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High-performance liquid chromatography of methamphetamine and its related compounds in human urine following derivatization with fluorescein isothiocyanate

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Abstract

A high-performance liquid chromatographic method with fluorescence detection for the determination of methamphetamine and its related compounds is reported. Methamphetamine, amphetamine, norephedrine, *p*-hydroxymethamphetamine and 1-phenylethylamine as an internal standard were extracted from human urine, derivatized with fluorescein-4-isothiocyanate, and then separated on a reversed-phase column within 36 min. The fluorescence intensity of the effluent was monitored at excitation and emission wavelengths of 496 and 518 nm, respectively. Calibration curves were confirmed to be linear up to at least 100 pmol on the column with a correlation coefficient (*r*) of 0.994–0.999 for the target compounds. The detection limits (*S/N*=3) were 55–105 fmol per 20- μ l injection. The method was successfully applied to urine samples taken from methamphetamine addicts. © 1997 Elsevier Science B.V.

Keywords: Methamphetamine; Amphetamine; Norephedrine; *p*-Hydroxymethamphetamine; Fluorescein isothiocyanate

1. Introduction

Although methamphetamine (MP), an indirectly acting adrenomimetic amine with a high abuse potential is a Drug Enforcement Administration (schedule 2) controlled substance, the illegal synthesis in private laboratories is a major street supplier. In Japan, MP is the most frequently abused drug, and about 15 000 persons were arrested for MP offences, and over 160 kg of the drug were seized in 1992 [1].

In the human body, MP is expected to undergo three metabolic pathways, namely N-demethylation,

aromatic hydroxylation and aliphatic hydroxylation at the methylene group next to the benzene ring. After oral administration of MP, the metabolites which were found in human urine were the unchanged drug, amphetamine (AP), *p*-hydroxymethamphetamine (*p*-HMP), norephedrine (NE), *p*-hydroxyamphetamine (*p*-HAP) and *p*-hydroxynorephedrine (*p*-HNE) [2]. Therefore, in order to meet the requirements of pharmacokinetics and forensic studies, a sensitive and selective analytical method should be developed. Several methods have been introduced, among those, thin layer chromatography (TLC) after labeling with a fluorescent reagent [3], polarization fluoroimmunoassay [4,5], gas chromatography (GC) [6,7], gas chromatography–mass spectrometry (GC–MS) [8,9], and high-performance

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liquid chromatography (HPLC) with fluorescence or chemiluminescence detection [10–15].

In this study, fluorescein-4-isothiocyanate (FITC), a fluorescence derivatization reagent, that has been reported to react with amino acids and proteins [16–18] to give derivatives showing strong fluorescence was tested to determine MP, AP, NE, and *p*-HMP in human urine using the internal standard (I.S.) 1-phenylethylamine (PEA).

2. Experimental

2.1. Materials and reagents

FITC was purchased from Dojindo Laboratories (Tokyo, Japan). MP hydrochloride, NE, and PEA (I.S.), were obtained from Dainippon Pharmacy (Osaka, Japan), Aldrich (Milwaukee, WI, USA), and Nacalai Tesque (Kyoto, Japan), respectively. AP sulphate and *p*-HMP sulphate were synthesized in our laboratory according to the literature [19,20], these compounds gave satisfactory elemental analyses and MS data. Dansyl-Cl (Dns-Cl) and acetonitrile (HPLC grade) were purchased from Wako (Osaka, Japan). All other chemicals were of analytical reagent grade. Water was deionized and passed through a pure line WL21P (Yamato Kagaku, Tokyo, Japan) water purification system.

Standard solutions ($1 \cdot 10^{-2}$ M) of MP and its related compounds were prepared by dissolving and then diluting these compounds in water. These standard solutions were kept in the refrigerator at 4°C and were stable at least for 1 month.

Spiked urine samples were prepared by adding the proper volume of standard MPs solution to fresh, drug-free human urine collected from healthy subjects. Urine samples from MP addicts were kindly supplied by the Forensic Laboratory of Nagasaki Prefectural Police Headquarters, and were kept at –20°C until needed.

