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Ion-Pair High-Performance Liquid Chromatographic Analysis of Sulpyrine and Its Metabolites in Rabbit Plasma

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A simple, specific and sensitive ion-pair high-performance liquid chromatographic analysis was developed for aminopyrine, sulpyrine and their metabolites (4-methylaminoantipyrine, 4-aminoantipyrine, 4-acetylaminoantipyrine and 4-formylaminoantipyrine) in rabbit plasma. Following deproteinization with acetonitrile and separation on an octadecylsilane-bonded silica column (250 × 4 mm, JASCO Fine SIL C-18), the eluted components were detected with a multi-wavelength ultraviolet monitor at 260 nm. The mobile phase consisted of 22% (v/v) acetonitrile and 78% (v/v) of an aqueous solution containing 10 mM KH_2PO_4 and 1.24 mM tetra-*n*-butylammonium bromide, adjusted to pH 4.5 with 0.1 M phosphoric acid solution. Hexobarbital was employed as an internal standard. Based on 0.1 ml of plasma, the detection limits were 0.35 μM for 4-acetylaminoantipyrine, 0.35 μM for 4-formylaminoantipyrine, 0.7 μM for sulpyrine, 1 μM for 4-aminoantipyrine, and 2 μM for 4-methylaminoantipyrine at the signal-to-noise ratio of 5 : 1.

Keywords——sulpyrine; sulpyrine metabolite; methylaminoantipyrine; aminoantipyrine; acetylaminoantipyrine; formylaminoantipyrine; ion-pair high-performance liquid chromatography; plasma concentration

Sulpyrine (I) has been widely used as an analgesic and antipyretic drug. The following compounds are known to be the main metabolites in human urine:[1] 4-methylaminoantipyrine (II), 4-aminoantipyrine (III), 4-acetylaminoantipyrine (IV) and 4-formylaminoantipyrine (V).

Several methods for the quantitative analysis of I in pharmaceutical preparations or aqueous solution have been reported, including colorimetric methods,[2] a thin-layer chromatographic method,[3] and high-performance liquid chromatographic (HPLC) methods.[4] These methods are unsuitable for simultaneous determination of I and its metabolites in plasma. Several investigators[5,6] reported the determination of these compounds in urine or muscle of the rat. Unfortunately, it was difficult to determine the parent compound in plasma because I was easily split into II and hydroxymethanesulfonate in water, reaching an equilibrium.[7] Tamura[6] *et al.* used an HPLC method to determine I and its metabolites (II—IV) in rat muscle, but the separation of the peaks and the sensitivity do not seem to be sufficient to detect these compounds in plasma, and no information on V was given.

In this paper, a simple, specific and sensitive ion-pair HPLC method was established for the separation and quantification of I, its metabolites (II—V) and aminopyrine (VI) in rabbit plasma. The sensitivity and accuracy were determined and an application of the method is described.

Experimental

Materials——All chemicals and reagents were of analytical grade unless otherwise indicated. Compounds I and

VI were of JP quality. Compounds III and IV (Wako Pure Chemical Industry) and hexobarbital (VII) (Tokyo Kasei Kogyo) as an internal standard were purchased. Compound II was kindly supplied by Daiichi Co., Ltd., and V was synthesized by formylation of III.[8]

Apparatus——The high-performance liquid chromatograph used in this study consisted of a model 635S liquid chromatograph and a model 635M multi-wavelength ultraviolet (UV) monitor (Hitachi, Ltd., Tokyo, Japan). The column (250 × 4 mm, i.d.) was packed with 5-μm porous silica bonded with octadecylsilane (Fine SIL C-18, Japan Spectroscopic Co., Tokyo, Japan), and was protected by a precolumn (23 × 3.8 mm, i.d.) of 30-μm glass beads bonded with octadecylsilane (Co-pell ODS, Whatman, Inc., Clifton, U.S.A.). The precolumn was periodically renewed (about every 60 injections) to ensure effective separation and adequate sensitivity in the analysis.

