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DETERMINATION OF PHENYLPYRUVIC ACID IN URINE AND SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A highly sensitive and simple method for the determination of phenylpyruvic acid in urine and serum is described which employs high-performance liquid chromatography with fluorescence detection. Phenylpyruvic acid, after extraction with ethyl acetate, is reacted with 4'-hydrazino-2-stilbazole in aqueous methanol to give the corresponding fluorescent hydrazone which is separated by reversed-phase chromatography on μBondapak Phenyl. The lower limits of detection are 25 and 32 pmol for phenylpyruvic acid in 0.2 ml of urine and serum, respectively. This sensitivity permits the determination of the acid in urine of normal adults and newborn infants.

INTRODUCTION

It is well known that phenylpyruvic acid (PPA) is greatly increased in serum and urine of patients with phenylketonuria. Several methods have been reported for the determination of PPA in biological samples. Spectrophotometric methods [1—5] are not sensitive and selective for PPA. Gas chromatographic methods [6, 7] and gas chromatographic—mass spectrometric methods [8, 9] are sensitive and selective, but not simple to perform. A high-performance liquid chromatographic (HPLC) method with UV detection for the determination of PPA in urine, based on the pre-column derivatization of PPA with 2,3-diaminonaphthalene, has been reported [10]. However, this method is not so sensitive and requires a long time for the derivatization. Although HPLC methods with fluorescence detection have also been described for the sensitive determination of 2-oxo acids based on the derivatization with 4-bromomethyl-7-methoxycoumarin [11] and with N^1-methylnicotinamide chloride

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[12], these methods have not been applied to PPA in urine and serum.

Recently, we found that the reaction of PPA with 4'-hydrazino-2-stilbazole (4H2S; fluorogenic reagent for 2-oxo acids and other carbonyl compounds) to give the corresponding hydrazone [13] was enhanced by the addition of methanol to the reaction mixture, the resulting fluorescence being stabilized for a long time. The hydrazone could be separated from the products from other carbonyl compounds present in biological samples by reversed-phase HPLC. We thus developed a highly sensitive and simple HPLC method with fluorescence detection for the determination of PPA in human urine and serum.

EXPERIMENTAL

Materials and reagents

All chemicals were of analytical-reagent grade, unless otherwise noted. 4H2S dihydrochloride was purchased from Tokyo Kasei Ind. Co. (Tokyo, Japan). Double-distilled water and solvents were used. Urine and serum samples of adults were obtained from normal volunteers in our laboratory. Urine samples from newborn infants were supplied from Kyushu University Hospital.

Apparatus

A Mitsumi liquid chromatograph equipped with a 7120 syringe-loading sample injector and a Shimadzu FLD-1 fluorescence detector fitted with a coated mercury lamp (emitting light, ca. 300—400 nm; maximum intensity of the light, 360 nm) and an EM-4 secondary cut-off filter (cutting out the light of wavelengths shorter than 430 nm) was used. The column was μBondapak Phenyl (particle size, 10 μm; 300 × 3.9 mm I.D.; Waters Assoc., Milford, MA, U.S.A.). This column can be used for more than 500 injections with only a small decrease in the theoretical plate number when washed with aqueous methanol (1 : 1, v/v) at the flow-rate of 1 ml/min for ca. 10 min after everyday analyses. The fluorescence spectra of the column effluents were measured with an Hitachi MPF-4 spectrofluorimeter in 10 × 10 mm cells. The slit-widths in terms of wavelengths were set at 10 nm in both the exciter and the analyser. The spectra are uncorrected.

Procedure

To 0.2 ml of urine or serum placed in a 10-ml centrifuge tube, 1.0 ml of water and a mixture of benzene and ethyl acetate (1 : 1, v/v) were added. The mixture was shaken for 3 min and centrifuged. The organic layer was discarded, then the aqueous layer was acidified, with ca. 0.15 ml (3 drops) of concentrated hydrochloric acid, to pH 1 or less, followed by addition of 0.5 g of sodium chloride. The mixture was extracted with 1.0-ml portions of ethyl acetate (twice) by 5-min shaking and centrifugation. The extracts were combined and concentrated to dryness in vacuo at room temperature. To the residue, 1.0 ml of water, 0.5 ml of 0.5 *M* ammonium chloride solution (pH 4.0, adjusted with 0.1 *M* hydrochloric acid) and 0.5 ml of 1.2 m*M* 4H2S dihydrochloride solution in methanol (freshly prepared) were added. The mixture was warmed at 50°C for 10 min in the dark to develop fluorescence and cooled in ice—water. Within 2 h, an aliquot (50 μl) of the reaction mixture was applied