The 20 mM phosphate buffer (pH 7.9) used in the mobile phase was prepared by diluting a mixture of 0.1 M Na_2HPO_4 – NaH_2PO_4 (pH 7.9) to the proper volume. The 0.2 M carbonate buffer (pH 9.0) used for the derivatization reaction was prepared by adjusting the pH of 0.2 M NaHCO_3 with the proper

volume of 0.2 M Na_2CO_3 to get a pH of 9.0. The 0.5 M borate buffer (pH 10.5) used in the extraction was prepared by dissolving the proper amounts of boric acid and sodium hydroxide in water.

2.2. Pretreatment of urine samples

MP and its metabolites were extracted from urine as follows: to 2 ml of urine or diluted urine sample in a test tube, 20 μl of $1 \cdot 10^{-4}$ M PEA in water were added as an I.S., followed by the addition of 400 μl conc. HCl, the mixture was heated at 80°C for 1 h, cooled with tap water, and neutralized with 600 μl of 25% liquid ammonia. A 5-ml volume of Na_2CO_3 (10% w/v), 2 ml of 0.5 M borate buffer (pH 10.5), and 2 ml of chloroform–isopropanol (3:1, v/v) were added successively. The mixture was vortex mixed for 1 min, then centrifuged at 1500 g for 10 min at 12.5°C. The organic layer was taken, and the extraction was repeated once more. After combining, 100 μl of the organic phase were transferred to a screw-capped reaction vial, followed by the addition of 10 μl acetic acid and dried up under a stream of N_2 gas at room temperature. The resultant residue was redissolved in 50 μl 0.2 M carbonate buffer (pH 9) to be used for labeling with FITC.

This procedure has been used to measure the total concentrations of the metabolites in urine including *p*-HMP conjugates, therefore, to measure the free portion of *p*-HMP, the hydrolysis step with conc. HCl was not carried out.

2.3. Labeling with FITC

To 50 μl of sample in 0.2 M carbonate buffer (pH 9.0) in a screw-capped vial, 50 μl of 10 mM FITC in ethanol were added, mixed and placed in the dark at 80°C for 15 min, then 20 μl of the reaction mixture were injected onto the column.

2.4. Measurement of fluorescence

A 650-10S (Hitachi, Tokyo, Japan) with a 10-mm quartz cell was used to determine the fluorescence properties of FITC derivatives.

2.5. HPLC system

The HPLC system consisted of two HPLC pumps (LC10AS; Shimadzu, Kyoto, Japan), a 7125 injector with a 20- μ l loop (Rheodyne, Cotati, CA, USA), an analytical column (250 $\times$ 4.6 mm I.D., 5 μ m: Daisopak SP-120-5-ODS, Daiso, Osaka, Japan), an RF-550 spectrofluorometer (Shimadzu, Kyoto, Japan), set at λ_{ex} and λ_{em} of 496 and 518 nm, respectively, and an R-01 recorder (Rikadenki, Tokyo, Japan). Stainless steel tubing (0.5 mm) was used in all flow lines.

The separation of FITC derivatives was carried out by using eluent A (80:20, v/v) and eluent B (76:24, v/v) 20 mM phosphate buffer–acetonitrile (pH 7.9) as the mobile phase. Eluent A was pumped for 16 min, and then immediately changed to eluent B at a flow-rate of 0.8 ml min⁻¹ for both. The eluents were premixed, passed through a JG type 0.2- μ m (Nihon Millipore, Yonezawa, Japan) membrane filter and degassed just prior to use.

2.6. Standard calibration curves

Fresh, drug-free human urine containing $1 \cdot 10^{-6}$ M of the I.S., was spiked with MP, AP, NE and *p*-HMP to provide final concentrations in the range $1.5 \cdot 10^{-8}$ – $1 \cdot 10^{-5}$ M per compound. These samples were analyzed as described above. Standard calibration curves for each compound were obtained by plotting the peak height ratio of the compound to that of the internal standard against its concentration.

2.7. Validation of the method

The proposed method was evaluated to assess within-day and between-day variations. Fresh, drug-free human urine containing I.S. was spiked at concentrations of $1.5 \cdot 10^{-6}$ M of MP and AP, and $1 \cdot 10^{-6}$ M of NE and *p*-HMP. Four samples or more were analyzed day by day as described above over a period of 9 days.

To the spiked urine, aliquots of MPs standard solution were added to provide final concentrations of 2, 4, and $8 \cdot 10^{-6}$ M of *p*-HMP, and NE, and $3 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ M of AP and MP. Three samples of each concentration were analyzed as described above.

