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Determination of *p*-hydroxymandelic acid enantiomers in urine by high-performance liquid chromatography with electrochemical detection[1]

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Abstract

High-performance liquid chromatography (HPLC) with electrochemical detection using a chiral ligand-exchange column was developed for the enantioselective determination of *p*-hydroxymandelic acid (HMA), a metabolite of synephrine, with high sensitivity. A good linear relationship between current ratio and amount was noted for 0.5–500 pmol HMA, with a correlation coefficient of 0.999 for each HMA enantiomer. The relative standard deviation (R.S.D.) was 1.6% at 100 pmol *d*-HMA and 2.2% at 100 mpol *l*-HMA. The detection limit of each HMA enantiomer was 0.5 pmol (signal to noise ratio, S/N = 3). By this method, HMA in *Citrus unshiu* and in urine following the ingestion of *C. unshiu* was determined. Although no HMA was found in *C. unshiu*, *d*- and *l*-HMA were present in urine after the ingestion of *C. unshiu*. The time courses of HMA and conjugated synephrine enantiomers excreted in urine following the ingestion of *C. unshiu* for 24 h could be monitored. This method should prove applicable to the study of synephrine metabolism. © 1997 Elsevier Science B.V.

Keywords: *p*-Hydroxymandelic acid; Enantioselective determination; Synephrine; High-performance liquid chromatography; Chiral ligand-exchange column

1. Introduction

Synephrine is a component of herbal drugs, Chinese medicines, citrus plants and other substances [1,2]. Synephrine has sympathomimetic activity expressed as vasoconstriction, blood pressure elevation, bronchial muscle relaxation and so on [3]. Gjessing and Armstrong [4] reported the main metabolite of synephrine to be *p*-hydroxymandelic acid (HMA). Synephrine may possibly undergo oxidation by monoamine oxidase to HMA in vivo. Synephrine is a chiral compound and present in nature only as the *l*-synephrine form [5]. thus,the amount and chiral activity of HMA excreted in urine after the ingestion of synephrine are points of considerable interest. However, a method for the exact determination of HMA enantiomers excreted in urine has yet to be established.

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Several methods for HMA determination have been reported and include differential pulse voltammetry [6], gas chromatography coupled with mass spectroscopy [7], high-performance liquid chromatography (HPLC) with electrochemical detection [8], gas chromatography coupled with thin-layer chromatography [9], for determining HMA in food and biological samples such as urine, blood and brain extract. But in no case has enantioselective determination been possible and derivatization is required in some cases. Thus, a simple means for the enantioselective determination of HMA with high sensitivity should be established for the study of HMA. For this purpose, the Authors considered HPLC with electrochemical detection using a chiral ligand-exchange column to be potentially very useful. This HPLC with electrochemical detection was developed in this study for the determination of HMA enantiomers in *C. unshiu* and urine following the ingestion of *C. unshiu*.

2. Experimental

2.1. Reagents and materials

p-Hydroxy-*dl*-mandelic acid was purchased from Tokyo Kasei Kogyo, *dl*-synephrine from Sigma Chemical and *m*-hydroxybenzoic acid (*m*-HBA) from Wako Pure Chemical. All other chemicals were of reagent grade and were obtain commercially. *Citrus unshiu* fruit was obtained commercially from markets in Hachioji, Japan.

2.2. Apparatus and HPLC conditions

The HPLC system consisted of a Jasco 880-PU pump (Jasco, Tokyo, Japan), 8125 injector fitted with a 5-μl injection loop (reodyne, Cotati, USA) Sumichiral OA-6000 column (150 × 4.6 mm i.d., Sumika Chemical Analysis Service, Osaka, Japan), LiChrospher 100 RP-18 (75 × 4 mm i.d., E. Merck, Darmstadt, Germany) and EDP-1 electrochemical detector (Kotaki, Japan). A 100 RP-18 column was placed between OA-6000 column and electrochemical detector. The mobile phase was water containing 1 mM copper(II) acetate and 20 mM ammonium acetate methanol (99:1, v/v) and the flow rate was 1.0 ml min^{-1}. The detection potential was 1.0 V vs. Ag/AgCl. *m*-Hydroxybenzoic acid served as the internal standard. clean-up of urine samples was made by Extrelut® column (E. Merck, Darmstadt, Germany).