Chromatographic Conditions——The mobile phase consisted of 22% (v/v) acetonitrile and 78% (v/v) of an aqueous solution containing 10 mM KH_2PO_4 and 1.24 mM tetra-*n*-butylammonium bromide (TBA), adjusted to pH 4.5 with 0.1 M phosphoric acid solution. The flow rate was maintained at 1.5 ml/min at a pressure of 140—160 kg/cm^2. The eluted components were monitored at 260 nm. The chromatography was carried out at 25 ± 5 °C.

Sample Preparation——A sample of 100 μl of plasma was pipetted into a glass test tube, and 10 μl of sodium bisulfite aqueous solution (15 mg/ml) was immediately added. The mixture was left to stand in an ice bath until extraction. After the addition of 1 ml of acetonitrile containing hexobarbital (9 μg/ml), the mixture was mixed for 10 s, and centrifuged at 2000 × *g* for 1 min. One ml of the clear supernate was pipetted into another glass test tube, and evaporated to dryness under reduced pressure (about 15 mmHg) at ambient temperature. The tube was covered with Parafilm and stored at −20 °C until analysis. All extraction procedures were immediately carried out after sampling, because I and III are unstable to heat and water. The residue in the test tube was redissolved in 70 μl of the mobile phase immediately before the analysis. A 50 μl aliquot of this solution was injected into the chromatograph.

Standard Curves——A standard solution containing IV and V at a concentration of 50 μM, and I—III at a concentration of 100 μM, was prepared in methanol. A suitable volume of this methanol solution was transferred into a test tube. Then, the methanol was evaporated off under reduced pressure and 100 μl of plasma was added to each test tube. After mixing for 7 min in ice bath to dissolve the compounds, the samples were immediately extracted as described under "Sample Preparation." The standard solution was always freshly prepared.

Stability Test of Sulpyrine in Plasma——Plasma samples containing 30 μM I were kept in an ice bath. At suitable times up to 60 min, aqueous sodium bisulfite solution was added to the plasma sample and the mixture was immediately extracted as described above.

Animal Experiment——Compound I was administered intravenously *via* the medial vein of one ear of male white rabbits, 3.0—3.3 kg, and blood samples were collected from the marginal vein of the other ear at intervals for 3 h. The samples were kept at 0 °C. Plasma sample was separated from blood by centrifugation at 2000 × *g* for 2 min and a 100 μl aliquot was transferred to another test tube. The plasma sample was analyzed by the procedure described above.

Results and Discussion

Extraction

Compound I is easily decomposed to II in water, but is fairly stable at pH 4—8.[7] Therefore, a simple and quick extraction at neutral pH is required. Thus we chose the deproteinization method with acetonitrile and evaluated the effects of sodium bisulfite, which is known to stabilize I, on the stability of I during extraction. Addition of sodium bisulfite to the samples was necessary to prevent the decomposition of I during extraction and

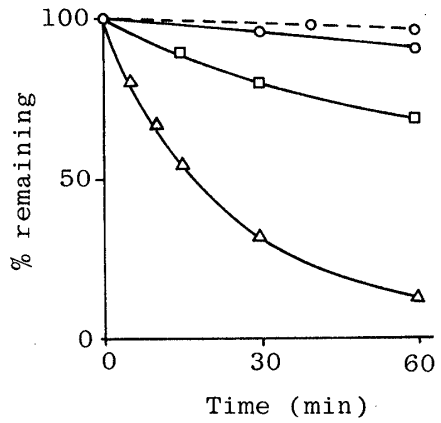

Fig. 1. Stability of Sulpyrine in Plasma with or without Sodium Bisulfite

○, 0 °C; □, 20 °C; △, 37 °C; ----, with sodium bisulfite; ——, without sodium bisulfite.

evaporation. When this was not done, almost all of I decomposed. Figure 1 shows the effects of sodium bisulfite and temperature on the stability of I in rabbit plasma. The best results were obtained with sodium bisulfite at 0 °C; more than 98% of the initial I remained unchanged after 60 min. There was an optimum concentration of sodium bisulfite; larger amounts decreased the reproducibility and the recovery, and smaller amounts allowed I to decompose.

