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Note

High-performance liquid chromatographic determination of etilefrine in human plasma using combined solid-phase and organic solvent extraction and electrochemical detection

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Etilefrine hydrochloride, 2-ethylamino-1-(3-hydroxyphenyl)ethanol hydrochloride (Fig. 1), is an amine with sympathomimetic activity that has been used for many years in the treatment of orthostatic disorders [1]. It undergoes considerable first-pass metabolism passing through the gut wall and shows inter-individual variation in bioavailability [2]. It is important, therefore, to monitor the plasma levels in clinical research. Gas chromatography with mass spectrometry [3] and liquid scintillation counting of the radioactively labelled drug [4] have been the principal methods used to determine etilefrine in plasma and urine for pharmacokinetic studies. The high-performance liquid chromatographic (HPLC) methods are accurate and selective, but the sensitivity for etilefrine is not sufficient to enable the use of UV detection.

Recently HPLC coupled with electrochemical detection has been widely used for the determination of drugs in biological fluids, because of its enhanced selectivity and sensitivity [5]. This paper describes a reversed-phase HPLC

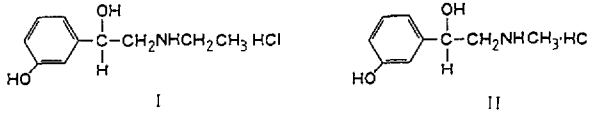

Fig. 1. Structures of etilefrine hydrochloride (I) and the internal standard (II).

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method, with amperometric detection, for the determination of etilefrine in human plasma and its application to a pharmacokinetic study.

EXPERIMENTAL

Materials

Etilefrine hydrochloride and Effortil® tablets were provided by Iwaki Pharmaceutical (Tokyo, Japan) and Tanabe Pharmaceutical (Osaka, Japan), respectively. *l*-Phenylephrine hydrochloride (internal standard, I.S., Fig. 1) and HPLC-grade methanol were purchased from Wako (Osaka, Japan). Bonded-phase extraction columns containing aromatic sulphonic acid for cation exchange were purchased from Baker (Phillipsburg, NJ, U.S.A.). Other reagents and chemicals were of analytical-reagent grade.

Cyclic voltammetry

The apparatus used was an HA-301 potentiostat–galvanostat (Hokuto Denko, Tokyo, Japan). The potential range was set from +20 mV to +1120 mV versus an Ag/AgCl reference electrode with a glassy carbon working electrode. The solvent system consisted of methanol–0.1 *M* phosphate buffer (pH 4.0) (1:8, v/v). The scan rate was 10 mV/s.

HPLC conditions I for recovery experiments

The chromatographic system consisted of a Shimadzu Model LC-4A high-performance liquid chromatograph equipped with a UV detector set at 272 nm (Shimadzu, Osaka, Japan). A stainless-steel column (250 mm × 4.6 mm I.D.) packed with 5 μm particle size Cosmosil C_{18} (Nakarai Chemicals, Kyoto, Japan) was used at 25°C. Methanol–0.1 *M* phosphate buffer (pH 4.0) (1:4, v/v) was employed as the mobile phase at a flow-rate of 1.0 ml/min.

HPLC conditions II for etilefrine in human plasma

The chromatographic system consisted of a Shimadzu Model LC-4A high-performance liquid chromatograph and Shimadzu Model L-ECD-6A amperometric detector equipped with a glassy-carbon electrode at +950 mV. Signals from this detector were monitored on a two-channel chart recorder. A stainless-steel column for preliminary experiments was used at 20°C. Methanol–0.1 *M* phosphate buffer (pH 4.0) (1:8, v/v) was employed as the mobile phase at a flow-rate of 1.0 ml/min.

