TECHNICAL NOTE

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Achiral and Chiral Quantification of Methamphetamine and Amphetamine in Human Urine by Semi-micro Column High-Performance Liquid Chromatography and Fluorescence Detection

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ABSTRACT: In this paper, miniaturized achiral and chiral high-performance liquid chromatographic procedures for the determination of methamphetamine and amphetamine in human urine are described. After a simple pretreatment of human urine (i.e., 10 μL of urine or diluted urine were acidified and dried-up under N_2 at room temperature) and fluorescence derivatization with 4-(4,5-diphenyl-1*H*-imidazol-2-yl)-benzoyl chloride under mild conditions (pH 9.0, 10 min at room temperature), the derivatives were isocratically separated on a semi-micro ODS column with Tris-HCl buffer (0.1 M, pH 7.0): acetonitrile (45 + 55 v/v) at a flow rate of 0.2 mL/min or their enantiomers were separated on a semi-micro OD-RH column with sodium hexafluorophosphate (0.3 M aq.): acetonitrile (44 + 56 v/v) at a flow rate of 0.1 mL/min as the mobile phase. Wide-ranged calibration curves were obtained with detection limits for the achiral and chiral analyses in the atto and femtomol levels, respectively, per injected volume. Satisfactory within- and between-day reproducibility data were obtained with both the methods with the highest relative standard deviation being 9.6%. The methods were applied to the determination of methamphetamine and amphetamine in human urine samples and the concentrations determined by the two methods were well correlated ($r = 0.994$).

KEYWORDS: forensic science, methamphetamine, achiral and chiral analyses, human urine, semi-micro column, fluorescence, 4-(4,5-diphenyl-1*H*-imidazol-2-yl) benzoyl chloride

Methamphetamines (MPs) are substances that produce an intense alertness and false confidence in the user's sense of perseverance, energy, and mental abilities, hence, abusers become talkative, excited, and feel capable of working tirelessly with special insights. Abusers who illegally use this group of related drugs that stimulate the central and peripheral nervous systems tend to be antisocial and exhibit aggressive or violent behavior. The S(+)-enantiomer of methamphetamine (MP) is more potent as a stimulant than its antipode (1), hence, it is the most widely abused drug in Japan and detected together with its metabolite, S(+)-amphetamine (S(+)-AP) in biosamples of abusers. On the other hand, R(−)-enantiomers of MP and AP might be detected in biosamples of Parkinson's disease patients on selegiline, [R(−)-*N*-methyl-*N*-(1-phenyl-2-propyl)-2-propinylamine; *l*-Deprenyl] therapy as this compound is known to be metabolized in the human body into R(−)-enantiomers of MP and AP among other metabolites (2). However, the determination of these compounds and their enantiomers in biological fluids and tissues that has become a routine practice in forensic laboratories and criminal justice agencies is still challenging due to ever-increasing need to simple, selective and highly sensitive analytical method that can be performed within a reasonable time.

Scaling down a liquid chromatographic system is an advantageous approach that not only does increase the sensitivity due to less diluting effect by the mobile phase, but it also leads to a significant reduction of solvents and samples volume which is important in case of expensive chemicals and less available biological samples such as brain microdialysate or sweet. Moreover, the time required for analysis is relatively shorter than that of conventional HPLC. Having proved these advantages, semi-micro columns of 1.0 to 2.0 mm i.d. typically, have been utilized in the analyses of several substances in biological samples including adenosine and its metabolites (3), colchicine (4), opioid peptides (5), neurotransmitters such as γ-aminobutyric acid (6), aspartate, glutamate (7), and dopamine (8). Among the illicit drugs, cocaine in brain microdialysate (8), MP (9) and its enantiomers (10) in urine have been also determined using semi-micro HPLC columns.