For comparison, 14 urine samples containing the I.S. were spiked with AP and MP at concentrations ranging from $1 \cdot 10^{-6}$ M to $9.5 \cdot 10^{-6}$ M per compound, and analyzed simultaneously with the proposed FITC method and also with Dns-Cl method. The dansylation procedure, HPLC conditions, as well as the λ_{ex} and λ_{em} of dansyl derivatives were as reported in the literature [13].

3. Results and discussion

3.1. Measurement of fluorescence

The fluorescence properties of FITC were studied by diluting FITC and the MP reaction mixture in different solvents. The strongest fluorescence intensity was observed when the reaction mixture was diluted in aqueous solutions of a miscible organic solvent at pH values >7, on the other hand, there were no significant changes in the maximum excitation and emission wavelengths. The fluorescence intensity in organic solvents mixtures was very weak and did not exceed 0.05% of that in the aqueous solutions at best.

3.2. Derivatization with FITC

The derivatization reaction (Fig. 1) was optimized using $5 \cdot 10^{-6}$ M standard solution of MP and its related compounds. The maximum yield (relative fluorescence intensity, RFI) as peak height was obtained at 80°C for 15 min or at 55°C for 30 min (Fig. 2). The early eluted derivatives (i.e. *p*-OH metabolites) were better separated when the reaction mixture was heated at elevated temperature (i.e. 80°C) since there was a significant reduction in the reagent's blank fluorescence. When FITC concen-

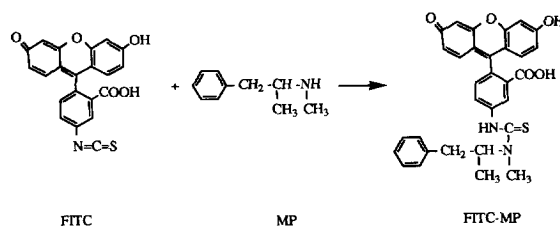


Fig. 1. Reaction scheme for labeling of MP with FITC.

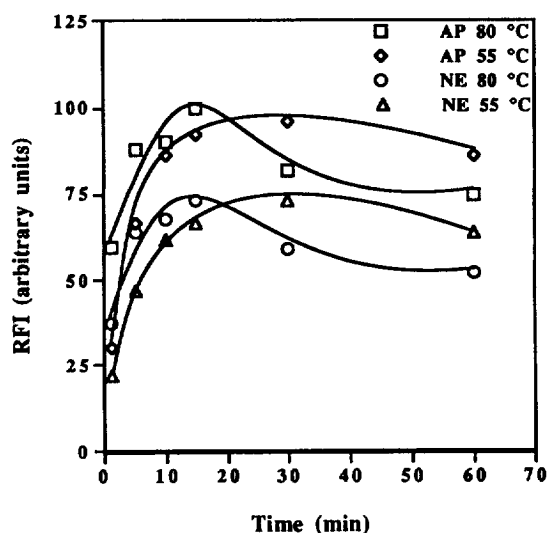


Fig. 2. Effect of the reaction time on the derivatization yield of AP and NE with FITC at 80°C and 55°C.

trations over a range of 1.25–40 mM were examined, the maximum and constant RFIs were observed at the concentration of 10 mM or greater. Among several buffers at various concentrations and pH values which were used to dissolve the sample, the use of carbonate buffer showed the largest RFI at a pH range 8.5–9.5 (Fig. 3) while its concentration in

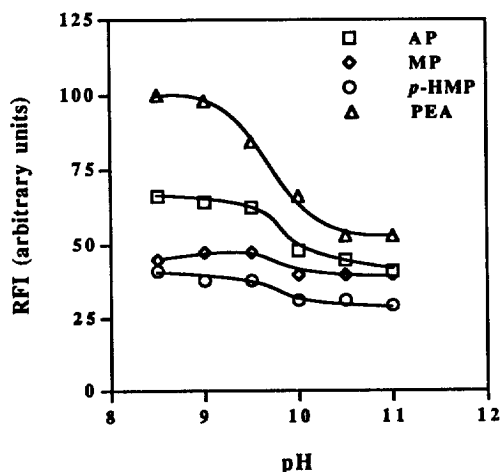


Fig. 3. Effect of the pH of carbonate buffer (0.2 M NaHCO₃–Na₂CO₃) on the derivatization yield of MP, AP, *p*-HMP and PEA with FITC.

the range 25–200 mM showed no significant effect. Regardless whether solvent was acetone, DMSO, DMF or 0.2 M carbonate buffer (pH 9.0), FITC reacted with MP, but the highest derivatization yield was obtained when it was dissolved in ethanol. Accordingly, the derivatization reaction conditions were selected to be as follows: temperature and time, 80°C for 15 min, 10 mM FITC in ethanol and sample in 0.2 M carbonate buffer (pH 9.0). The derivatives were confirmed to be stable for at least 24 h at room temperature.