Recovery experiments for the solid-phase extraction column

In order to examine the eluting conditions of etilefrine from the cation-exchange column, etilefrine samples were applied to a solid-phase extraction column containing 500 mg of bonded-phase aromatic sulphonic acid in a 3-ml polypropylene column, prewashed with 10 ml of distilled water and followed

by 10 ml of methanol and a further 10 ml of 0.1 *M* ammonium acetate (pH 8.6). Samples were eluted from the column with 5 ml of 1 *M* hydrochloric acid containing 1 *M* sodium chloride, and 100-μl aliquots were injected into the HPLC column under conditions I above.

Determination of etilefrine in human plasma

Plasma (2.0 ml) was mixed with 0.5 ml of I.S. (153.8 ng/ml) and 0.5 ml of 0.2% (w/v) bovine serum albumin (BSA). The mixture was passed through a solid-phase aromatic sulphonic acid column prewashed with 10 ml of distilled water and then 10 ml of 0.1 *M* ammonium acetate (pH 8.6), followed by 10 ml of methanol at 25°C. After interferences in the plasma had been removed by washing with 10 ml of 0.1 *M* ammonium acetate (pH 8.6), etilefrine and I.S. were eluted with 5 ml of 1 *M* hydrochloric acid containing 1 *M* sodium chloride. The eluate was extracted with 5 ml of ethyl acetate after the addition of 3 ml of 2 *M* sodium hydroxide and 3 g of sodium chloride. Anhydrous sodium sulphate (0.5 g) was added to the organic layer, which was then shaken for 1 min. After centrifugation for 10 min at 1000 *g*, 4 ml of the supernatant were evaporated in a centrifugal evaporator at 55°C. The residue was dissolved in 50 μl of methanol, and 10 μl of the solution were injected into the HPLC column under conditions II above.

Human subjects and drug administration

Ten healthy male adult subjects were fasted for 12 h before administration. Three Effortil tablets (15 mg as etilefrine hydrochloride) were administered to each subject orally with 200 ml of tap water. Blood samples were withdrawn into heparinized tubes 10, 20, 30, 60, 120 and 240 min after oral administration. Samples were centrifuged immediately to obtain plasma fractions, which were stored at −20°C until analysis.

RESULTS AND DISCUSSION

Cyclic voltammograms

In order to characterize the electrochemical properties of etilefrine, chromatographically assisted cyclic voltammograms are necessary. As shown in Fig. 2, etilefrine is oxidized in the potential range from +20 mV to +1120 mV. Under the above chromatographic conditions, etilefrine can be detected at +1004 mV, where its potential curve reaches a maximum. However, we chose +950 mV for the determination of etilefrine in plasma, because at +1004 mV an unstable baseline was observed owing to the high background current.

Extraction and purification by cation-exchange column and organic solvent

In order to remove interfering compounds from plasma, the solid-phase cation-exchange column that retained etilefrine was washed with 10 ml of 0.1 *M*