Recently, we have been aiming at developing easy-to-perform highly sensitive analytical methods for the determination of MPs and their enantiomers in biosamples. HPLC with fluorescence de-

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tection after derivatization with 4-(4,5-diphenyl-1*H*-imidazol-2-yl) benzoyl chloride (DIB-Cl) as a labeling reagent was suitable as MP and AP or their enantiomers could be determined in human urine and hair at femtomol levels (11–13). Moreover, the derivatization reaction proceeded under mild conditions (10 min at room temperature, pH 9.0) and resulted in derivatives that are stable for 24 h at least when kept at room temperature in the dark (12,13). In this study, we describe two miniaturized HPLC methods with fluorescence detection for the achiral and chiral determination of MP and its major metabolite, AP. Both the methods were successfully applied to determine these compounds in urine samples of abusers' and a Parkinson's disease patient on selegiline therapy.

Experimental

Materials and Reagents

DIB-Cl was synthesized according to our previous procedure (14). S(+)-MP hydrochloride was obtained from Dainippon Pharmacy (Osaka, Japan). R(−)-AP and S(+)-AP were a kind gift from Professor T. Nagano (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan) and R(−)-MP was kindly supplied by Dr. S. Ikeda (Kyushu District Narcotics Central Office, Ministry of Health and Welfare, Fukuoka, Japan). Acetonitrile (HPLC grade) and Sodium hexafluorophosphate ($NaPF_6$) were purchased from Wako (Osaka, Japan) and Tokyo Kasei Kogyo (Tokyo, Japan). Tris-(hydroxymethyl) aminomethane (Tris) was obtained from Sigma (St. Louis, MO, USA). Water was deionized and passed through a pure line WL21P (Yamato Kagaku, Tokyo, Japan). All other chemicals were of analytical reagent grade and used as received.

Standard stock solutions of MP and AP enantiomers were prepared by dissolving suitable amounts of these compounds in water to give a final concentration of 10.0 mM per compound. These solutions were kept at 4°C and were stable for at least 6 months. Working solutions were prepared by dilution with $NaHCO_3$-Na_2CO_3 buffer (10.0 mM, pH 9.0), hereafter referred to as carbonate buffer, and in water for standard samples derivatization and to spike the urine samples, respectively.

Control urine samples were collected from healthy volunteers in our department. Spiked urine samples were prepared by adding aliquots of MPs standard solutions to the control urine. The Forensic Laboratory of Nagasaki Police Headquarters (Nagasaki, Japan) and Kyushu Kousei Nenkin Hospital (Kitakyushu, Japan) kindly supplied urine samples from MP abusers and a Parkinsonian on selegiline therapy, respectively. These samples were kept at −20°C until needed.

Urine Samples Pretreatment and Fluorescence Derivatization

Into a 3.5 mL amber glass, screw-capped septum reaction vial, 10 μL of urine or diluted urine were transferred. After adding 10 μL acetic acid, the mixture was evaporated to dryness under a gentle stream of N_2 gas at room temperature. To the residue, 10 μL carbonate buffer and 180 μL DIB-Cl in acetonitrile (0.1 mM) were successively added, vortex-mixed for few seconds and placed at room temperature for 10 min. To stop the reaction, 10 μL of liquid ammonia (28%) were added and the resultant mixture was subjected to HPLC analysis. For the standard samples, 10 μL of MPs in carbonate buffer were used instead of urine.

HPLC Systems and Operating Conditions

Except for the columns, the HPLC systems employed for the achiral and chiral analyses were the same and consisted of the following components of the Nanospace series (Shiseido, Tokyo, Japan): a Nanospace SI-1/2001 inert pump, a Nanospace SI-1/2003 autosampler set at a sample volume of 1 μL, a Nanospace SI-1/2004 column oven set at 35°C, a Nanospace SI-1/2013 fluorescence detector equipped with 7 μL flow cell and set at λex of 330 and λem of 440 nm and a Nanospace SI-1/2009 on-line degasser. All the units mentioned above used polyetheretherketone (PEEK)-made parts to avoid the contact of the mobile phase with any metal-made part. The recorder used was a model U-228-2P-500 (Nippon Denshi Kagaku, Tokyo, Japan). A 0.13 mm i.d. PEEK-made tubing (Shiseido) was used in all the flow lines. The columns used for analysis were a 250 × 1.5 mm i.d., 5 μm Capcell Pak C18 UG 120 S5 (Shiseido) and a 150 × 2 mm i.d., 5 μm Chiralcel OD-RH (Daicel Chemical Industries, Tokyo, Japan) for the achiral and chiral analyses, respectively.