to the chromatograph. The mobile phase was a mixture of 0.1 *M* hydrochloric acid, tetrahydrofuran and water (10:24:64, v/v; pH 2.0) and the flow-rate was 1.0 ml/min. The column temperature was ambient (ca. 25°C). The height of the peak at the retention time of 13 min was used for the quantitation. The amount of PPA was calibrated by means of the standard addition method: 1.0 ml of water added to the sample in the procedure was replaced by 1.0 ml of a PPA standard solution (0.50 nmol/l; prepared with the sodium salt of PPA; stable for more than 6 months at 5°C).

RESULTS AND DISCUSSION

HPLC conditions

Fig. 1 shows the chromatograms obtained with PPA solutions and the reagent blank. The peak observed at the retention time of 13 min is repro-

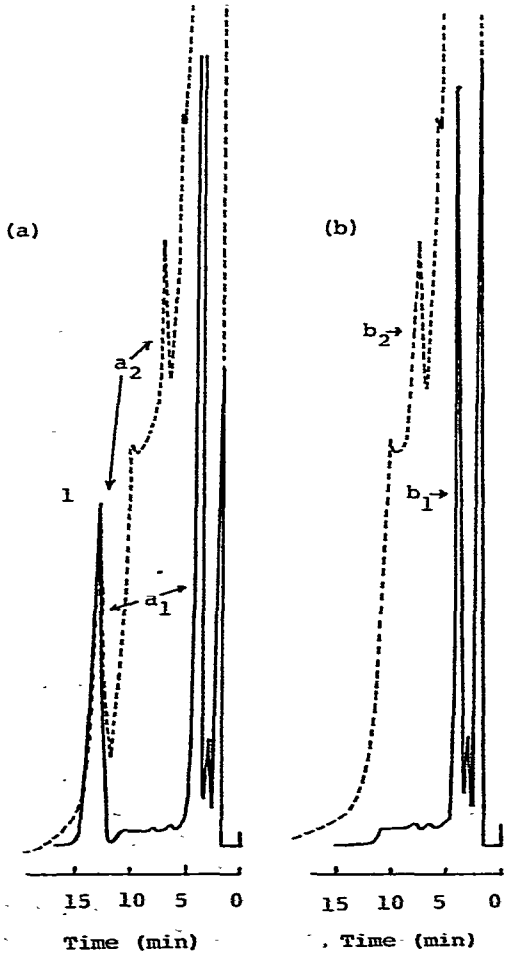

Fig. 1. Chromatograms of (a) 4H2S derivative of PPA, and (b) reagent blank. Aliquots (1.0 ml) of PPA solutions of 20 nmol/ml (a_1) and 0.5 nmol/ml (a_2), and of water for blank, were treated with 4H2S as in the procedure. Detector sensitivity: a_1 and b_1, 1; a_2 and b_2, 32. Peak 1 = PPA.

ducible and there is a linear relationship between the peak height and the amount of PPA under the prescribed conditions. The peak is not interfered with by the blank which is recorded as large peaks when the instrument sensitivity is set at a high level for the determination of PPA at sub-nanomol concentrations (Fig. 1, a_1 and a_2, peak 1).

The concentration of tetrahydrofuran in the mobile phase affects the separation of the peaks. At a concentration greater than 26%, the peak for PPA overlaps those of the blank, while a concentration of less than 20% causes delay in the elution with broadening of the peak; a concentration of 24% was selected for the procedure recommended. The 4H2S derivative of PPA fluoresces most intensely at pH 2 or less with a minimum blank fluorescence, and the column packing μBondapak Phenyl can be used in the limited range of pH 2—8. Therefore, a mobile phase of pH 2.0 was used in the recommended procedure.

Sample solutions for HPLC

Under the HPLC conditions described, 4H2S derivatives of biologically important carbonyl compounds examined (e.g. pyruvic, oxalacetic, 2-oxoglutaric, 2-oxobutyric, 2-oxocaproic, 2-oxoadipic, 2-oxoisovaleric, indolepyruvic, and *p*-hydroxyphenylpyruvic acids, formaldehyde, acetaldehyde, *n*-butylaldehyde, propionaldehyde, benzaldehyde, acetone and diacetyl) eluted much earlier or later than the retention time of 13 min. However, the peak for PPA overlaps that of the derivative of *p*-hydroxybenzaldehyde, which exists usually in a small amount in urine [14]; and the derivatives of isovaleraldehyde and vanillin elute closely to that of PPA (retention times, 12.3 and 12.7 min, respectively). These aldehydes give peak heights of less than 0.2% that of PPA at equimolar concentrations.

