.92 \times 10^{-6}$	$7.76 \times 10^{-6}$	$2.03 \times 10^{-5}$	$1.98 \times 10^{-7}$	$1.78 \times 10^{-6}$	ND
H3-36	$1.34 \times 10^{-3}$	$9.13 \times 10^{-5}$	$2.26 \times 10^{-6}$	$1.03 \times 10^{-5}$	$3.82 \times 10^{-5}$	$1.65 \times 10^{-7}$	$7.50 \times 10^{-7}$	$2.50 \times 10^{-6}$
H3-40	$1.24 \times 10^{-3}$	$7.23 \times 10^{-5}$	$7.39 \times 10^{-7}$	$5.70 \times 10^{-6}$	$1.09 \times 10^{-5}$	$1.80 \times 10^{-8}$	$2.70 \times 10^{-7}$	ND
H3-51	$3.72 \times 10^{-5}$	$1.70 \times 10^{-5}$	$2.63 \times 10^{-6}$	$3.42 \times 10^{-6}$	$4.46 \times 10^{-6}$	$1.54 \times 10^{-7}$	$2.37 \times 10^{-7}$	ND
H3-58	$3.18 \times 10^{-4}$	$6.93 \times 10^{-5}$	$1.69 \times 10^{-6}$	$8.15 \times 10^{-6}$	$1.04 \times 10^{-5}$	$1.93 \times 10^{-7}$	$4.12 \times 10^{-7}$	ND

ND, not detected.

relation found between EP and MA concentrations. Considering that EP is often contained in commercially available cough medicines, it is possible that EP in these samples might not be a metabolite of MA but rather a component originating from the cough medicine taken by the abusers.

Secondly, there tended to be a recognizable order in the concentrations of MA metabolites. Both H2-74 and H3-36 were not used, since NE is biotransformed from AP as well as EP. In samples other than H2-06, all six of the MA metabolites were determined in this decreasing order of concentration:  $[MA] > [AP] > [pOHMA + pOHMAG] > [NE] > [pOHAP + pOHAPG]$ . When glucuronides were separately calculated from unconjugates, the order was  $[MA] > [AP] > [pOHMAG] > [pOHMA] > [NE] > [pOHAPG] > [pOHAP]$  as shown in Fig. 5. Although the number of urine samples tested was not large and there were two minor exceptions:  $[pOHMA] > [pOHMAG]$  (H1-49) and  $[pOHAP] > [pOHAPG]$  (H2-33), it is noteworthy that the orders were the same in spite of differences in dose, sampling time, user type and other

information in Table I. If analytical results also bear out these tendencies, the reliability of the evidence will be even stronger.

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