The HPLC separations were carried out using Tris-HCl buffer (0.1 M, pH 7.0): acetonitrile (45 + 55 v/v) at a flow rate of 0.2 mL/min for the achiral analysis and 0.3 M $NaPF_6$ (aq.): acetonitrile (44 + 56 v/v) at a flow rate of 0.1 mL/min for the chiral analysis.

Calibration Curves and Method Reproducibility

Calibration curves were obtained by adding AP and MP standards to 10-fold diluted fresh, drug-free human urine in the range of 0.02–100 and 0.04–100 μM of AP and MP racemates, respectively for the achiral analysis whereas concentrations in the range of 0.1–50 and 0.4–200 μM per enantiomer of AP and MP, respectively, were used for the chiral analysis. Calibration curves were constructed by plotting the fluorescence intensity as peak height in arbitrary units against the concentration in urine (μM).

Within- and between-day variations were evaluated by using control human urine samples spiked with standard solutions to give the following concentrations: 0.25 and 2.5 of AP and 0.5 and 5.0 μM of MP for the achiral analysis. For the chiral analysis, 0.5 and 5.0 μM per enantiomer of AP and 2.0 and 20.0 μM per enantiomer of MP were used. These samples were analyzed as described.

Results and Discussion

In this investigation, we did not only aim at providing a procedure to screen human urine for the presence of MP and its major metabolite, AP, followed by an enantiomer-specific analysis to confirm whether it was lawfully administered, but we did also consider the requirements of routine forensic analyses including simplicity, speed, minimal use of samples and solvents besides others such as high sensitivity and selectivity. In our previous work (11–13), DIB-Cl derivatized MP and AP were separated on conventional size columns and could be fluorometrically detected with high sensitivity (i.e., femtomol amounts per injected volume). Therefore, in this paper, we describe two miniaturized HPLC systems for improved achiral and chiral analysis of DIB-MPs derivatives in human urine.

Achiral and Chiral HPLC Separations of DIB-MP and -AP

After urine samples pretreatment and fluorescence derivatization according to our previously optimized procedure (11–13), DIB-MP and -AP were well separated from the reagent blank and other compounds found in human urine within 10 min using the semi-micro ODS column. At a flow rate of 0.1 mL/min, another MP's metabolite, namely, *para*-hydroxy MP could be also simultaneously quantified and each HPLC run consumed about 20 min. However, the adaptation of a higher flow rate of 0.2 mL/min reduced the analysis time and DIB-AP and -MP were well separated and eluted at 7.8 and 8.9 min, respectively (Fig. 1).

