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METHOD FOR THE SIMPLIFIED ANALYSIS OF DEPROTEINIZED PLASMA AND URINARY ISOPROTERENOL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple method for the determination of isoproterenol in urine and plasma by high-performance liquid chromatography coupled with an automated trihydroxyindole method is described. No pre-purification procedures are required. The sensitivity was 0.2 pmol and the average recoveries of isoproterenol added to plasma and urine were 89% and 101%, respectively. The method has been applied successfully to clinical samples.

INTRODUCTION

Isoproterenol (ISP) has been used as a bronchodilator for the treatment of asthma and as an inotropic agent for certain cardiovascular disorders. Although improved β2-stimulators have begun to replace ISP as an anti-asthmatic drug, the inotropic action of ISP is still important for the treatment of low-

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output syndrome in children. However, its β-action causes changes in fatty metabolism, hormonal secretion and arrhythmia [1, 2]. There has been a report of sudden death of asthmatic patients who had been treated with ISP [3].

To find out the relationship between the plasma concentration of ISP and changes in fatty metabolism, hormonal secretion or arrhythmia, it is desirable to examine the blood level of ISP. However, no simple and reliable method for the determination of ISP for clinical application has yet been established.

For the determination of ISP in biogenic materials and for checking the pharmaceutical quality of ISP, several methods, such as fluorimetry [4], gas chromatography [5] and radioisotope methods [6], have been used. However, these methods require complicated procedures for the extraction of ISP from biological specimens. Because of this disadvantage, these methods are unsatisfactory with respect to speed, accuracy and reproducibility.

The purpose of this study was to establish a method for the direct determination of ISP in deproteinized plasma and urine by high-performance liquid chromatography (HPLC) coupled with the trihydroxyindole (THI) method [7, 8].

EXPERIMENTAL

Apparatus

An LC-2 high-performance liquid chromatograph, an RF500LCA spectrofluorimeter, an R-12 recorder from Shimazu Seisakusho (Kyoto, Japan), a KMT-24 autosampler from Kyowa Seimitsu (Osaka, Japan) and a Technicon proportionating pump were used.

Strong cation-exchange columns (Zipax-SCX, particle size 30 μm, 150 cm × 2.1 mm I.D. for the main column, 15 cm × 2.1 mm I.D. for the guard column) from Shimazu Seisakusho were used.

Reagents

ISP was purchased from Sigma (St. Louis, MO, U.S.A.). O-Methylisoproterenol was supplied by Nikken Chemical Industry (Tokyo, Japan). Sodium phosphate (monobasic), hydrochloric acid, ethylenediaminetetraacetic acid (EDTA), potassium hexacyanoferrate(III), perchloric acid, ascorbic acid, sodium bisulphite and sodium hydroxide were of analytical-reagent grade from Wako (Osaka, Japan).

Reagent preparation

All aqueous reagent solutions were prepared with water distilled twice in glass. A stock standard solution of ISP (4.7 m*M* ISP) was prepared by dissolving 1 g of ISP in 1 l of 0.1 *M* hydrochloric acid containing 10 m*M* sodium bisulphite. A working standard solution was prepared by 100-fold dilution of the stock standard solution with 0.1 *M* hydrochloric acid containing 10 m*M* sodium bisulphite. Further 100-fold and 1000-fold dilutions of the working standard solution were made daily with 0.1 *M* hydrochloric acid containing 5 m*M* ascorbic acid as a calibration standard for urinary and

plasma ISP, respectively. As reagents for oxidizing, reducing and intensifying the fluorescence in the THI method, 4 m*M* potassium hexacyanoferrate(III) dissolved in 0.5 *M* phosphate buffer (pH 6.8), 6 m*M* ascorbic acid and 8 *M* sodium hydroxide solution were used. As the mobile phase in HPLC, 0.25 *M* monobasic sodium phosphate solution (pH 4.3) was used.

Sample preparation

Plasma. A 5-ml volume of healthy human blood was collected and immediately transferred into a plastic tube containing 5 mg of solid EDTA, disodium salt. The blood was mixed by gentle inversion of the tube, and then centrifuged at 3000 *g* for 10 min at 4°C. The plasma (2.0 ml) was mixed with 200 μl of 4 *M* perchloric acid and vortex-mixed vigorously for 1 min, then centrifuged at 6000 *g* for 20 min at 4°C. To the deproteinized plasma, 20 μl of the working standard solution of various concentrations were added. The final concentration of ISP in the plasma ranged from 1 to 240 pmol/ml.