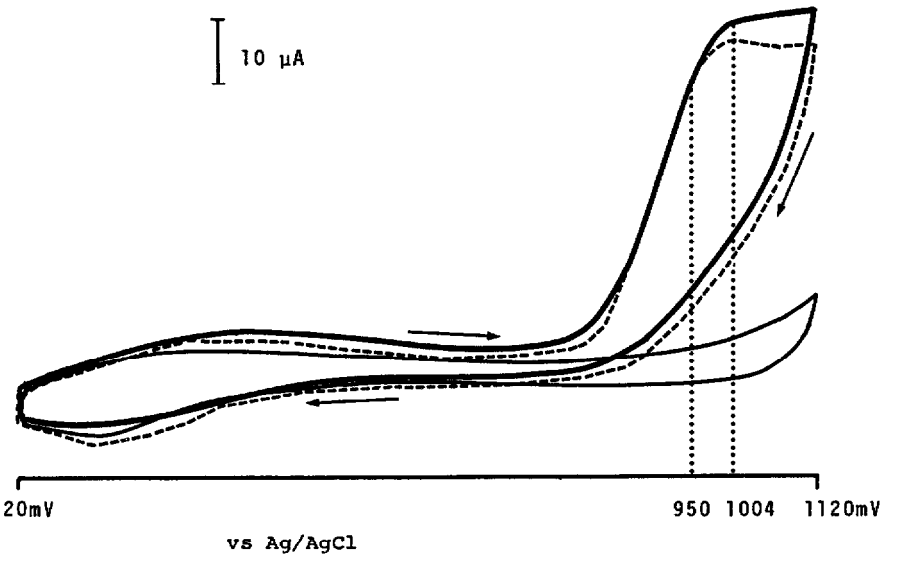

Fig. 2. Cyclic voltammograms of etilefrine (——), I.S. (---) and background (——)

ammonium acetate (pH 8.6). The etilefrine was quantitatively recovered with 5 ml of 1 *M* hydrochloric acid containing 1 *M* sodium chloride effluent from the column (Fig. 8). However, the 1 *M* hydrochloric acid content of the eluting solution showed interference in the chromatograms under HPLC conditions II (Fig. 4). This interference was also observed with acidic solutions such as 1 *M* sulphuric acid, 1 *M* nitric acid and 1 *M* phosphoric acid. A purification of etilefrine in the plasma was therefore carried out by a two-step procedure of solid-phase extraction on a cation-exchange column and extraction with an organic solvent. As described above, sample clean-up was achieved by extraction with ethyl acetate, following elution from the cation-exchange column. The results did not show any interference after the organic extraction, as indicated in Fig. 4.

Chromatography

The retention times of etilefrine and I.S. were 8.0 and 5.5 min, respectively. Fig. 5 shows that the plasma components did not interfere in the determination of etilefrine.

Calibration graph and reproducibility

A calibration graph, obtained by plotting the ratio of the peak height of etilefrine to that of the I.S against known drug concentration, was linear in the range 1.1–43.8 ng/ml. The equation for the calibration graph was $y=14.01x+0.30$, where y is plasma concentration of etilefrine (ng/ml) and x is ratio of peak height, and the coefficient of correlation (r) was 0.999. The

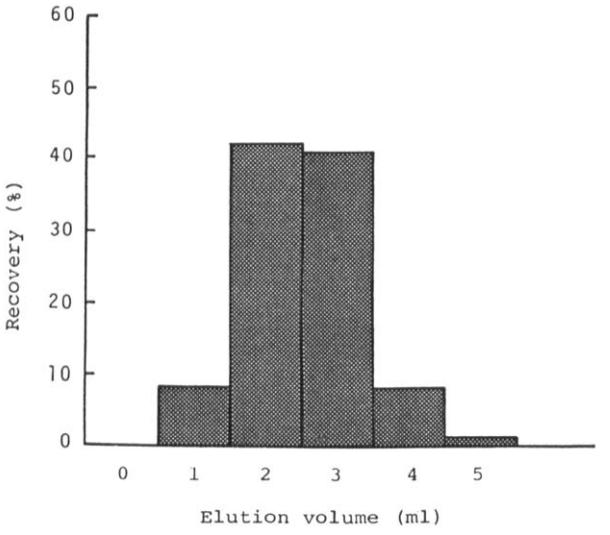

Fig. 3. Elution profiles of etilefrine by 1 *M* hydrochloric acid containing 1 *M* sodium chloride from the solid-phase cation-exchange column.

