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Note

Assay of the optical isomers of methamphetamine and amphetamine in rat urine using high-performance liquid chromatography with chiral cellulose-based columns

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Methamphetamine (MAMP) and amphetamine (AMP), which are strong central nervous system stimulants, exist as isomers (*d* and *l*) and a racemate (*dl*). Several methods have been proposed for the analysis of these optical isomers, in which the diastereoisomers treated with optically active reagent have been measured by gas chromatography (GC) or high-performance liquid chromatography (HPLC) [1–3]. In our previous report we described an analytical method for the determination of the optical purity of MAMP using an HPLC column packed with a chiral cellulose-based column [4]. However, these studies did not lead to significant improvements in peak resolution, analysis time and simultaneous analysis of MAMP and AMP isomers. In order to investigate the possibility of the stereoselective metabolism of MAMP and AMP, we present here a simple and rapid HPLC method for the simultaneous analysis of MAMP and AMP isomers in rat urine, using two different chiral cellulose-based columns.

EXPERIMENTAL

Reagents

The *d*- and *l*-isomers of MAMP·HCl were obtained from Dainippon Pharmaceutical (Osaka, Japan). The *dl*-MAMP·HCl was synthesized using the

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method of Caldwell et al. [5]. The sulphates of *d*-, *l*- and *dl*-AMP were synthesized according to the method of Blackburn and Burghard [6]. Benzoyl chloride was obtained from Wako (Tokyo, Japan) and N-*n*-propylaniline (NnPA) from Tokyo Kasei (Tokyo, Japan). *n*-Hexane and 2-propanol were both from Merck (Darmstadt, F.R.G.).

Administration of MAMP and sampling of urine

We used male Wistar rats weighing 200 g. The rats were administered orally 15 mg of *dl*-MAMP·HCl per kg body weight dissolved in saline. Urine was collected for 4 h after the administration. The controls were administered saline alone. The animals were kept individually in suitable metabolism cages.

Isolation of methamphetamines and benzoyl derivatives

To 0.5 ml of rat urine containing methamphetamines were added 0.1 ml of NnPA (internal standard, I.S.; 25.0 μg/ml), 6 ml of carbonate buffer (pH 10.0)

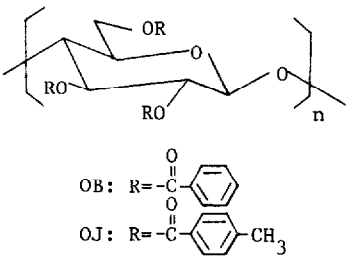

Fig. 1. Structures of two chiral cellulose-based stationary phases (Chiralcel OB and OJ) coated on silica gel.

TABLE I

HPLC SEPARATION OF BENZOYL DERIVATIVES OF *dl*-MAMP AND *dl*-AMP

Column, Chiralcel OB or OJ; mobile phase, hexane–2-propanol (9:1, v/v); flow-rate, 1.0 ml/min; column temperature, 40 °C; detection, UV at 220 nm; sensitivity, 0.032 a.u.f.s. Combined use of OB and OJ columns: mobile phase, hexane–2-propanol (9 1, v/v); flow-rate, 1.4 ml/min; column temperature, 48 °C; detection, UV at 220 nm; sensitivity, 0.032 a.u.f.s.

Compound	Column	Resolution (R_s)	Retention time (min)		Ratio of optical isomers (l/d)
			d	*l*	
MAMP	OB	1.42	13.1	16.5	1.01
	OJ	0.63	11.8	12.4	1.19
	OB+OJ[a]	1.46±0.02	13.1±0.20	15.0±0.10	1.01±0.02
AMP	OB	5.54	30.6	16.2	1.00
	OJ	0.40	16 9	16.3	0.71
	OB+OJ[a]	4.55±0.03	24.4±0.10	17.0±0.10	1.00±0.02

[a]Mean ± S.D., $n=6$.