3.3. Pretreatment of urine samples

Compared to the solid-phase extraction, a convenient and a low-cost liquid–liquid extraction procedure has been reported [15], in which MP and its metabolites including the polar ones were extracted into chloroform–isopropanol (3:1, v/v). Although free *p*-hydroxy metabolites are extractable by this procedure, the conjugates which cannot be extracted as such from urine, should be hydrolyzed either enzymatically [15] or by acid hydrolysis [12] prior to extraction. In case of the samples which are expected to have high concentrations of MP and its metabolites, the urine could be diluted with water if needed.

3.4. HPLC separation

MP and its metabolites were separated on a reversed-phase column with a mixture of 20% acetonitrile in 20 mM phosphate buffer (pH 7.9) which was changed quickly at 16 min to 24% acetonitrile in the same buffer. The best separation was obtained by the use of a basic (pH 7.9) elution buffer. Typical chromatograms of a standard mixture, control human urine and a urine sample from a MP addict are shown in Fig. 4. The FITC derivatives of *p*-HMP, NE, PEA, AP, and MP were eluted at 15, 16.8, 26.6, 32, and 35.2 min, respectively. The peaks corresponding to FITC–MP, –AP, –*p*-HMP and –NE in the chromatogram of the standard mixture (Fig. 4A) were found in the urine sample of the MP addict (Fig. 4C). No interfering peaks were observed at the retention times of the FITC derivatives of MP and its metabolites in the control human urine (Fig. 4B).

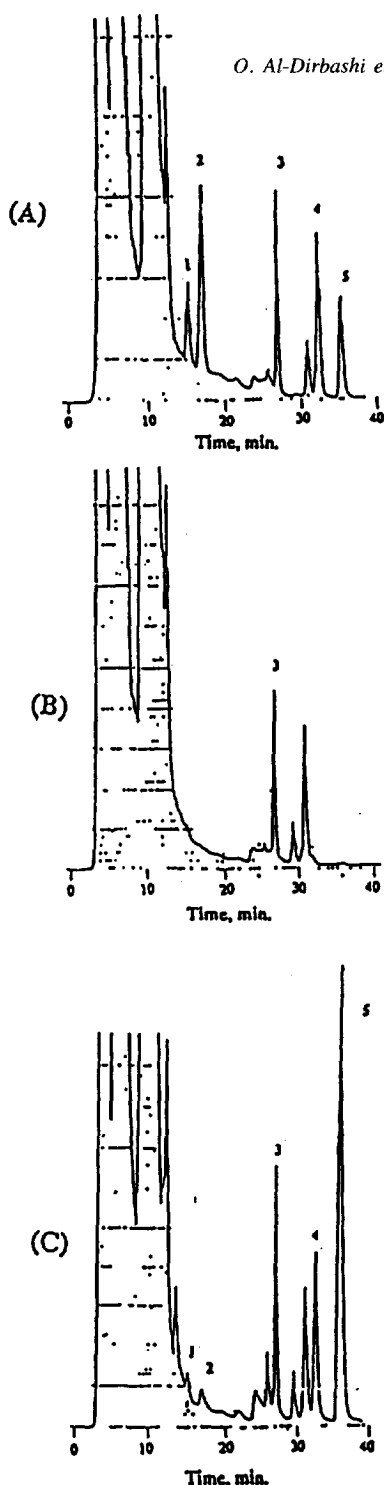


Fig. 4. Typical chromatograms of (A) standard sample of FITC derivatives sample amount on column (pmol): (1) *p*-HMP, 5 (2) NE, 9 (3) PEA (I.S.) (4) AP (5) MP, 6.5 pmol each. (B) 10-fold diluted control human urine. (C) 10-fold diluted MP addict urine. For other experimental conditions refer to the text.

