Direct Separation and Determination of Synephrine Enantiomers by High-Performance Liquid Chromatography with Electrochemical Detection

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High-performance liquid chromatography (HPLC) with electrochemical detection using a chiral ligand-exchange column (Sumichiral OA-5000) and a mobile aqueous phase containing 1 mM copper(II) acetate and 20 mM ammonium acetate (pH 6.4) was applied to the direct separation and determination of synephrine enantiomers. The calibration curve for each enantiomer showed good linearity ($r=0.999$) over a concentration range of 0.2—100 μM with a detection limit of 0.2 μM (signal-to-noise ratio (S/N)=3). As far as reproducibility was concerned, the relative standard deviation (R.S.D.) was 2.6% at 20 μM *d*-synephrine and 2.4% at 20 μM *l*-synephrine. The degradation and optical isomerization of *l*-synephrine in *Citrus unshiu* were caused by ultraviolet (UV) light or heat. The synephrine enantiomers in citrus plants, crude drugs and Chinese medicines were determined by the present method.

Key words synephrine; enantioseparation; citrus plants; HPLC; electrochemical detection

Synephrine was first developed as a synthetic sympathomimetic drug and has been shown to exhibit pharmacological activities such as vasoconstriction, blood pressure elevation, and bronchial muscle relaxation.[1] Synephrine is a chiral compound and the structures of its enantiomers are shown in Fig. 1. Stewart *et al.* isolated *l*-synephrine from the leaves and juice of citrus plants.[2] Some citrus plants, such as *Auruntii nobilis Pericarpium* (Japanese name, Chinpi 陳皮) and *Auruntii fructus Immaturus* (Japanese name, Kijitsu 枳実), have been used as crude drugs and Kinoshita *et al.* have isolated synephrine from such crude drugs.[3] Synephrine in crude drugs from citrus plants has been determined by HPLC using an electrochemical detector with high sensitivity.[4] Patil *et al.* found that synephrine enantiomers had different pharmacological activities[5] and, therefore, the determination of the synephrine enantiomers in these crude drugs is very important. Although, the separation of synephrine enantiomers has been accomplished by different methods such as capillary gas chromatography and HPLC,[6–8] the determination of the synephrine enantiomers in these crude drugs has not been investigated previously. In measuring the synephrine enantiomers in a sample with a complicated composition, derivatization of the enantiomers is not desirable.

In this study, direct determinations of the synephrine enantiomers in citrus plants, crude drugs and Chinese medicines were conducted using HPLC on a chiral ligand-exchange phase with electrochemical detection.

Experimental

Plant Materials Citrus plants were obtained commercially from markets in Hachioji, Japan. Crude drugs were purchased from Kusuri Nippon Do (Hachioji, Japan) and powders of Chinese medicine extract from Tsumura Co., Ltd.

Chemicals *d,l*-Synephrine was purchased from Sigma Chemical Co., *l*-phenylephrine from Tokyo Kasei Kogyo Co., copper(II) acetate from E. Merck and ammonium acetate from Wako Pure Chemical Industries, Ltd. *N*-Methyltyramine was kindly supplied by the Institute for Chinese Materia Medica (Beijing, China).

Apparatus The HPLC system consisted of a Jasco 880-PU pump (Jasco, Japan), an 8125 injector fitted with a 5 μl injection loop (Reodyne, U.S.A.), a Sumichiral OA-5000 column (250 × 4.6 mm i.d., Sumika Chemical Analysis Service, Japan) and an EDP-1 electrochemical detector (Kotaki, Japan). An octadecyl silica (ODS) column (LiChrospher 100 RP-18, 75 × 4 mm i.d., E. Merck, Germany) was also used for the determination of samples.

Sample Preparation A pulverized sample (5 mg) was extracted with 2 ml water under ultrasonication at room temperature for 10 min. After centrifugation, the supernatant solution was transferred to a test tube. The precipitate was re-extracted in the same manner. After this supernatants were combined and 1 ml 0.2 mM *l*-phenylephrine as an internal standard solution and 5.8 ml water were added to the supernatant to give 10 ml sample solution. After filtering this solution through a 0.45 μm membrane filter, 5 μl aliquots of the sample solution were injected into the HPLC system.