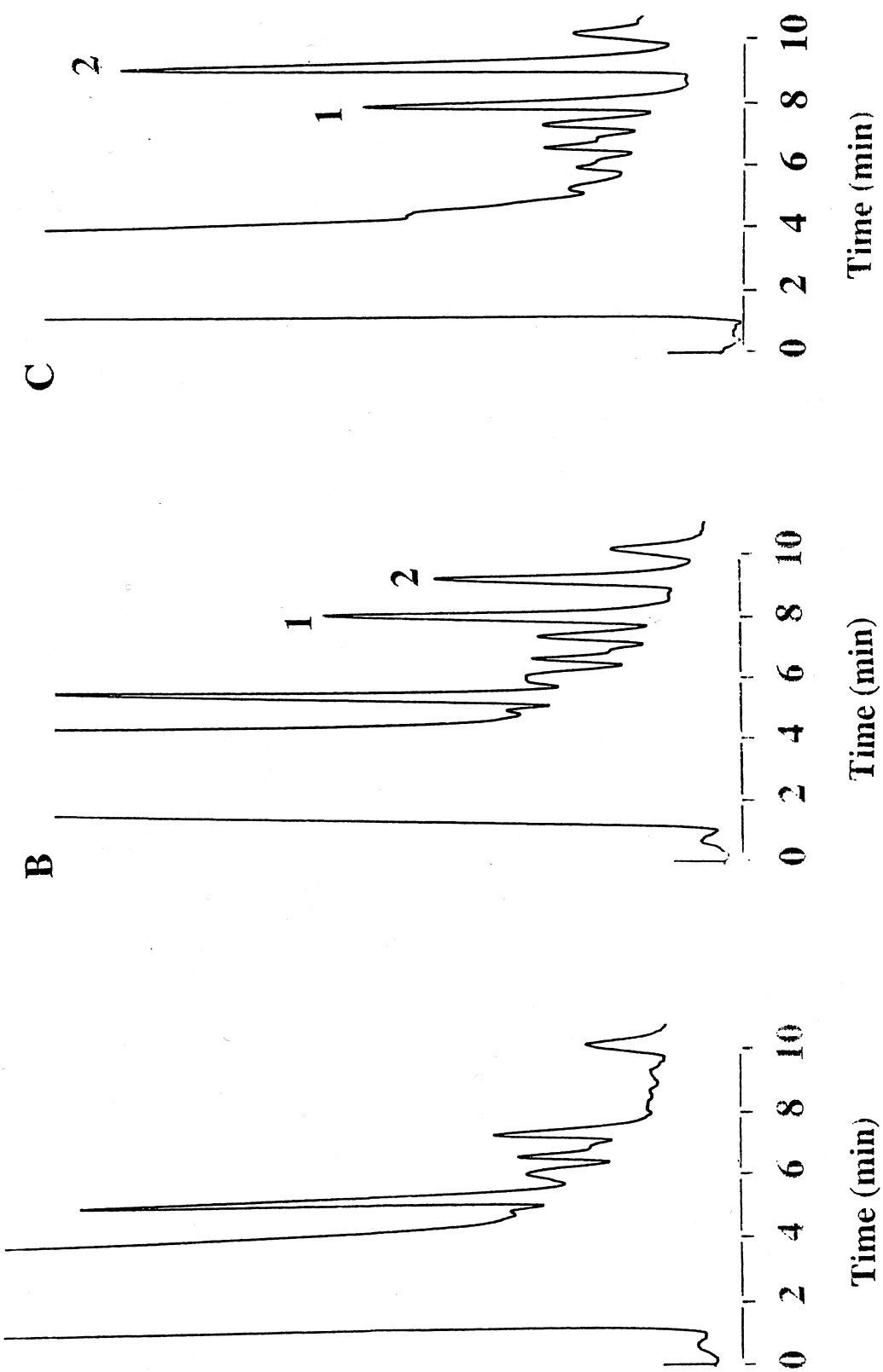

FIG. 1—*Typical chromatograms with fluorescence detection obtained by the achiral method for 10-fold diluted control human urine (A); 10-fold diluted control human urine spiked with 1 μM AP (50 fmol on column) and 2 μM MP (100 fmol on column) (B) and 40-fold diluted abuser urine sample (C). Peaks: 1) DIB-AP and 2) -MP. For experimental details, refer to the text.*