3.5. Calibration curves and detection limits

The calibration curves were prepared by spiking a fresh, drug-free human urine with MP and its metabolites. The linear relationships between the peak height ratio of the target compounds to the I.S. and sample concentration were obtained ($r=0.994$ – 0.999) in the concentration range of $1.5 \cdot 10^{-8}$ – $1 \cdot 10^{-5}$ M for AP, MP, NE and $2.5 \cdot 10^{-8}$ – $1 \cdot 10^{-5}$ for *p*-HMP. The equations obtained were as follows: $y=0.082x-0.016$ for AP, $y=0.061x-0.024$ for MP, $y=0.028x+0.011$ for *p*-HMP and $y=0.049x+0.002$ for NE, where y is the peak height ratio of the target compounds to the I.S., and x is the concentration in pmol/20- μ l injection. The lower limits of detection ($S/N=3$) for MP and AP were 55 fmol/20- μ l injection ($5.5 \cdot 10^{-9}$ M in urine), and 90 ($9 \cdot 10^{-9}$ M in urine), 105 fmol/20- μ l injection ($1.05 \cdot 10^{-8}$ M in urine) for *p*-HMP and NE, respectively.

3.6. Validation of the method

Within-day and between-day variation data of the proposed method are reported in Table 1. The relative standard deviation (% R.S.D.) values of a within-day precision study ($n=6$) were less than 2.6%, and those of the between-day study over 9 days ($n=5$) were less than 7.1% for all the target compounds at the concentrations of $1 \cdot 10^{-6}$ M of *p*-HMP, NE and $1.5 \cdot 10^{-6}$ M of AP and MP.

The quantitation of MP and AP in 14 spiked human urine samples obtained by the proposed method were checked against another method reported in the literature [13], (i.e. HPLC–fluorescence after Dns-Cl derivatization). The calibration curves of Dns-AP and -MP were constructed by plotting the fluorescence intensity as peak height against the concentration in urine, and were confirmed to be linear over the concentration range of $1 \cdot 10^{-7}$ – $1 \cdot 10^{-5}$ M, with a correlation coefficients (r) of 0.999 of both compounds. As shown in Fig. 5, the concentrations obtained by the present method were well correlated with those determined by the Dns-Cl method. The coefficient of correlation between the two methods were 0.996 and 0.994 for AP and MP respectively. The advantage of FITC over Dns-Cl as a fluorophore, is due to the fact that its λ_{ex} (i.e. 496 nm) is very close to the output of argon-ion laser

Table 1

Within- and between-day reproducibility of the analysis of MP and its metabolites in human urine

Compound	Conc. added ($\cdot 10^{-6}$ M)	Within-day reproducibility ($n=6$)			Between-day reproducibility ($n=5$)		
		Mean ($\cdot 10^{-6}$ M)	S.D.	R.S.D. (%)	Mean ($\cdot 10^{-6}$ M)	S.D.	R.S.D. (%)
<i>p</i> -HMP	1.00	1.01	0.02	2.6	1.02	0.07	7.1
NE	1.00	1.20	0.01	2.1	1.14	0.05	5.3
AP	1.50	1.66	0.01	1.7	1.64	0.02	2.4
MP	1.50	1.73	0.02	2.0	1.69	0.05	3.8

S.D.=standard deviation.

R.S.D.=relative standard deviation.