and 15 ml of distilled water. The mixture was injected onto an Extrelut column (Merck). The column was allowed to stand for 20 min, then 40 ml of hexane–ethyl acetate (9:1, v/v) were used to elute MAMP and AMP isomers. The eluate was mixed with 3 ml of 0.1 *M* H_2SO_4 and 0.5 g of NaCl, stirred for 20 min and centrifuged at 1000 *g* for 5 min. The lower layer was vigorously stirred in 3 ml of 2.5 *M* NaOH and 20 μl of benzoyl chloride for 30 min, and the mixture was extracted with 1.5 ml of chloroform. The chloroform layer was washed twice with 5.0 ml of distilled water and evaporated to dryness at 40°C. The

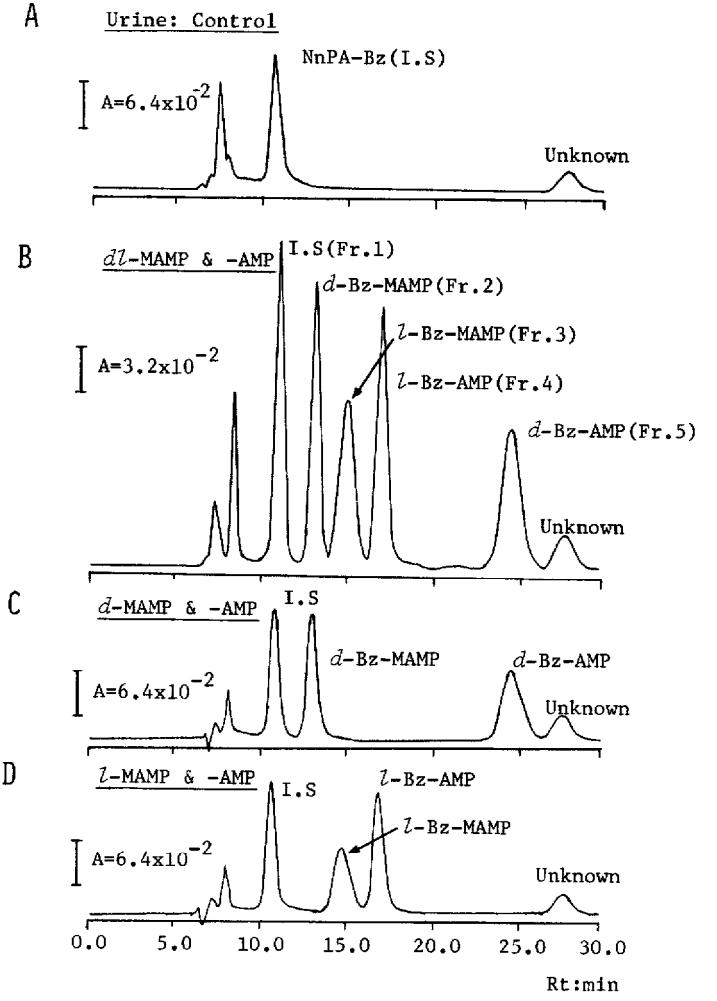

Fig. 2. HPLC elution profiles of *dl*-benzoyl-MAMP and *dl*-benzoyl-AMP in rat urine obtained using the combined columns: (A) containing NnPA (I.S.); (B) containing NnPA (I.S.), *dl*-MAMP and *dl*-AMP; (C) containing NnPA (I.S.), *d*-MAMP and *d*-AMP; (D) containing NnPA (I.S.), *l*-MAMP and *l*-AMP.