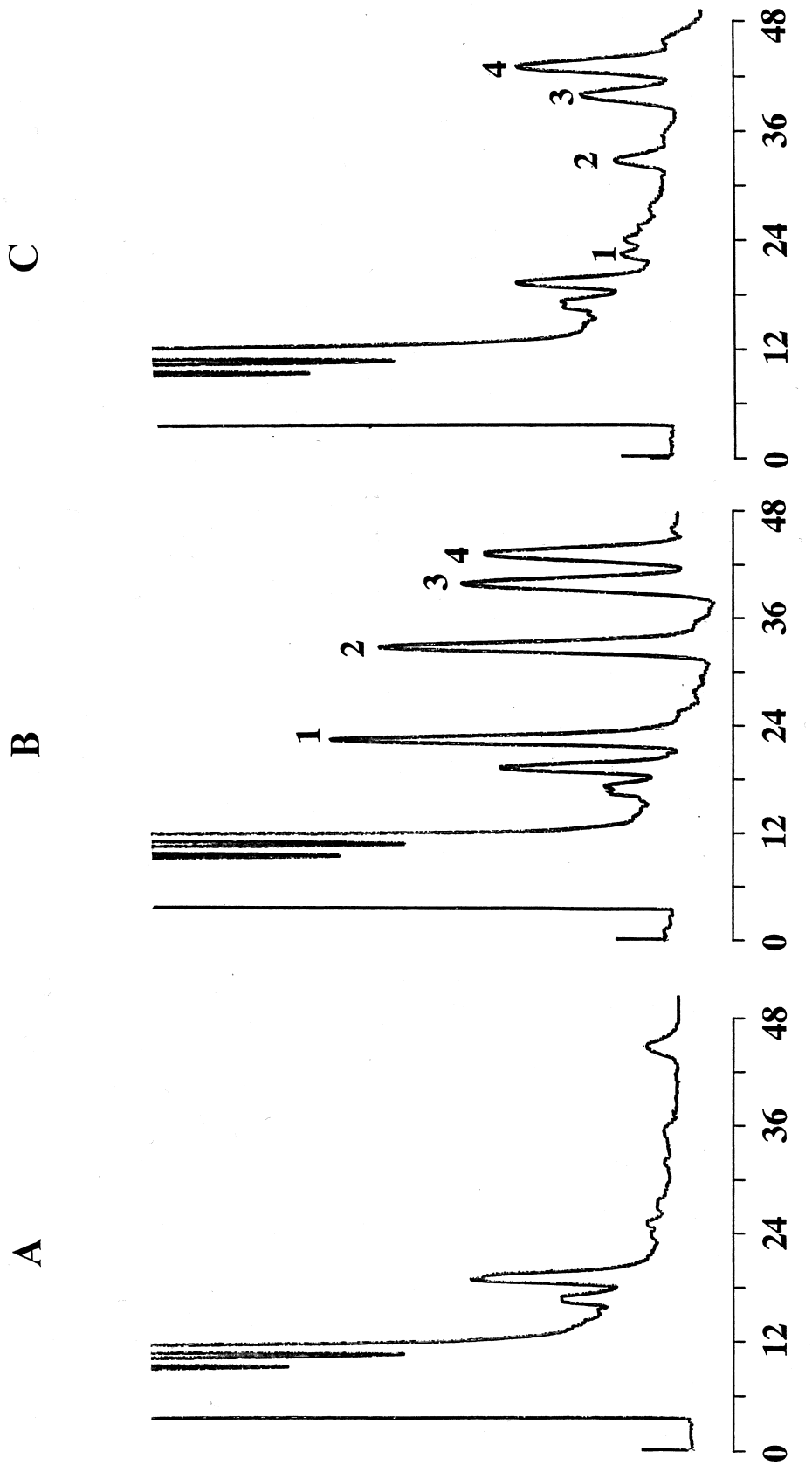

FIG. 2—*Typical chromatograms with fluorescence detection obtained by the chiral method for 10-fold diluted control human urine (A); 10-fold diluted control human urine spiked with 5 μM per enantiomer of AP (250 fmol on column) and 20 μM per enantiomer of MP (1 pmol on column) (B) and 10-fold diluted abuser urine sample (C). Peaks: 1) DIB-R(−)-AP, 2) −S(+)-AP, 3) −S(+)-MP and 4) −R(−)-MP. For experimental details, refer to the text.*

Previously, we reported the separation of MPs enantiomers as DIB-Cl derivatives on a conventional size (250 × 4.6 mm i.d., 10 μm) Chiralcel OD-R column packed with cellulose tris (3,5-dimethylphenyl carbamate) coated silica connected to the outlet of an ODS (250 × 4.6 mm i.d., 5 μm) column which was necessary for separating S(+)-AP and S(+)-MP that co-eluted when the Chiralcel OD-R column was used alone with a mobile phase composed of propan-2-ol-acetonitrile-disodium hydrogenphosphate (20 mM)-citric acid (10 mM) buffer (pH 4.5) containing 50 mM $NaPF_6$ (1 + 59 + 40 v/v/v) (12). In this experiment, a semi-micro (150 × 2 mm i.d., 5 μm) Chiralcel OD-RH column, packed with cellulose tris (3,5-dimethylphenyl carbamate) coated silica, was employed with an aqueous 0.3 M $NaPF_6$-acetonitrile (44 + 56 v/v) as a mobile phase. R(−)-AP, S(+)-AP, S(+)-MP, and R(−)-MP were eluted at 21.6, 32.4, 39.0, and 42.0 min, respectively, provided that a far superior resolution (Rs) of DIB derivatives of enantiomers of MP (1.2) and AP (3.8) in comparison with our previous data (12) was achieved (Fig. 2).