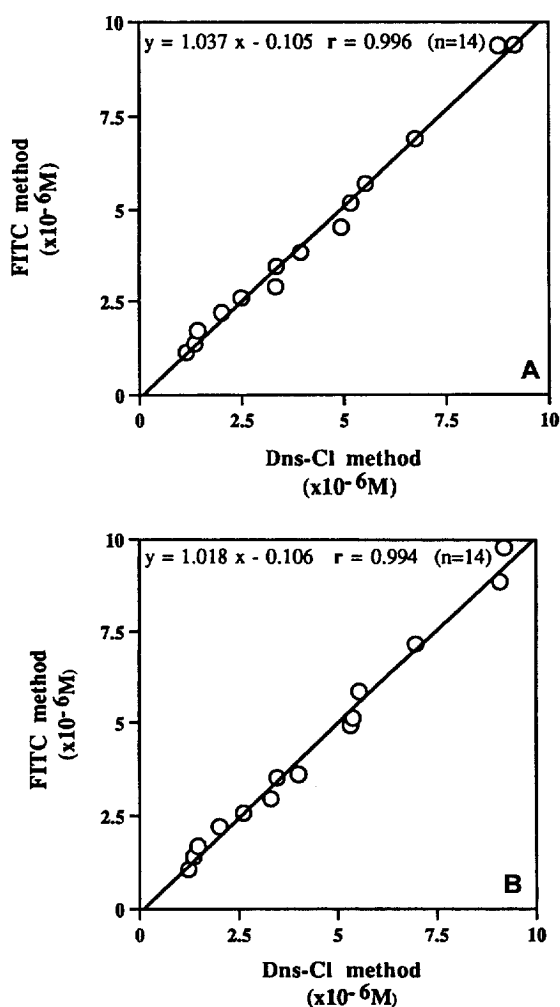


Fig. 5. Correlation between the concentrations of AP and MP in human urine ($n=14$) determined by the FITC method and by Dns-Cl method. (A) AP; (B) MP.

light emission (i.e. 488 nm), therefore it might show higher sensitivity using the highly sensitive laser induced fluorescence detection.

In an attempt to examine the accuracy of the proposed method, the urine spiked with the MPs for within and between-day studies which was kept in the refrigerator at 4°C for 2 weeks, was further spiked with aliquots of standard MP solutions to provide another 3 different concentrations of each compound. As shown in Table 2, the recoveries of all the compounds at the 3 different concentrations were between 90–104.6% and the absolute percentage differences between the added and the found concentrations were in the range of 1–8%.

According to these results, the proposed method showed satisfactory linear relationship between the RFI and the sample concentration in addition to sufficient precision and accuracy, as well as good correlation with another method reported in the literature [13]. Furthermore, the sensitivity of the proposed method is higher than that of GC with FID or ECD [6,7] and GC-MS [8,9]. In comparison with the immunological methods [4,5], which are not particularly specific [8], the proposed method is superior since the MPs are structurally quite simple, closely related to some compounds produced in the body and to several others that are commonly used for the treatment of common cold and allergy symptoms [21]. On the other hand, the methods utilized HPLC with chemiluminescence detection showed higher sensitivity [10–15], so this method may be more sensitive using peroxyoxalate chemiluminescence, or laser induced fluorescence detection.

Table 2
Recovery rates and quantitation of MP and its metabolites in spiked human urine

Compound	Concentration in urine ^a ($\cdot 10^{-6}$ M)	Concentration added ($\cdot 10^{-6}$ M)			Concentration found ^b (S.D.) ($\cdot 10^{-6}$ M)			Bias ^c (%)			Recovery (%)		
		A	B	C	A	B	C	A	B	C	A	B	C
<i>p</i> -HMP	1	1	3	7	1.98 (0.19)	3.93 (0.12)	7.49 (0.30)	−1	−1.8	−6.4	90	98.2	93.6
NE	1	1	3	7	2.09 (0.19)	3.68 (0.33)	7.47 (0.31)	+4.5	−8	−6.6	104.5	92	93.4
AP	1.5	1.5	3.5	8.5	2.83 (0.23)	4.73 (0.34)	9.35 (0.39)	−5.7	−5.4	−6.5	94.3	94.6	93.5
MP	1.5	1.5	3.5	8.5	2.93 (0.22)	5.23 (0.17)	10.32 (0.46)	−2.3	+4.6	+3.2	97.7	104.6	103.2

^a This urine was spiked first to be used in within- and between-day variation studies, and after 2 weeks was further spiked to provide three different concentrations (A, B, and C).

^b Average concentrations of 3 samples with S.D. in parentheses.

^c Bias (%) = (found − total added/added) × 100.

3.7. Assay of MP and its metabolites in MP addicts urine samples

To evaluate the applicability of the proposed method, samples from MP addicts were analyzed. In order to determine the major metabolites (i.e. MP and AP), the urine samples were diluted 4-fold (sample 4) to 100-fold (samples 1, 3, and 5), and accordingly, the net volumes of urine needed were 20 μ l for samples 1, 3 and 5, while 100, 500, and 200 μ l were used in case of samples 2, 4 and 7 respectively. Sample 6 which was examined without dilution, was confirmed to be free of MP and its metabolites. To determine the less available metabolites (i.e. *p*-HMP and NE), a 4-fold dilution was satisfactory. After extraction, derivatization, and injection onto the column, six out of seven samples

were confirmed to be MP positive. Table 3 shows the concentrations of MP, AP, NE, and *p*-HMP in MP abusers' urine. The concentrations of MP metabolites determined in this experiment are following the same decreasing order suggested in a previous report [15], in which the concentration of MP > AP > *p*-HMP > *p*-HMP-C > *p*-HMP > NE (Fig. 6).