Urine. To 10 ml of freshly voided healthy human urine, 100 μl of concentrated hydrochloric acid and then 10 μl of the working standard solution of various concentrations were added. The final concentration of ISP ranged from 20 pmol/ml to 4.8 nmol/ml.

For the assay of plasma and urinary ISP in patients who had been administered ISP after cardiac surgery, samples were treated as mentioned above.

Analytical procedure

A flow diagram of the assay system is shown in Fig. 1. The sample solution was injected automatically into the column at intervals of 30 min (injection volume: 200 μl of deproteinized plasma or 10 μl of urine). The column was eluted at a flow-rate of 1.0 ml/min and the column temperature was

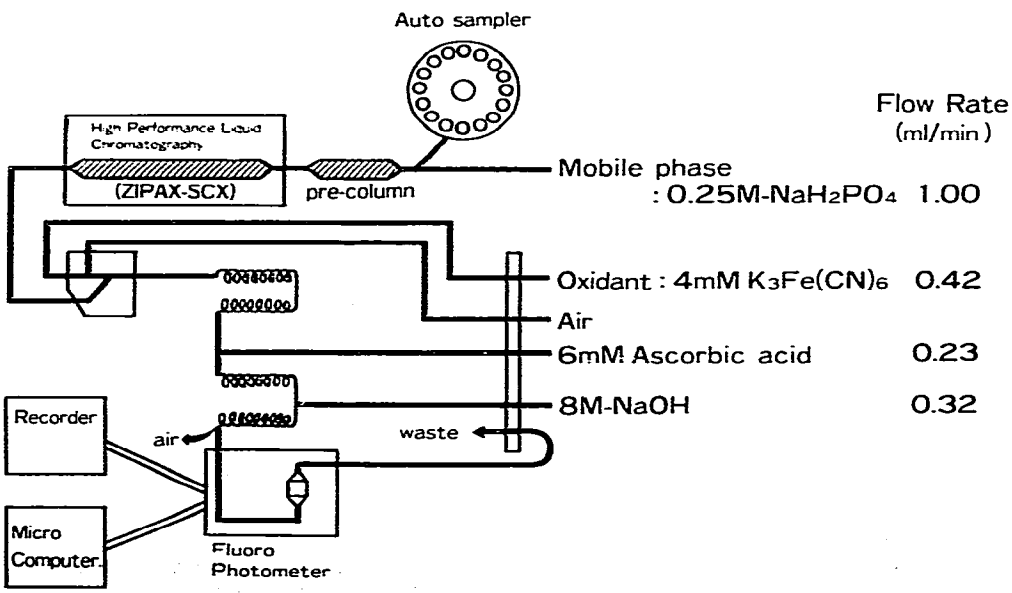

Fig. 1. Analytical flow diagram.

maintained at 40°C. ISP in the column eluate was converted into trihydroxyindole derivatives automatically and the fluorescence intensity was measured with a spectrofluorimeter (excitation wavelength 400 nm, fluorescence wavelength 510 nm) equipped with a recorder. The fluorescence intensity was calculated from the peak height.

RESULTS

Typical chromatograms of the standard solution, a plasma sample and a urine sample are shown in Fig. 2. The retention time of ISP was 24 min. ISP was well separated from interfering substances in urine and deproteinized plasma. The peak of metanephrine appeared at 18 min in the urine sample. The peak of noradrenaline (retention time 10 min) and adrenaline (12 min) were overlapped by unknown interfering substances.

Concerning the metabolites of ISP, O-methylisoproterenol (main intermediate metabolite of ISP) appeared in the chromatogram at 69 min only when a large dose was applied and did not affect the ISP peak. In addition, no substances interfering with the ISP peak were found on application of either urine or deproteinized plasma from patients who had been treated with ISP. Further, we investigated possible interferences in the assay system due to drugs used in intensive care units. These drugs included other catecholamines, α,β-blockers, antibacterial drugs, several vitamins, narcotics and sedatives. None of them interfered with the ISP peak.