2.3. C. unshiu sample preparation

C. unshiu fruit was treated to give exocarp, mesocarp, endocarp. and sarcocarp portions, which were each homogenized and extracted with water under ultrasonication at room temperature for 10 min. After centrifugation, the residue was re-extracted in the same manner and the supernatants were combined. Internal standard and water were added to the supernatant thus obtained to give the desired volume of sample solution, which was subsequently filtered through a 0.45 μm membrane filter prior to injection into the HPLC system.

2.4. Preparation of urine sample

2.4.1. Subjects

Four volunteers in good physical health participated in the present study. Subject A was male, aged 35 and weighing 57 kg, subject B a male, aged 25 and weighing 65 kg, subject C a male, aged 23, weighing 62 kg and subject D, a male, 24 years old and weighing 90 kg.

2.4.2. Urine collection

The subjects adhered to a plant-free diet for 3 days. HMA is an endogenous compound continuously excreted into the urine [4]. at the end of the 3-day period, HMA was detected in the urine. The diet-controlled urine served as a blank. All four subjects then consumed about 250 g *C. unshiu* containing sarcocarp and endocarp. Urine was collected 11 times for 24 h, adjusted to pH 1 with HCl and stored at −30°C until use.

2.4.3. Sample preparation

Urine was adjusted to pH 2.2 with 0.2 M citrate buffer. 0.5 ml of the solution was applied onto the Extrelut® column. At 15 min, the HMA enantiomers were eluted with 10 ml ethyl acetate fol-

lowed by evaporation of the elute. The residue was dissolved in 1 ml water and 200 pmol *m*-HBA acid as the internal standard were added. Then 5-μl aliquots of the solution were injected into the HPLC system for enantiomer determinations.

3. Results and discussion

3.1. HMA enantiomer determination

In a ligand-exchange HPLC column, mobile phase conditions such as solvent, pH and metal ion concentration are significant factors for enantioseparation of the peaks on the chromatogram [10]. The stationary phase of Sumichiral OA-6000 column consists of a coordination compound of copper(II) ions and a chiral ligand as (*R*,*R*)-tartaric acid mono-(*R*)-1-(α-naphthyl)ethylamide [11]. Separation is based on the formation of copper(II) complexes with solutes, such as HMA enantiomers, to give transient equilibria. To obtain the largest possible separation factor, examination was made as to the effect of pH and copper ion(II) concentration. At too high a concentration, the lifespan of the ligand-exchange column was reduced. Copper ion(II) concentration in the mobile phase was thus made 1.0 mM. Increase in separation was also noted with pH. Above pH 6.5, a copper(II) hydroxide precipitate formed in the mobile phase. The pH of the mobile phase was thus adjusted to 6.4 (Fig. 1)

Fig. 2 shows the hydrodynamic voltammograms of the HMA enantiomers. HMA was oxidized at potentials more positive than 0.6 V (Ag/AgCl was used as reference throughout). Two oxidation waves were observed in the voltammograms, one at 0.85 V and the other, at 1.1 V. By cyclic voltammetry and coulometry, two electrons and two protons were found to participate in the first oxidation step of HMA. The ultraviolet absorption spectra of HMA and its electrolytic products after constant potential electrolysis at 1.0 V suggested that the phenolic hydroxyl group of HMA could be oxidized. Above 1.0 V, the reproducibility of HMA peak height in the chromatogram became less, possibly due to contamination of the electrode surface by some other