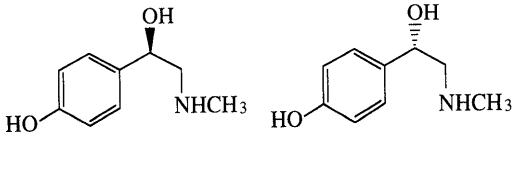
Decoction of Chinese Medicines A pulverized sample (50 mg) was decocted with 50 ml water for 40 min at 100 °C. After cooling, the decoction was centrifuged. Then 5 ml 0.2 mM *l*-phenylephrine and water were added to the supernatant to give 50 ml sample solution.

Reflux Extraction of Chinese Medicines A pulverized sample (50 mg) was refuxed with 50 ml water for 12 h at 100 °C. After cooling, the extract was centrifuged. Then 5 ml 0.2 mM *l*-phenylephrine and water were added to the supernatant to give 50 ml sample solution.

Results and Discussion

Enantioselective Separation of Synephrine The enantioselective separation of synephrine was carried out on a Sumichiral OA-5000 column using an aqueous buffer containing copper(II) ion as a mobile phase. This mobile phase also allowed the electrochemical detection of synephrine with high sensitivity and selectivity.

In such ligand-exchange HPLC, the pH and copper(II) ion concentration in the mobile phase affect enantiomer separation.[9] In order to optimize the mobile phase, the pH and copper(II) ion concentration were changed to keep the chiral column stable. Figure 2 shows the effects of the pH and copper(II) ion concentration in the mobile phase on the separation factor (α) of the synephrine enantiomers.



d-Synephrine *l*-Synephrine

Fig. 1. Structures of the Synephrine Enantiomers

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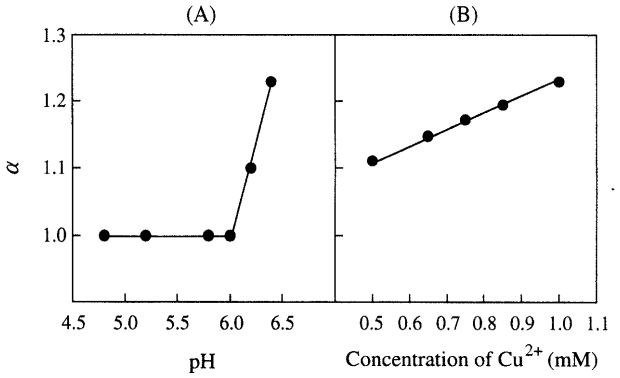

Fig. 2. Effects of pH and Copper(II) Ion Concentration in the Mobile Phase on the Elution of the Synephrine Enantiomers

HPLC conditions: (A) aqueous mobile phase containing 1 mM copper(II) acetate and 20 mM ammonium acetate (pH 4.8—6.4); column, Sumichiral OA-5000 (250 × 4.6 mm i.d., 5 μm); flow rate, 0.8 ml/min; applied potential, 1.0 V *vs.* Ag/AgCl; injection volume, 5 μl; (B) aqueous mobile phase containing 0.5—1 mM copper(II) acetate and 20 mM ammonium acetate (pH 6.4); other HPLC conditions are the same as in (A).

On a chromatogram obtained below pH 6.0, there was no separation of the synephrine enantiomers, *i.e.* α equals 1. Above pH 6.0, the α value increased proportionally with increasing pH. However, the mobile phase formed a precipitate at pH 6.5 or above, so the mobile phase could not have a pH higher than 6.4. The stationary phase for such chromatography consists of a coordination compound of copper(II) ion and the chiral ligand, *N*,*S*-dioctyl-(D)-penicillamine.[10] The separation is based upon the difference in coordination formation ability of the solutes, such as synephrine enantiomers, and the ligand with copper(II) ion. The equilibrium of the formation of such copper(II) complexes is affected by pH, due to competition for the complexing anion by hydrogen ion. Therefore, the adequate separation cannot be obtain at a lower pH. The α values also increased proportionally with increasing copper(II) ion concentration. Based on these results, the optimum composition of the mobile phase were determined as 1 mM copper(II) acetate and 20 mM ammonium acetate in aqueous solution (pH 6.4).