Fig. 3 shows the relationship between the injection volume with and without pH adjustment and relative fluorescence intensity (the pH was adjusted to 4.5 with 2 *M* potassium hydroxide solution). A linear correlation was

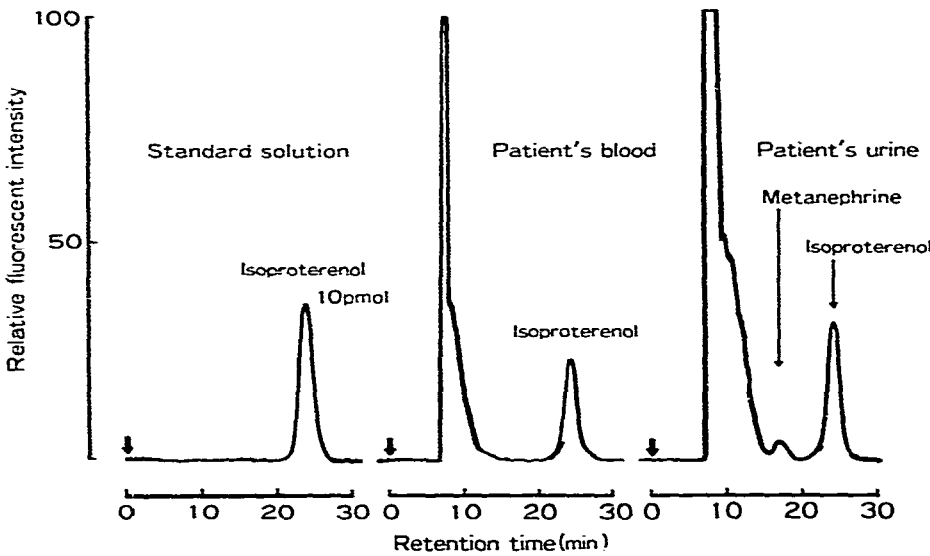

Fig. 2. Chromatography of ISP. Deproteinized plasma and urine samples were taken from a patient who had been administered isoproterenol (734 pmol/kg/min, 20 h) after cardiac surgery. Sample size: 200 μl of deproteinized plasma, 10 μl of urine and 200 μl of standard solution, respectively.

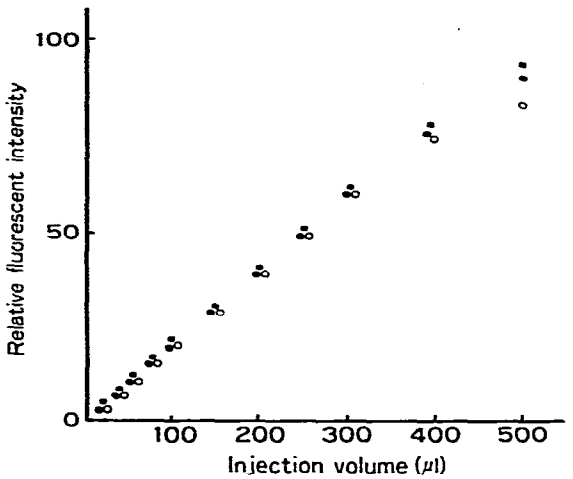

Fig. 3. Correlation between injection volume and fluorescence intensity. Deproteinized plasma containing isoproterenol (50 pmol/ml) with and without pH adjustment was subjected to HPLC. Injection volume ranged from 25 to 500 μl (pH adjustment of deproteinized plasma was effected with 2 *M* potassium hydroxide solution. •, Standard (pH 1.0); ○, deproteinized plasma (pH 0.5); ▪, deproteinized plasma (pH 4.5, adjusted).

observed from 10 to 300 μl without pH adjustment, and up to larger injection volumes with pH adjustment.

There was a linear relationship between relative fluorescence intensity and amount of ISP added from 0.2 to 48 pmol both for urine (10-μl injection) and deproteinized plasma (200-μl injection, without pH adjustment).

An amount of 0.4 pmol of ISP added to deproteinized plasma was detected at a signal-to-noise ratio of 4. Thus, the minimal sensitivity for determination of plasma ISP was 0.2 pmol.

Intra-assay variation was examined by the analysis of five samples of three sorts of doses both in urine and deproteinized plasma. The mean coefficient of variation (C.V.) was 3.2% for deproteinized plasma ISP and 1.2% for urinary ISP (Table I).