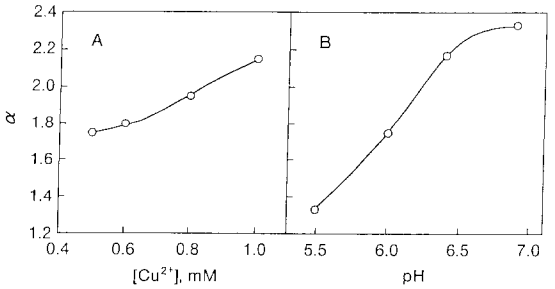

Fig. 1. Effects of copper(II) ion concentration and pH in the mobile phase on the separation factor of HMA enantiomers. HPLC conditions: (A) mobile phase, water containing 0.5–1.0 mM copper(II) acetate and 20 mM ammonium acetate (pH 6.4)—methanol (99:1, v/v); column, Sumichiral OA-6000 (150 × 4.6 mm i.d.) and Lichrospher 100RP-18 (100 × 4.6 mm i.d.) arranged in series; flow rate, 1.0 ml min^{-1}; applied potential, 1.0 V vs. Ag/AgCl; (B) mobile phase, water containing 1.0 mM copper(II) acetate and 20 mM ammonium acetate (pH 5.5–6.8)—methanol (99:1, v/v); other HPLC conditions as in (A).

products at more positive potentials than 1.0 V and/or oxidation of electrode surface itself. At 1.0 V the first oxidation wave showed the highest peak. Thus a detection potential of 1.0 V was used.

Fig. 3 presents a typical chromatogram of a standard mixture of dl-HMA containing *m*-HBA as the internal standard. When the flow rate of the mobile phase was 1.0 ml min^{-1}, the retention

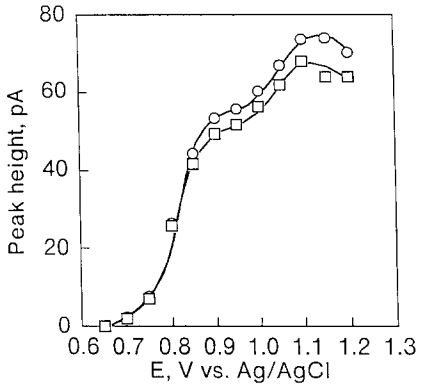

Fig. 2. Hydrodynamic voltammograms of HMA enantiomers (○, *l*-HMA; □, *d*-HMA). HPLC conditions mobile phase, water containing 1.0 mM copper(II) acetate and 20 mM ammonium acetate (pH 6.4)—methanol (99:1, v/v); other HPLC conditions as in Fig. 1.

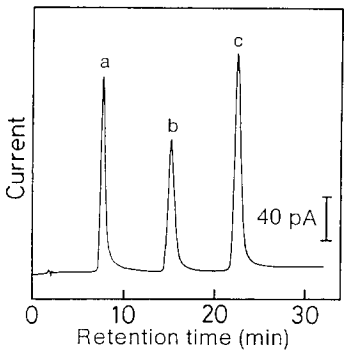

Fig. 3. Chroamtogram of a standard mixture of *dl*-HMA and *m*-hydroxybenzoic acid (internal standard). Peaks: (a) *d*-HMA (100 pmol); (b) *l*-HMA (100 pmol); (c) *m*-hydroxybenzoic acid (200 pmol). HPLC conditions: applied potential, 1.0 V vs. Ag/AgCl; other HPLC conditions as in Fig. 2.

times of *l*-HMA and *d*-HMA, and *m*-HBA were 8, 15 and 22 min, respectively. The separation factor for the HMA enantiomers was 2.2 and resolution, 3.76.

Peak current ratios relative to the internal standard on the chromatograms for standard HMA were plotted against amounts of the HMA enantiomers. A good linear relationship was noted between peak current ratio and amount at 0.5–500 pmol HMA injected, with a correlation coefficient of 0.999 for each HMA enantiomer. The relative standard deviation (R.S.D.) was 1.6% at 100 pmol *d*-HMA and 2.2% at 100 pmol *l*-HMA. The detection limit of each HMA enantiomer was 0.5 pmol (signal to noise ratio, S/N = 3).