Chromatograms of Synephrine Enantiomers Figure 3 shows a hydrodynamic voltammogram of synephrine. Synephrine was oxidized to give its quinoid form at a potential above 0.8 V *vs.* Ag/AgCl. When the potential was maintained at 1.2 V *vs.* Ag/AgCl for a long time, the peak current of synephrine decreased. This may be due to the roughness of the glassy carbon electrode surface in the electrochemical detector. Therefore, a detection potential of 1.0 V *vs.* Ag/AgCl was adopted. Figure 4 shows a chromatogram of a standard mixture of *d*,*l*-synephrine and *l*-phenylephrine as an internal standard which was separated by the chromatographic system. When the mobile phase was pumped at a flow rate of 0.8 ml/min, the retention times of *d*-synephrine, *l*-synephrine, and *l*-phenylephrine were 8.6, 9.8 and 21.4 min, respectively. The α and resolution (Rs) values for both synephrine enantiomers were 1.23 and 1.23, respectively.

Chromatograms of a series of solutions containing the internal standard and various concentrations of synephrine enantiomers were obtained. Peak current ratios relative to the internal standard were plotted against the

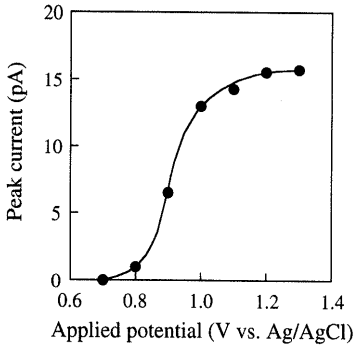

Fig. 3. Hydrodynamic Voltammogram of Synephrine

HPLC conditions: aqueous mobile phase containing 1 mM copper(II) acetate and 20 mM ammonium acetate (pH 6.4); other HPLC conditions are the same as in Fig. 2.

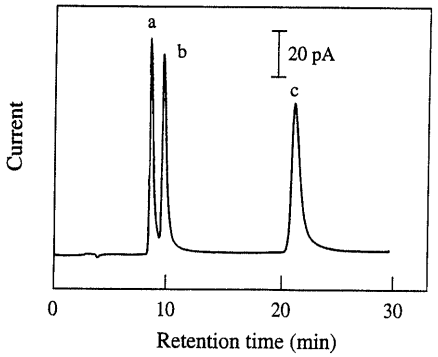

Fig. 4. Chromatogram of a Standard Mixture of *d*,*l*-Synephrine and *l*-Phenylephrine

Peaks: a, *d*-synephrine (20 μM); b, *l*-synephrine (20 μM); c, *l*-phenylephrine (20 μM); HPLC conditions: applied potential, 1.0 V *vs.* Ag/AgCl; other HPLC conditions are the same as in Fig. 3.

concentration of synephrine enantiomers. A good linear relationship between concentration and peak current ratio was found over the range 0.2—100 μM with a correlation coefficient of 0.999 for each synephrine enantiomer. The relative standard deviation was 2.6% at 20 μM *d*-synephrine ($n=10$) and 2.4% at 20 μM *l*-synephrine ($n=10$). The detection limit of each synephrine enantiomer was 0.2 μM ($S/N=3$), *i.e.* 170 pg per injection.

Determination of Synephrine Enantiomers in Citrus Plants The extraction of synephrine from the peel of citrus plants was attempted using methanol or water as an extraction solvent. The synephrine content in the solutions of both solvents was about the same. Thus, water extraction of the sample by ultrasonication was adopted in this study.

Figure 5 shows a chromatogram of a sample extract of *Citrus unshiu*. There was no interference with *l*-synephrine peak. However, the retention time of *d*-synephrine was the same as that of *N*-methyltyramine which is often present in citrus plants. Therefore, an ODS column was connected in series before the chiral separation column to separate *d*-synephrine and *N*-methyltyramine.

Figure 6 shows a chromatogram of a standard mixture of *d*,*l*-synephrine, *N*-methyltyramine, and *l*-phenylephrine in which peaks a, b, c, and d correspond to *d*-synephrine, *l*-synephrine, *N*-methyltyramine, and *l*-phenylephrine, respectively. The α and Rs values for both enantiomers of synephrine were 1.23 and 1.09, respectively. A good linear

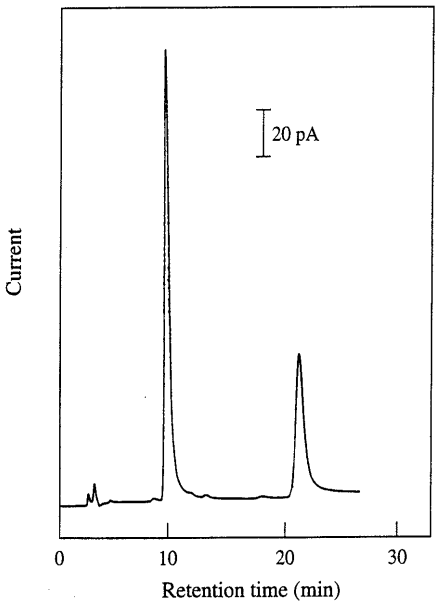

Fig. 5. Chromatogram of a Sample Solution Extracted from *Citrus unshiu*

HPLC conditions are the same as in Fig. 4.