TABLE I

INTRA ASSAY AND INTER ASSAY OF DEPROTEINIZED PLASMA AND URINARY ISOPROTERENOL

Assay	Parameter	Deproteinized plasma isoproterenol			Urinary isoproterenol		
Intra-assay	$\bar{X}$ (pmol)	9.4	4.8	1.9	19.5	9.8	4.8
	n	5	5	5	5	5	5
	S.D. (pmol)	± 0.2	± 0.1	±0.1	± 0.1	± 0.1	± 0.1
	C.V. (%)	± 2.1	± 2.1	±5.3	± 0.5	± 1.0	± 2.1
Inter-assay	$\bar{X}$ (pmol)	9.4	1.8		19.3	4.8	
	n	10	10		10	10	
	S.D. (pmol)	± 0.4	± 0.1		± 0.5	± 0.1	
	C.V. (%)	± 4.3	± 5.6		± 2.6	± 2.1	

TABLE II

RECOVERY OF ISOPROTERENOL FROM PLASMA AND URINE

Values of recoveries obtained by adding standard compound to plasma and urine samples.

No.	Plasma isoproterenol			Urinary isoproterenol		
	Added (pmol)	Measured* (pmol)	Recovery (%)	Added (pmol)	Measured* (pmol)	Recovery (%)
1	37.9	34.3	91	37.9	38.9	103
2	18.9	16.6	88	18.9	19.0	101
3	9.5	8.3	87	9.5	9.5	100
4	4.7	4.2	89	4.7	4.8	102
5	2.4	2.1	88	2.4	2.4	100

*Average of duplicate assay.

Inter-assay variation was measured by using samples stored in a refrigerator for 10 days at 4°C. The mean C.V. was 5.0% for deproteinized plasma ISP and 2.4% for urinary ISP (Table I).

When several doses of ISP (2.4—37.9 pmol) were added to both plasma and urine, the recovery of ISP from plasma ranged from 87 to 91% and that from urine from 100 to 103% (Table II).

It was possible to measure the ISP concentration in blood and urine from patients (n=12) who had received ISP (23.7—734.0 pmol/kg/min) after cardiac surgery. The plasma concentration of ISP ranged from 1.3 to 28.7 pmol and that in urine from 41.2 pmol/ml to 1.10 nmol/ml.

DISCUSSION

The retention time of ISP is longer than that of other catecholamines and related substances when they are applied to Zipax-SCX [7]. In addition, we found by the present HPLC method coupled with the THI method that most fluorescent interfering substances in urine or deproteinized plasma appeared in the early phase of the chromatograms and the peak of ISP appeared clearly without any interfering peaks. The peak of O-methylisoproterenol appeared at a later phase of the chromatogram. Therefore, urinary or deproteinized plasma samples could be injected directly. In addition, we did not use an internal standard for calibration, but only used an external standard because of the good recovery and low coefficient of variation.

To measure low concentrations of ISP, a large injection volume was necessary as well as enhancement of fluorescence. As shown in Fig. 3, if the sample pH was adjusted to higher values, the sample volume could be increased. However, in this study, the separation of ISP was performed as simply as possible. Therefore, we curtailed the pH adjustment and 200 μl of deproteinized plasma without pH adjustment was chosen as the sample of blood to be applied. As the concentration of urinary ISP is high [9], 10 μl of urine was enough for the measurement.

Although the sensitivity of the method was adequate for our purpose,

a situation where it is necessary to measure lower concentrations of ISP might arise. To improve the sensitivity, several parameters can readily be altered, for example, increased sample volume and improvement of the detection method. The sample volume will be increased by pH adjustment and by an increase in the capacity factor of the column. The latter will be improved by increasing the theoretical plate number of Zipax-SCX. For an improved detection method, there are more sensitive THI methods [10] and a more sensitive fluorescence method for catecholamines [11]. The final question is how many times a column can be used even if direct injection of the sample is applied. We found we could analyse more than 200 samples with one column and exchanged the pre-column after every 200 samples.

The proposed method requires only deproteinization of plasma as the pre-treatment procedure and, because of its speed, accuracy and reproducibility, the method is suitable for clinical application.

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