3.2. Determination of HMA in C. unshiu

The hydrophillic fraction was extracted from sarcocarp and endocarp 24 g *C. unshiu* by ultrasonication and a 5-μl aliquot of the supernatant was injected into the HPLC system. No HMA peak appeared on the chromatogram, thus demonstrating HMA in *C. unshiu* to be less than 10 pmol in 100 g sarcocarp and endocarp of *C. unshiu*.

3.3. Monitoring of HMA enantiomers in urine after ingestion of C. unshiu

Urine samples were obtained from the four subjects after the ingestion of *C. unshiu*. Fig. 4 shows typical chromatograms of solutions extracted from the blank (A) and sample (B) urine. There was no interference from urine extracts with peaks of the HMA enantiomers. A total of 10–40 μM *l*-HMA was detected in the blank urine and *d*- and *l*-HMA, in the sample urine. Even when the urine was heated at 100°C for 15 min at pH 1 with HCl, no change in peak height of the HMA enantiomers could be detected. HMA enantiomers in urine are thus shown to be present not in the conjugated form but the free form.

Synephrine present in *C. unshiu* was previously shown only to be in the free *l*-synephrine form, while synephrine excreted in urine after the ingestion of *C. unshiu* was present as conjugated synephrine enantiomers [12]. Although no HMA was found in *C. unshiu*, *d*- and *l*-HMA were present in urine following the ingestion of *C. unshiu*. Much L-synephrine is present in *C. unshiu* and thus may possibly be the source of HMA and conjugated synephrine enantiomers excreted in urine.

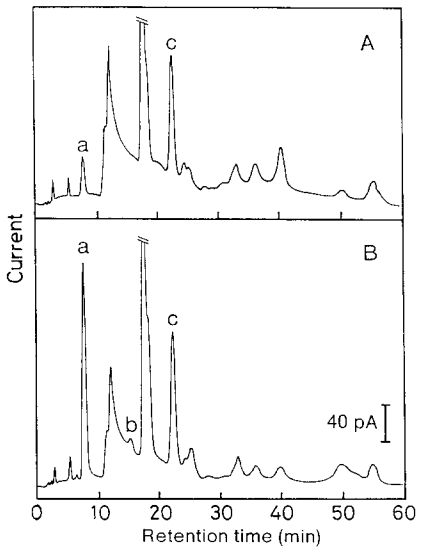

Fig. 4. Chromatograms of solutions extracted from blank (A) and sample (B) urine following the ingestion of 252 g *C. unshiu*. The urine was obtained from a healthy subject (male, 35 years old, 57 kg). Peaks and HPLC conditions as in Fig. 3.