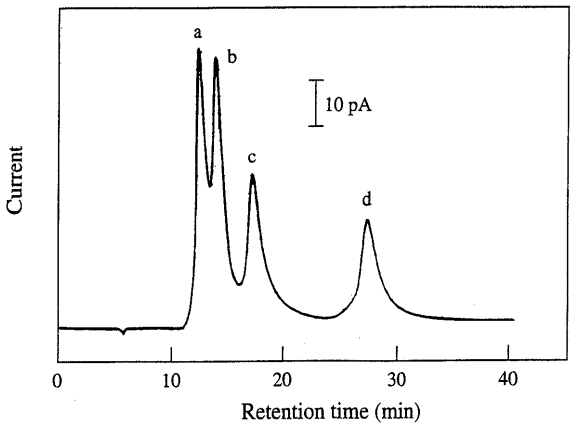

Fig. 6. Chromatogram of a Standard Mixture of *d,l*-Synephrine, *N*-Methyltyramine and *l*-Phenylephrine

Peaks: a, *d*-synephrine (50 μM); b, *l*-synephrine (50 μM); c, *N*-methyltyramine (25 μM); d, *l*-phenylephrine (20 μM). HPLC conditions: aqueous mobile phase containing 1 mM copper(II) acetate and 50 mM ammonium acetate (pH 6.4)–methanol (99 : 1); column, LiChrospher 100 RP-18 (75 × 4 mm i.d., 5 μm), Sumichiral OA-5000 (250 × 4.6 mm i.d., 5 μm); flow rate, 0.7 ml/min; applied potential, 1.0 V *vs.* Ag/AgCl; injection volume, 5 μl.

relationship between concentration and peak current ratio was found over the range 0.5—100 μM with a correlation coefficient of 0.999 for each synephrine enantiomer. The relative standard deviation was 1.7% at 20 μM *d*-synephrine and *l*-synephrine ($n=10$). The detection limit of each synephrine enantiomer was 0.2 μM ($S/N=3$), *i.e.* 170 pg per injection.

The concentrations of synephrine enantiomers in various samples were then determined. Table 1 shows the measured synephrine enantiomer content in the peel of citrus plants. The recovery of 2 μM *d,l*-synephrine from these citrus plants was 96—104%. The *d*-synephrine content was less than 0.001% in all the samples examined. When 56.2 mg *Citrus unshiu* was used for the determination of the enantiomers by HPLC using only a Sumichiral OA-5000 column, the content of *d*-synephrine was less than 0.0001% in *Citrus unshiu*.

Table 1. Content of Synephrine Enantiomers in Citrus Plants

Peel of citrus plant	Content (%)		Recovery (%)[a]	R.S.D. (%)[b]
	d-Synephrine	*l*-Synephrine		
Citrus unshiu	—	0.395	96.5	2.3
C. natsudaidai	—	0.322	97.0	2.2
C. sinensis	—	0.202	96.0	2.2
C. limon	—	0.005	103.8	3.0
C. junos	—	0.067	96.7	2.8
C. sudachi	—	0.154	98.5	2.7

a) *d,l*-Synephrine added: 2×10^{-6} M. *b*) $n=5$. —, not detected (<0.001%).