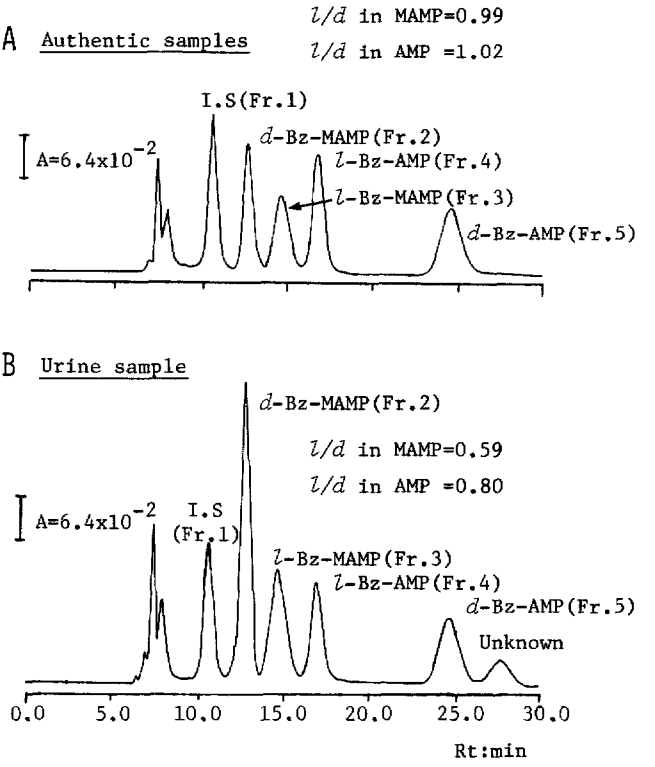

Fig. 3. Chromatograms of (A) authentic samples of *dl*-benzoyl-MAMP and *dl*-benzoyl-AMP and (B) MAMP and AMP isomers in a rat urine sample collected for 4 h after oral administration of *dl*-MAMP.

residue was dissolved in 200 μl of hexane–2-propanol (9:1, v/v) and then analysed. The above procedure was also used for the calibration of MAMP and AMP isomers.

HPLC analysis

The HPLC apparatus used was a UV-8000, CCPN equipped with SIC Chromatocorder 12, manufactured by Toyo Soda (Tokyo, Japan). Chiralcel OB and Chiralcel OJ columns (25 cm×4.6 mm I.D.) (Fig. 1) were purchased from Daicel (Tokyo, Japan). The column temperature wa 48°C. The mobile phase was hexane–2-propanol (9:1, v/v) and the flow-rates were 1.0 and 1.4 ml/min. The wavelength used for the detection was 220 nm. The chromatographic parameters are given in Table I and illustrated in Figs. 2 and 3.

RESULTS AND DISCUSSION

Investigation of the analytical method

The resolution (R_s) obtained under the conditions described in our previous

report [4] was as follows: *dl*-acetyl-MAMP = 0.92 and *dl*-acetyl AMP = 0.39. Hayes et al. [3] reported an HPLC method for chiral amines, including diastereometric derivatives (N-trifluoroacetyl-*l*-propyl) of MAMP and AMP isomers. However, *d*- and *l*-MAMP were not as well separated.

We have, therefore, developed an improved and simplified method for the separation of these stereoisomers. Using *dl*-benzoyl-MAMP and *dl*-benzoyl-AMP as separation markers, R_s, the *l*/*d* ratio and the retention time were examined on Chiralcel OB or OJ columns used alone or in combination. The results are summarized in Table I. The Chiralcel OB column separated *dl*-benzoyl-MAMP ($R_s = 1.42$) and *dl*-benzoyl-AMP ($R_s = 5.54$). These values demonstrated a complete separation of the *d*- and *l*-isomers. In a simultaneous analysis, *l*-benzoyl-MAMP and *l*-benzoyl-AMP were eluted with a retention time difference of only 0.3 min. When the racemates of MAMP and AMP were analysed independently, the *l*/*d* ratio was 1.01 for MAMP and 1.00 for AMP. Both values corresponded to the theoretical value of 1.00. Using the Chiralcel OJ column, the separation profile of MAMP and AMP racemates showed four peaks (Table I), but the R_s of each of these showed a partial separation. The *l*/*d* ratio was 1.19 for *dl*-MAMP and 0.71 for *dl*-AMP; both of these values deviate from the theoretical value of 1.00. The combined use of the Chiralcel OB and OJ columns increased the resolution of peaks, as can be seen from Table I. Thus, the combined use of Chiralcel OB and OJ columns allowed complete separation of the stereoisomers of MAMP and AMP at 48°C.