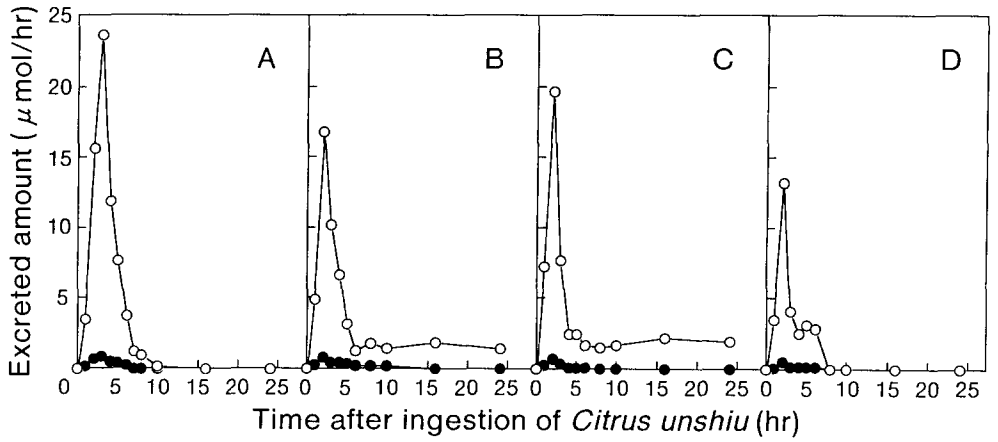

Fig. 5. Amounts of HMA enantiomers (○, *l*-HMA; ●, *d*-HMA) excreted in the urine after the ingestion of *C. unshiu* for four subjects. Values obtained by subtracting HMA in blank urine are shown. The urine samples were from subject A (male, 35 years old, 57 kg) after the ingestion of 252 g *C. unshiu* (A), subject B (male, 25 years old, 65 kg) after the ingestion of 240 g *C. unshiu* (B), subject C (male, 23 years old, 62 kg) after the ingestion of 230 g *C. unshiu* (D) ingestion.

The time courses of the excretion of HMA enantiomers in the urine of the four subjects are shown in Fig. 5. Values obtained by subtracting HMA in blank urine are thought to be the HMA due to the ingestion of *C. unshiu* and thus used as excreted amount in the following. The ingestion of *C. unshiu* gave rise to the presence of HMA enantiomers in the urine. The excretion of HMA was at a maximum 2–3 h later. Although the excreted amount of the HMA differs from that of the conjugated synephrine, the shape of each time course curve of HMA in Fig. 5 was essentially the same as that of synephrine [12].

Table 1 shows amounts of the HMA and synephrine enantiomers excreted in 24 h urine after the ingestion of *C. unshiu*. Assuming all *l*-synephrine ingested to have been extracted from *C. unshiu* pulp and transferred into blood, 14–31%

Table 1
Amounts of *l*-synephrine present in *C. unshiu* and conjugated synephrine and HMA enantiomers excreted in urine after the ingestion of *C. unshiu*.

Subject	Amount of *l*-synephrine in *C. unshiu* (μmol)	Excreted amount[a] (μmol)			
		Conjugated *l*-synephrine	Conjugated *d*-synephrine	*l*-HMA[b]	*d*-HMA[b]
A	222.2	18.80 (8.46%)	2.61 (1.17%)	68.33 (30.75%)	3.08 (1.39%)
B	180.6	12.62 (6.99%)	1.32 (0.73%)	49.10 (27.19%)	2.76 (1.53%)
C	173.1	6.74 (3.89%)	0.52 (0.30%)	48.14 (27.81%)	1.67 (0.96%)
D	207.0	22.01 (10.63%)	3.31 (1.51%)	29.30 (14.15%)	1.35 (0.65%)

[a] Ratio to the amount of *l*-synephrine in *C. unshiu*.
[b] Values obtained by subtracting HMA in blank urine.

may be considered to have been converted to *l*-HMA, ca. 1% to *d*-HMA, 4–10% to conjugated *l*-synephrine and ca. 1% to conjugated *d*-synephrine in vivo. HMA content in most cases exceeded that of synephrine. However, the ratio of these compounds differed according to the subject. The amounts of HMA and synephrine in urine following *C. unshiu* ingestion can thus be easily compared. The present method is thus applicable to the study of synephrine metabolism.

4. Conclusion

HPLC with electrochemical detection using a chiral ligand-exchange column was found to be adequate for the enantioselective determination of HMA with high sensitivity. By this method, HMA was determined in urine following the ingestion of *C. unshiu*. Although no HMA was present in *C. unshiu*, *d*- and *l*-HMA were observed in urine after the ingestion of *C. unshiu*. The time courses of HMA and conjugated synephrine enantiomers excreted in urine after the ingestion of *C. unshiu* for 24 h could be monitored. This method is thus shown to be useful for studying synephrine metabolism.

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