4. Conclusions

FITC which has not previously been used to derivatize MP and its related compound in an HPLC system has shown sufficient linearity, sensitivity, selectivity, repeatability, correlation with currently accepted methods, accuracy and precision. It was used to determine MP and its related compounds in a

Table 3
Concentrations of MA and its metabolites in suspected urine samples

Sample	Age	Sex	Concentration in urine (M)				
			<i>p</i> -HMP ^a	<i>p</i> -HMP-C ^{a,c}	NE ^a	AP ^b	MP ^b
1	44	M	$2.7 \cdot 10^{-6}$	$1.5 \cdot 10^{-5}$	$4.0 \cdot 10^{-6}$	$5.1 \cdot 10^{-5}$ (2.7)	$1.0 \cdot 10^{-3}$ (5.9)
2	31	F	$1.1 \cdot 10^{-6}$	$4.5 \cdot 10^{-6}$	$3.2 \cdot 10^{-7}$	$4.3 \cdot 10^{-6}$ (3.3)	$7.8 \cdot 10^{-5}$ (2.4)
3	37	M	$5.2 \cdot 10^{-6}$	$3.8 \cdot 10^{-6}$	$1.3 \cdot 10^{-6}$	$1.8 \cdot 10^{-5}$ (5.4)	$2.5 \cdot 10^{-4}$ (8.8)
4	32	M	$3.1 \cdot 10^{-7}$	$1.6 \cdot 10^{-7}$	$1.5 \cdot 10^{-7}$	$1.3 \cdot 10^{-6}$ (8.5)	$1.3 \cdot 10^{-6}$ (13.0)
5	32	M	$2.1 \cdot 10^{-5}$	$3.8 \cdot 10^{-5}$	$1.2 \cdot 10^{-5}$	$9.7 \cdot 10^{-5}$ (7.2)	$8.3 \cdot 10^{-4}$ (3.1)
6	38	M	ND ^d	ND	ND	ND	ND
7	44	F	$1.6 \cdot 10^{-6}$	$1.9 \cdot 10^{-5}$	$8.4 \cdot 10^{-7}$	$9.5 \cdot 10^{-6}$ (0.5)	$3.7 \cdot 10^{-5}$ (5.4)

^a Average of duplicates.

^b Average of triplicates and parentheses indicate the % RSD.

^c Conjugates of *p*-HMP.

^d Not detectable.

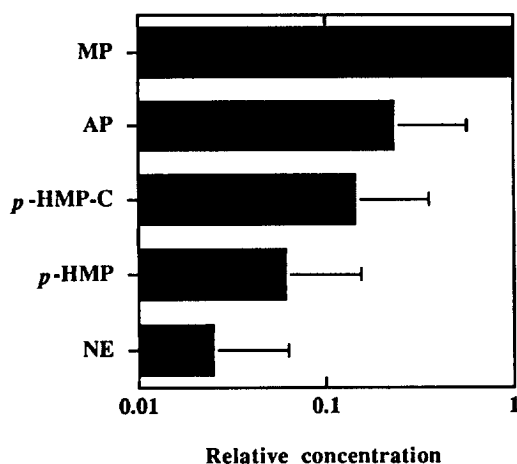


Fig. 6. Relative concentrations of MP metabolites in addicts' urine samples ($n=6$). MP concentration was considered to be unity. Each column and horizontal bar represent the mean and S.D., respectively.

polarization fluoroimmunoassay without a separation step [4,5], but in order to achieve a sensitive quantitative analysis the separation is necessary. Its long λ_{ex} and λ_{em} decrease the possibility of interference with other compounds found in biological samples which emit at shorter wavelengths. Furthermore, the present method might be much more sensitive for the determination of primary and secondary amines by using laser induced fluorescence detection.

Since the overall analytical procedure of the proposed method is simple, rapid and of low-cost, it may be helpful to those who are interested in pharmacokinetics, forensic and toxicological studies.

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