A number of other compounds examined (e.g. L-α-amino acids, sugars, aliphatic and aromatic amines, carboxylic acids, aldehydes and ketones, phenols, steroids and many others), all of them of biological importance, gave no peak on the chromatogram when added to urine and serum at a concentration of 100 nmol/ml or greater.

Water-diluted urine and serum are washed with benzene and ethyl acetate prior to the extraction of PPA in the procedure recommended. If this washing is omitted, the peak for PPA overlaps slightly with those due to unknown substances present in urine and serum samples. PPA can be extracted from strongly acidified urine and serum with ethyl acetate in the presence of a saturating concentration of sodium chloride.

The reaction of 2-oxo acid with 4H2S has been carried out in aqueous solution [13]. Methanol added to the reaction mixture enhances the reaction of PPA with the reagent and stabilized the resulting fluorescence (Fig. 2) in the concentration range of 20—30%; 25% was used in the procedure. Dimethylsulfoxide and dimethylformamide caused a decrease in the fluorescence development (Fig. 2). 4H2S was dissolved in the methanol. The reagent gives the most intense fluorescence at a concentration greater than ca. 1 m*M*; 1.2 m*M* was used as a sufficient concentration.

The fluorescence reaction proceeds most effectively at about pH 4. This was achieved by using 0.5 *M* ammonium chloride solution of pH 4.0. Formic acid solution at pH 4.0, when used in place of the ammonium chloride solution as

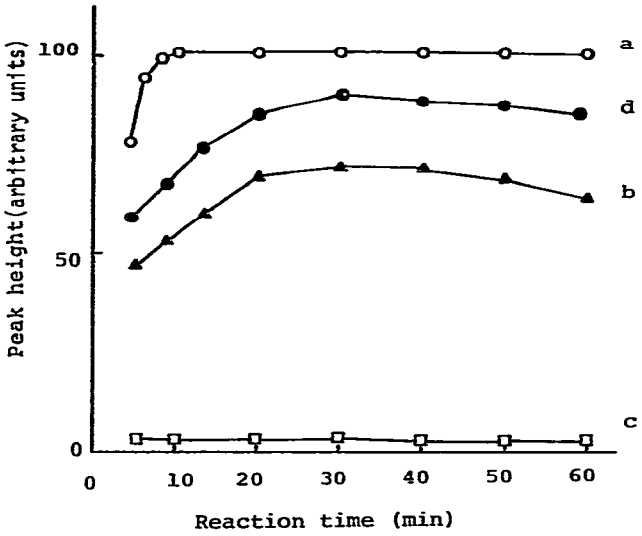

Fig. 2. Effect of solvent and reaction time on the fluorescence development. Portions (1.0 ml) of PPA solution (10 nmol/ml) were treated as in the procedure for the fluorescence development with (a) 4H2S dissolved in methanol, (b) dimethylsulfoxide, (c) dimethylformamide, and (d) water, for various reaction times.

previously described [13], caused some interfering peaks in the chromatogram. Higher temperature allows the fluorescence to develop more rapidly. At the recommended temperature, 50°C, the fluorescence intensity (peak height) reaches a maximum after warming for 10 min or more (Fig. 2). The fluorescence is unstable in daylight, and so the reaction should be carried out in the dark. The resulting fluorescence is stable for more than 2 h at room temperature.

Determination of PPA in urine and serum

Fig. 3 shows typical chromatograms obtained with normal urine and normal serum spiked with PPA according to the procedure. Small peaks observed at the retention time of 23 and 30 min for normal urine and a peak at 26 min for normal serum were unidentified. These peaks do not interfere with the quantitation of PPA in the biological samples.

The fluorescence excitation (maximum, 401 nm) and emission (maximum, 544 nm) spectra of the effluent from peak 1 in Fig. 3a were identical with those of the effluent from peak 1 in the chromatogram for PPA solution (Fig. 1a). A four-times methanol-diluted effluent of peak 1 in Fig. 3a (apparent pH 2.7) had the same fluorescence spectrum as that of peak 1 in Fig. 1a (excitation and emission maxima, 403 and 449 nm, respectively). When a mixture (ca. 1:1, v/v) of both effluents was subjected to HPLC on a LiChrosorb RP-18 (particle size, 5 μm; Japan Merck, Tokyo, Japan) column (150 × 4 mm I.D.; packed as previously described [15]; column temperature, 25°C) with 0.1 *M* hydrochloric acid—tetrahydrofuran—water (10:25:65, v/v) as mobile phase (flow-rate, 0.7 ml/min), a single peak was obtained at the retention time of 8.6 min. These observations indicate that the component of peak 1 in Fig. 3 is due undoubtedly to PPA from the urine sample.