Examples of HPLC analysis

The chromatograms obtained by combined use of Chiralcel OB and OJ columns at 48°C are shown in Fig. 2. Fig. 2A is the chromatogram of the control urine, showing no peaks corresponding to *d*- and *l*-benzoyl-MAMP and *d*- and *l*-benzoyl-AMP. The peak at 27.8 min originated from an unknown substance in urine. Fig. 2B shows the results for a mixture of 0.1 ml of 50.0 μg/ml *dl*-MAMP, 0.1 ml of 50.0 μg/ml *dl*-AMP and 0.5 ml of the control urine. *d*-MAMP and *l*-MAMP, these metabolites and *d*- and *l*-AMP were eluted in 25 min. The retention time was reduced by 50% (ca. 25 min) compared with our previous method [4] and Gunne's report [2]. The detection sensitivity for both MAMP and AMP isomers was 25 ng, and the standard curves of MAMP or AMP isomers after calibration with NnPA were linear, passing through the origin. The R_s and the *l*/*d* ratio were 1.46 and 0.99, respectively, for *dl*-MAMP and 4.55 and 1.02, respectively, for *dl*-AMP. In Fig. 2C (for *d*-MAMP and *d*-AMP) and Fig. 2D (for *l*-MAMP and *l*-AMP), the chromatograms indicated no other stereoisomers than the above.

Determination of MAMP and AMP isomers in urine of rats

GC and radioimmunoassay RIA methods have been employed for the analysis of MAMP and AMP isomers in human and animal urine [1,2,7]. However,

these methods have disadvantages. In GC, the heat-stability of MAMP and AMP diastereomers and optically liquid phase cannot be ensured, and the optical purity of the optical reagent itself must be taken into consideration [8]. In RIA, antisera against the *l*- and *d*-forms could not easily be obtained.

The HPLC assay using chiral cellulose-based columns allows simultaneous analysis and quantification of MAMP and AMP isomers in urine. Fig. 3 shows chromatograms of the authentic samples and of the extract of rat urine collected for 4 h after administration of *dl*-MAMP. Each HPLC fraction (Fr. 1–5) in Fig. 3B was also identified as NnPA, MAMP and AMP isomers of standard substances by GC and gas chromatography–mass spectrometry. All stereoisomers under study were completely separated. In the urine sample, the *l*/*d* ratio of MAMP isomers decreased from 0.99 to 0.59, and the metabolite AMP appeared at an *l*/*d* ratio of 0.80 (Fig. 3B). The amount excreted in the urine (μg per 4 h per rat) was 17.65 for *d*-MAMP, 10.43 for *l*-MAMP, 6.98 for *l*-AMP and 8.73 for *d*-AMP. The *l*/*d* ratio of the total MAMP and AMP isomers was 0.66. The greater decrease of *l*-MAMP compared with *d*-MAMP may have resulted from more rapid hydroxylation of *l*-MAMP.

CONCLUSION

An HPLC assay using two chiral cellulose-based columns for MAMP and AMP isomers was developed. MAMP isomers excreted in rat urine were analysed by combined use of Chiralcel OB and OJ columns, which offered good peak resolution, *l*/*d* ratio and retention time. Mixtures of MAMP and AMP could be separated simultaneously. The HPLC analysis time was less than 25 min, and the minimum detection limit was 25 ng per 20 μl. In the experiments with *dl*-MAMP administered to rats, *l*-isomers of MAMP and AMP were more rapidly metabolized than the *d*-isomers. This shows the stereoselective metabolism of MAMP.

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