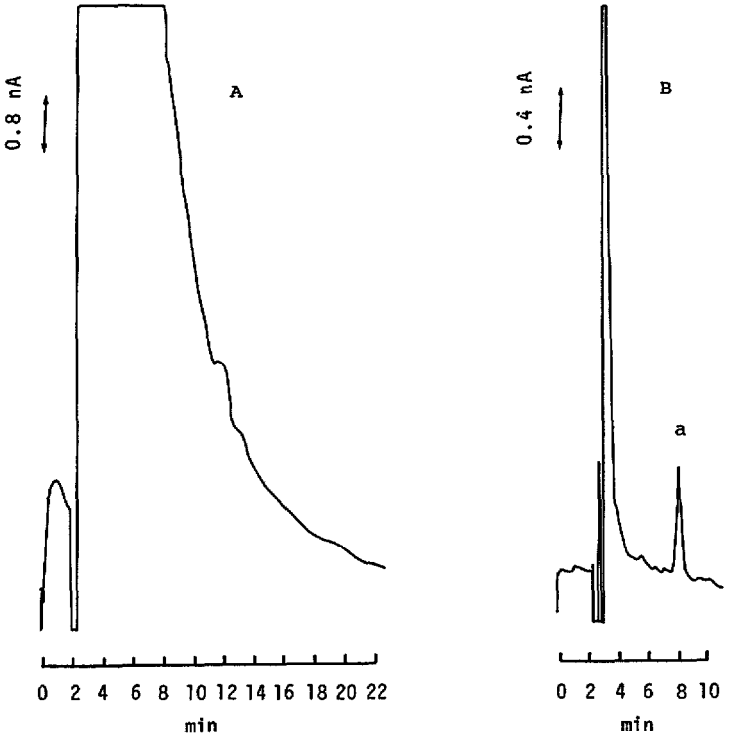

Fig. 4. Chromatograms of human plasma extracted with (A) the solid-phase cation-exchange column and (B) the combination of the solid-phase cation-exchange column and ethyl acetate. Peak a=etilefrine.

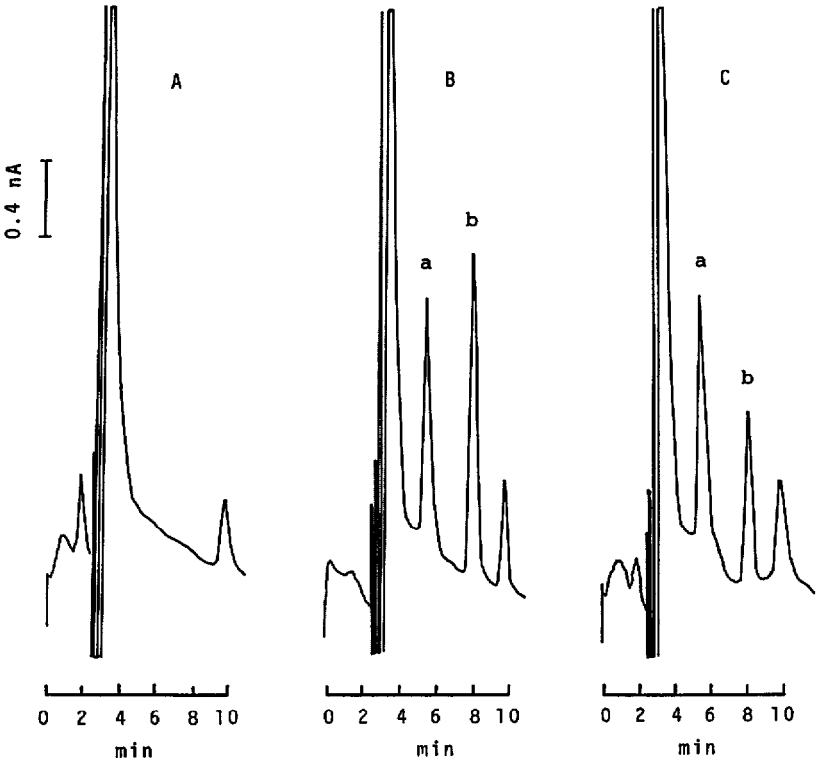

Fig. 5. Chromatograms of (A) drug-free plasma, (B) drug-free plasma spiked with 105.3 ng/ml etilefrine and 153.8 ng/ml I.S. and (C) a plasma sample collected 20 min after oral administration of 15 mg as etilefrine hydrochloride (three Effortil tablets) to a healthy human subject. Peaks: a = I.S.; b = etilefrine.