Calibration Curves, Detection Limits and Reproducibility

Table 1 summarizes the calibration curves parameters obtained by the proposed achiral and chiral HPLC methods. Excellent linearity ($r = 1.000$) over wide ranges calibration curves that cover the practical concentrations of MP and AP in abusers' urine were obtained in both the methods provided that dilution of >10-fold of urine was needed in many of the cases. Limits of detection achieved with the achiral method were better than those of the chiral method (i.e., sub fmol and fmol level on column, respectively) due to the additive effect of peak broadening (k' values of the last eluted peak were 7 and 12 for the achiral and chiral methods, respectively) that affected the peak height, and mobile phase composition and pH that reduced the fluorescence intensity in case of chiral analysis. However, our methods are still several orders of magnitude more sensitive than those of semi-micro HPLC-UV detection (9,10).

Within- and between-day variations were assessed using AP and MP spiked human urine at two different concentrations. These concentrations were set to be 5 and 50 times larger than the lower limit of quantitation in case of the chiral analysis and 12.5 and 125 times in case of the achiral analysis. As shown in Table 2, satisfactory reproducibility data were obtained with both the methods at the relatively high and low concentrations with a maximum relative standard deviation (% RSD) of 9.6%.

Application of the Methods on Practical Urine Samples

The applicability of the two developed methods was assessed using urine samples collected from a patient who received selegiline

TABLE 1—*Calibration curves parameters and detection limits obtained by the proposed achiral and chiral HPLC methods.*

Compound	Concentration Range (μM)	Regession Equation*	Correlation Coefficient	Detection Limit† (fmol/injection)
		Achiral		
AP	0.02–100.00	y = 13.066 x + 3.549	1.000	0.14
MP	0.04–100.00	y = 4.929 x + 0.872	1.000	0.67
		Chiral		
R(−)-AP	0.10–50.00	y = 5.290 x + 0.698	1.000	1.25
S(+)-AP	0.10–50.00	y = 4.700 x + 0.004	1.000	0.95
R(−)-MP	0.40–200.00	y = 0.767 x − 0.813	1.000	5.00
S(+)-MP	0.40–200.00	y = 0.864 x + 0.688	1.000	4.30

* y is the fluorescence intensity as peak height in arbitrary units and x is the concentration in urine (μM).
† Signal-to-noise ratio of 3.

TABLE 2—*Within- and between-day reproducibilities of the analysis of AP and MP in spiked human urine obtained by the present methods.*

Compound	Concentration Added (μM)	Within-day			Between-day		
		Found (μM)	RSD* (%)	*n*	Found (μM)	RSD (%)	*n*
			Achiral				
AP	0.25	0.20	4.9	6	0.24	9.6	4
	2.50	2.90	2.7	7	2.53	6.1	5
MP	0.50	0.50	5.1	6	0.55	8.0	4
	5.00	6.21	2.1	7	5.34	5.5	5
			Chiral				
R(−)-AP	0.50	0.39	4.6	5	0.41	7.7	4
	5.00	5.35	6.9	5	5.48	8.7	5
S(+)-AP	0.50	0.54	6.9	5	0.52	6.9	4
	5.00	5.62	5.9	5	5.61	9.0	5
R(−)-MP	2.00	1.57	3.7	5	1.73	7.3	4
	20.00	19.69	2.8	5	20.76	9.4	5
S(+)-MP	2.00	1.85	4.4	5	1.97	6.8	4
	20.00	21.06	2.5	5	21.57	8.0	5

* Relative standard deviation.