Chromatography

When a simple mixture of acetonitrile and phosphate buffer (pH 4.5) was used as the mobile phase to separate II—V, I eluted before V at 2 min, and this early elution of I is undesirable because of overlap with other peaks due to biological materials. Thus, we used TBA as the counter-ion of I in order to move the elution position to behind IV. Figure 2 shows the effects of TBA concentration on the capacity factors for I—V. Increasing the concentration of TBA increased the retention time of I, decreased those of II and III, and did not change those of IV and V. These materials were well separated at 1.24 mM TBA. Figure 3 shows typical chromatograms from blank rabbit plasma and plasma with added I—VI. The chromatogram from blank plasma gave a few unidentified peaks, but did not contain peaks showing retention times similar to those of I—VII. The retention times of I, II, III, IV, V, VI and VII were 8.2, 11.4, 9.4, 5.2, 4.4, 14.5 and 18.0 min, respectively.

Standard Curves

Figure 4 shows the standard curves obtained by HPLC assay of plasma containing various amounts of I—V. There were good linear relationships between the peak height ratios of I—V to VII and the plasma concentrations of these compounds. The detection limits were 0.7 μM for I, 2 μM for II, 1 μM for III, and 0.35 μM for IV and V at the signal-to-noise ratio of 5 : 1. The precision of the HPLC method was determined by assaying four replicate plasma samples at three different concentrations (Table I).

Stability of HPLC Samples

We investigated the stability of I—V in HPLC samples stored at −20 °C after extraction

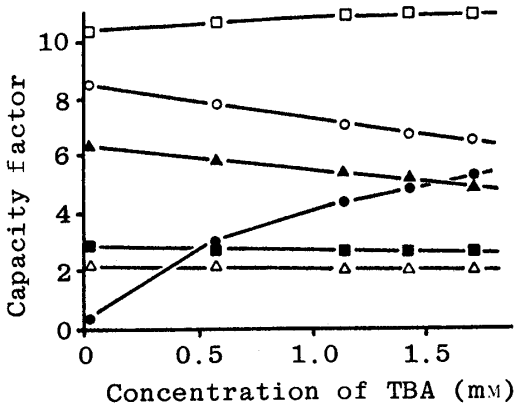

Fig. 2. Effects of Tetra-*n*-butylammonium Bromide on Capacity Factors

●, I; ○, II; ▲, III; ■, IV; △, V; □, VII (internal standard).

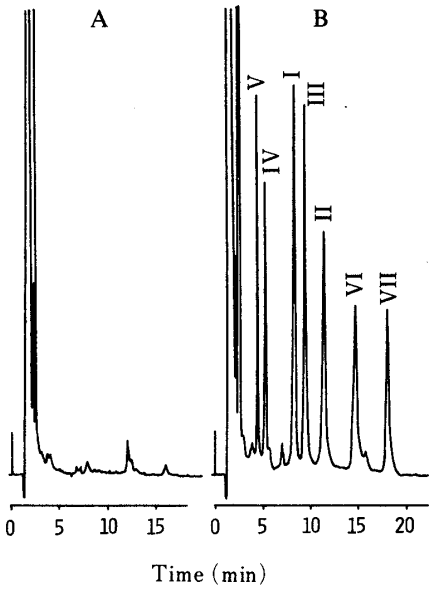

Fig. 3. Typical Chromatograms from Blank Rabbit Plasma and Plasma with Added Sulpyrine and Its Metabolites

A, blank plasma; B, plasma containing drug.