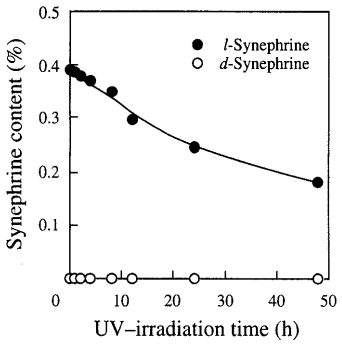

Fig. 7. UV-Irradiation of Synephrine

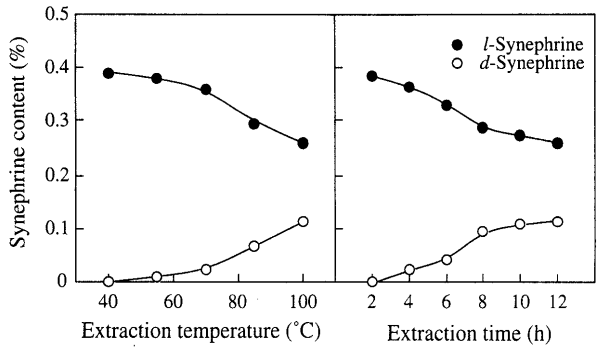

Fig. 8. Effects of Extraction Temperature and Extraction Time

Stability of Synephrine Extracted from *Citrus unshiu* An aliquot (5 ml) of an aqueous extract of 10 mg *Citrus unshiu* peel was placed in a quartz glass cell. The cell was exposed to 250 nm UV light at room temperature for various periods up to 50 min. After this exposure, the solutions were diluted and an internal standard solution was added. It is clear from Fig. 7 that *l*-synephrine in the sample solution was decomposed by UV light. The decomposition products may be generated through oxidation of synephrine, since no new peak appeared on the solution chromatogram after exposure. The half-life of *l*-synephrine decomposition was about 40 min. Therefore, the assay procedure has to be carried out avoiding exposure to UV light.

Generally, Chinese medicines have to be extracted using water with heating and so the heat stability of synephrine was also examined. A total of 50 mg *Citrus unshiu* peel was refluxed with 25 ml water at 100 °C for various periods or at various temperatures for 12 h. After refluxing, the solutions were diluted and an internal standard added. When *Citrus unshiu* peel was extracted under reflux for

Table 2. Content of Synephrine Enantiomers in Crude Drugs and Powders of Chinese Medicine Extract

Sample	Content (%)		Recovery (%)[a]	R.S.D. (%)[b]
	d-Synephrine	*l*-Synephrine		
Crude drug				
Chinpi	—	0.395	100.3	2.3
Kijitsu	—	0.215	98.4	2.1
Goshuyu	—	0.379	97.4	2.3
Powder of Chinese medicine extract				
Hangebyakujyutsu temma-to	—	0.070	95.4	2.3
Heiisan	—	0.109	98.9	2.2
Seihai-to	—	0.030	97.4	2.1
Shigyakusan	—	0.076	101.2	2.3
Bukuryoin-gou-hangekouboku-to	—	0.065	98.0	2.8
Unkei-to	—	0.079	103.3	2.5

a) *d,l*-Synephrine added: 2×10^{-5} M. *b*) $n=5$. —, not detected (<0.001%).

Table 3. Content of Synephrine Enantiomers in Chinese Medicines

Chinese medicine		Content (%)	
		d-Synephrine	*l*-Synephrine
Heiisan	Decoction[a]	—	0.112
	Reflux extract[b]	0.030	0.079
Shigyakusan	Decoction[a]	—	0.083
	Reflux extract[b]	0.025	0.061
Unkei-to	Decoction[a]	—	0.029
	Reflux extract[b]	0.009	0.015

a) Decocted for 40 min at 100 °C. *b*) Refluxed for 12 h at 100 °C. —, not detected (<0.001%).

2 h at 100 °C, the *d*-synephrine and *l*-synephrine content was 0.003% and 0.384%, respectively, i.e. the former was 0.8% of the latter. It is clear from Fig. 8 that *l*-synephrine in the sample solution was optically isomerized to *d*-synephrine as the temperature increased or the heating time was prolonged. Therefore, the assay procedure has to be carried out avoiding high temperatures and long periods of refluxing.

Determination of Crude Drugs, Chinese Medicines and Powders of Chinese Medicine Extract Table 2 shows the analytical results of the content of synephrine enantiomers in crude drugs and powders of Chinese medicine extract. The recovery of 20 μM *d,l*-synephrine from these samples was 95—104%. The *d*-synephrine content was less than 0.001% in all the samples examined.

Chinese medicines are extracted with decoctions to obtain an extract for drug administration and reflux extraction is also used.[11-14] In order to compare these two extraction techniques, the enantiomer content of extracts obtained by the two techniques was compared (Table 3). Although *l*-synephrine was the only enantiomer present in the decoction extracts for all the samples examined, both enantiomers were present in extracts obtained by refluxing. This implies that *l*-synephrine was optically isomerized to *d*-synephrine during refluxing. Since optical isomerization may cause changes in the pharmacological activities of synephrine[5] decoction is preferred for the extraction of Chinese medicines.

It is concluded that HPLC with electrochemical detection using a chiral ligand-exchange column is a simple and rapid method for the determination of synephrine enantiomers.

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