TABLE I

IINTRA-ASSAY PRECISION AND ACCURACY FOR THE DETERMINATION OF ETILEFRINE IN SPIKED HUMAN PLASMA

Concentration spiked (ng/ml)	Concentration found (mean ± S.D., $n=5$) (ng/ml)	Coefficient of variation (%)	Accuracy[a] (%)
1.1	1.1 ± 0.1	10.5	100
2.2	2.5 ± 0.4	14.7	114
8.8	8.8 ± 0.7	7.5	100
21.9	21.5 ± 0.5	2.4	98
43.8	44.0 ± 1.0	2.2	100

[a] $\text{Accuracy} = \frac{\text{found}}{\text{added}} \times 100\%$.

detection limit at a signal-to-noise ratio of 2.0 was 1.1 ng/ml, which is well below the concentration levels encountered in human plasma specimens after therapeutic dosing. Intra-assay reproducibility was determined by carrying out five determination from plasma samples spiked with a known amount of etilefrine. The data presented in Table I indicate that the coefficients of variation ranged from 2.2 to 14.7%.

Application to biological samples

The method was applied to a pharmacokinetic study of humans. Fig. 6 shows the mean plasma concentration versus time profile of etilefrine after a single oral dose of 15 mg as etilefrine hydrochloride (three Effortil tablets) to ten healthy humans. The mean values of the maximum plasma concentration of

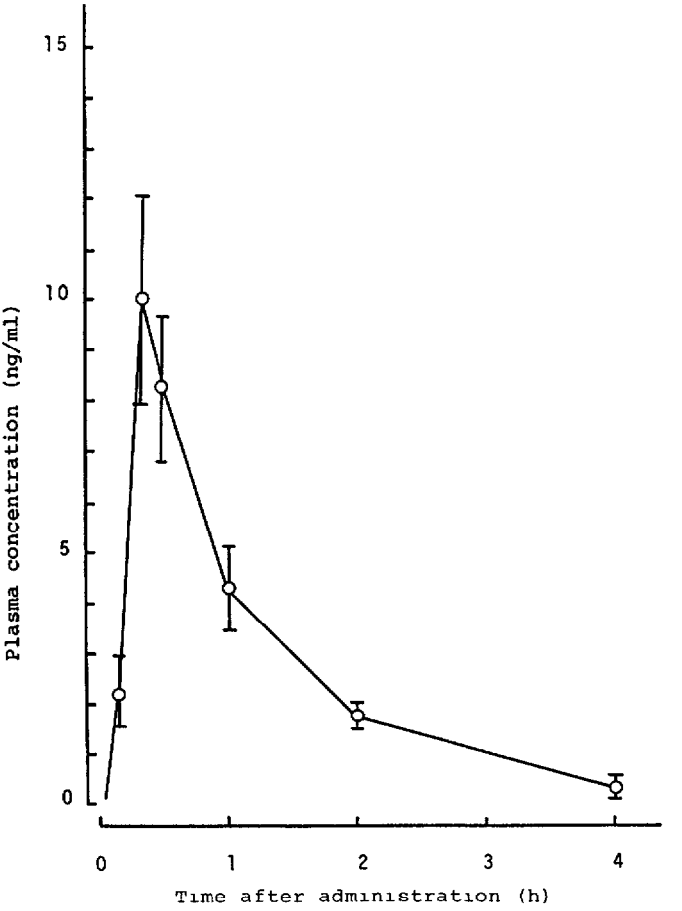

Fig. 6. Mean plasma concentration–time profile of etilefrine after oral administration of 15 mg as etilefrine hydrochloride (three Effortil tablets) to ten healthy humans.

10.1 ng/ml was attained 20 min after the administration, and thereafter etilefrine disappeared rapidly from the plasma with a half-life of 0.6 h.

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