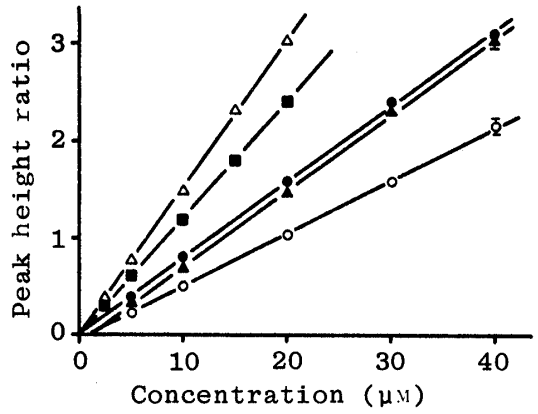

Fig. 4. Standard Curves for Determination of Sulpyrine and Its Metabolites in Plasma

●, I, $Y=7.67\times10^{-2}X+4.55\times10^{-2}$, $r=0.998$; ○, II, $Y=5.50\times10^{-2}X-7.03\times10^{-2}$, $r=0.998$; ▲, III, $Y=7.89\times10^{-2}X-7.71\times10^{-2}$, $r=0.997$; ■, IV, $Y=1.21\times10^{-2}X-2.13\times10^{-2}$, $r=0.999$; △, V, $Y=1.51\times10^{-2}X-4.66\times10^{-2}$, $r=0.999$. Y, peak height ratio; X, concentration (μM).

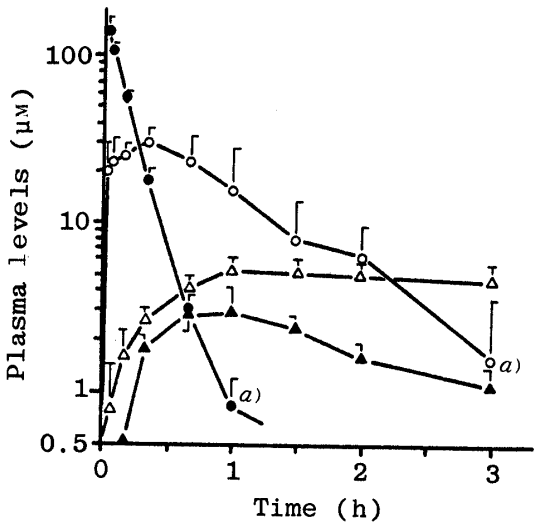

Fig. 5. Plasma Levels after Intravenous Administration of Sulpyrine in Rabbits ($n=3$)

Dose, sulpyrine 51.2 μmol/kg (=18 mg/kg).
a) Minimum detectable level.
Key: see Fig. 2.

TABLE I. Precision of Assays for Sulpyrine and Its Metabolites in Plasma

Compound	Plasma concentration (μM)		
	10[a]	20	40
I	9.95±0.43 (4.3)[b]	20.21±0.23 (1.1)	39.32±0.23 (0.6)
II	10.26±0.24 (2.3)	19.81±0.57 (2.9)	40.37±1.38 (3.4)
III	9.89±0.27 (2.7)	19.33±0.37 (1.9)	39.28±1.55 (1.6)
IV	9.87±0.42 (4.3)	20.10±0.52 (0.5)	—
V	9.75±0.27 (2.8)	19.89±0.26 (1.3)	—

a) Added amount.
b) Mean±standard deviation (coefficient of variation, %) of found amount in four determinations.

and evaporation. For I, IV and V, more than 99% of the initial amounts remained after they had been stored for 3 d. On the other hand, for II and III, about 94 and 75% remained, respectively. Therefore, the HPLC analysis was usually performed on the same day that the blood samples were drawn from the experimental animals, or at the latest by the next day.

Measurement of Plasma Concentration

A 51.2-μmol/kg dose of I was administered intravenously to three rabbits, and the time courses of the concentrations of I and its metabolites in plasma were measured by the present HPLC method. As shown in Fig. 5, there was a rapid fall-off of plasma I levels within 60—90 min, a rapid rise of II and a relatively slow rise of V and III; IV was not detected in the plasma samples.

The present study demonstrated that the proposed HPLC method is more specific and sensitive for I and its metabolites than other available methods.

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