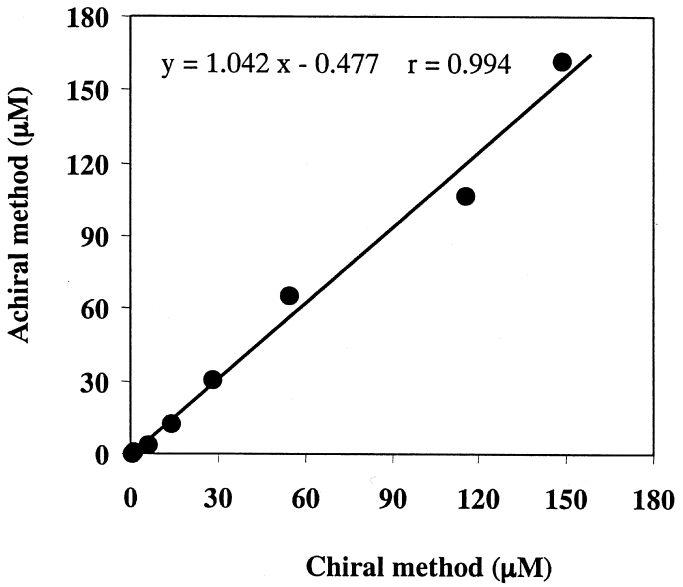

FIG. 3—*Correlation between the concentrations of AP and MP in human urine obtained by the proposed achiral and chiral HPLC methods (*n *= 8).*

as an anti Parkinson's disease medication and from suspected MP abusers. These samples were analyzed in triplicates and the maximum RSD among these measurements was 8.6%. Except for the Parkinsonian's sample, dilution of urine was needed and the net urine volumes used were in the range of 0.1–1.0 μL. MP was detected in all the samples with concentrations extending over a wide range (μ–mM) and AP was detected as its main metabolite. When these samples were analyzed by the enantiomer-specific method, R(−)-enantiomers of MP and AP were detected in the Parkinsonian's sample at concentrations of 1.2 and 0.7 μM, respectively. Interestingly, among the abusers' samples, one sample contained both the R(−)- and the S(+)-enantiomers of MP and AP which suggests that racemic MP is being abused in Japan as well. However, the S(+)-MP is the enantiomer which was reported to be the extensively abused form of this drug in Japan (15). As shown in Fig. 3, a satisfactory agreement between the concentrations of MP and AP determined by the proposed methods in four urine samples with a correlation coefficient of 0.994 and a slope of 1.042 ($n = 8$). In order to assess the accuracy of the developed methods, we plotted the results of the current chiral method against our previously published data (11,13) obtained for the same samples using conventional size HPLC systems and the following regression equation was achieved ($n = 8$): $y = 1.28 \times +7.49$ ($r = 0.993$), with x and y as the concentration (μM) obtained by the current chiral method and our previously published data, respectively. The accuracy of the results obtained previously (11) for AP ($n = 14$) and MP ($n = 13$) was evaluated by checking against another currently accepted method (16) and a satisfactory correlation was obtained for both the compounds.

Conclusion

In this paper, we described the possibility of miniaturization of our previously developed achiral and chiral HPLC methods to determine MP and AP in human urine as DIB-Cl derivatives. Using the semi-micro ODS column, these compounds could be separated in less than 10 min. This implies a significant reduction in solvents use with a total analysis time per sample (pretreatment + derivatization + HPLC) of less than half an hour. Mass sensitivity was also significantly improved and amol levels of AP and MP were detectable versus fmol levels in our previous procedure on a conventional size column (11). When a semi-micro OD-RH column was employed, not only a satisfactory enantiomeric resolution was obtained, but all the enantiomers were also well separated from each other and the reagent blank, therefore, the pre-separation on an ODS column was not necessary in comparison with our previous conventional enantioselective HPLC procedure (12). Besides their excellent sensitivity, selectivity and reproducibility, the significant reduction in the analysis time, solvents and samples volume made the present methods to be possible simple-to-perform alternatives for the routine analysis in the forensic and clinical application.

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