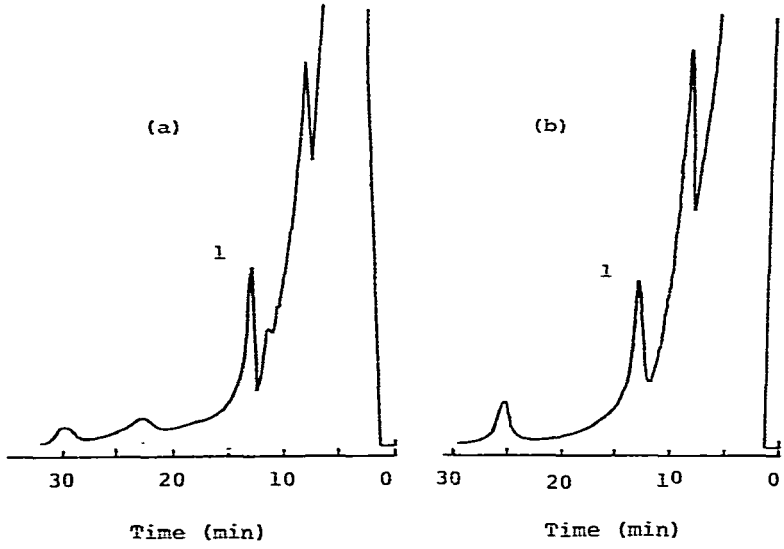

Fig. 3. Chromatograms of 4H2S derivative of PPA in (a) normal urine, and (b) normal serum (free from PPA) spiked with 0.5 nmol/ml PPA, obtained by the recommended procedure. Peak 1 = PPA. The concentration of PPA in urine was 0.58 nmol/ml.

A linear relationship was observed between the peak height and the amount of PPA added to urine or serum up to at least 4 nmol per 0.2 ml. The recoveries of PPA added to 0.2 ml of urine and serum in the amount of 0.5 nmol were 100 ± 3% and 78 ± 4% (mean ± standard deviation, $n = 15$ each), respectively. The recoveries were calculated from the determined values obtained with the fortified urine and serum samples and a PPA standard solution (0.5 nmol/ml) treated as in the procedure. The lower limits of detection for PPA in 0.2 ml of urine or serum were 25 and 32 pmol, respectively.

TABLE I

URINARY EXCRETION (24 h) OF PPA FROM HEALTHY PERSONS

Adults			Newborn infants*
Age (years)	Sex	PPA (nmol)	PPA (nmol)
22	m	800	1.48
22	m	500	1.54
23	m	920	4.36
28	m	550	3.31
29	f	990	8.59
30	f	970	4.05
30	m	860	5.54
30	m	520	13.26
32	m	500	19.15
33	m	450	2.74
Mean ± S.D.		710 ± 209	6.40 ± 5.44

*All female subjects, 2 days old.

The precision of the method was examined by performing ten separate determinations on urines containing 490 and 220 pmol per 0.2 ml PPA, and sera (free from PPA) spiked with 500 and 200 pmol PPA per 0.2 ml. The standard deviations were 15 and 8 pmol per 0.2 ml for PPA in urine, and 26 and 13 pmol per 0.2 ml for PPA in serum, respectively.

The concentration of PPA in normal urines of adults (22—33 years old, n = 10) and newborn infants (2 days old, n = 10) determined by this method were 0.68 ± 0.29 pmol per 0.2 ml and 0.66 ± 0.43 pmol per 0.2 ml (mean ± standard deviation), respectively. The amount of PPA in 24-h urines of these subjects are shown in Table I. PPA could not be detected in sera of normal adults (22—33 years old, n = 10) by this method. The results are the same as those reported by other workers [5, 16]. The concentration of PPA in sera of patients with phenylketonuria is very high (more than 25 nmol/ml [5]) and so can be easily determined by this method.

This study has provided the first HPLC method that permits the determination of PPA in normal urines. The method is simple to perform and may therefore be applied for routine use.

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