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DETERMINATION OF α-KETO ACIDS IN SERUM AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A simple, rapid and highly sensitive high-performance liquid chromatographic method for the determination of α-keto acids in serum and urine is described. In dilute hydrochloric acid, α-keto acids are converted by 1,2-diamino-4,5-dimethoxybenzene into highly fluorescent quinoxalinone derivatives. The derivatives are isocratically separated simultaneously within 14 min by reversed-phase chromatography on a Radial-Pak cartridge C_{18} and detected fluorimetrically. The limits of detection are 10—300 fmol in an injection volume of 10 μl (40—1200 pmol/ml of serum or urine). This sensitivity permits precise determination of several α-keto acids in 5 μl of serum or urine from healthy persons, and also the determination of phenylpyruvic acid in normal urine which cannot be simultaneously determined by other methods.

INTRODUCTION

α-Keto acids are important intermediates in the biosyntheses of amino acids, carboxylic acids and sugars. Certain α-keto acids exhibit greatly increased levels in sera and urines from patients with hereditary metabolic diseases such as

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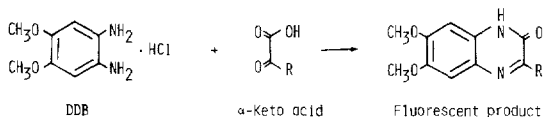

Fig. 1. Reaction of 1,2-diamino-4,5-dimethoxybenzene (DDB) with α-keto acid to produce 3-substituted 6,7-dimethoxy-2(1H)-quinoxalinone derivatives.

maple syrup urine disease, phenylketonuria and tyrosinosis. Thus, a simultaneous assay of α-keto acids in serum and urine may be useful for diagnosis and therapy of these diseases.

Many methods based on paper chromatography [1], gas–liquid chromatography [2–5] and high-performance liquid chromatography (HPLC) [6–11] have been reported for the determination of α-keto acids in serum and urine. Among these, HPLC methods with spectrophotometric and spectrofluorimetric detections have been widely employed because of their ease of operation. Spectrophotometric detection, based on the reaction of α-keto acids with 2,4-dinitrophenylhydrazine [6, 7] or *o*-phenylenediamine (OPD) [8–10], has a limited sensitivity and thus requires a large amount of serum and urine (more than 200 μl). A method with spectrofluorimetric detection using OPD as a fluorogenic reagent has been applied for the determination of α-keto acids in serum and urine [11]. However, it requires a rather complicated clean-up procedure and only permits the determination of a few α-keto acids in normal serum and urine because of the lack of selectivity and sensitivity. The method has been modified to enhance the selectivity and sensitivity [12]. Although the modified method can detect some α-keto acids in only 50 μl of serum, it still requires a complicated clean-up procedure using a hydrazide gel column and solvent extraction and the OPD derivatives should be separated with a gradient elution. The method has not been applied to urine samples.

We have reported that 1,2-diamino-4,5-dimethoxybenzene (DDB) reacts selectively with α-keto acids in an acidic solution to produce highly fluorescent products, 3-substituted 6,7-dimethoxy-2(1H)-quinoxalinone derivatives which can be separated by reversed-phase HPLC with isocratic elution (Fig. 1); the method is much more sensitive than that using OPD [13, 14]. This study aims to establish a simple HPLC method with fluorescence detection for the determination of α-keto acids in extremely small amounts of normal human serum and urine (5 μl) utilizing the above findings. α-Ketovaleric acid (KV) does not occur in human serum or urine, and thus was used as an internal standard.

EXPERIMENTAL

Reagents and solutions

All chemicals and solvents were of analytical-reagent grade, unless otherwise noted. Deionized and distilled water was used. The sodium salts of pyruvic acid (PY), α-ketobutyric acid (KB), KV, α-ketoisovaleric acid (KIV), α-keto-β-methylvaleric acid (KMV), α-ketocaproic acid (KC), α-ketoisocaproic acid (KIC), α-ketoglutaric acid (KG), phenylpyruvic acid (PP) and *p*-hydroxyphenylpyruvic acid (HPP), glyoxylic acid and DDB monohydrochloride were obtained as described previously [15].

DDB solution (5 m*M*) was prepared by dissolving 102 mg of DDB monohydrochloride in 100 ml of 0.5 *M* hydrochloric acid containing 0.7 *M* β-mercaptoethanol (stabilizer for DDB). The solution was used within 3 h.

Serum and urine sample solutions

Urine and serum specimens were obtained from healthy volunteers in our laboratories.

Serum sample solution. A 5-μl portion of serum was mixed with 5 μl of 40 nmol/ml KV as an internal standard and 90 μl of 0.8 *M* perchloric acid. The mixture was allowed to stand at room temperature (ca. 25°C) for 10 min and then centrifuged at 1000 *g* for 10 min. The supernatant was used as a serum sample solution.

Urine sample solution. Urine (24 h) was collected in the presence of 6 *M* hydrochloric acid (10 ml) and stored at 4°C. All α-keto acids examined were stable for at least two days in the acidified urine. To 5 μl of the urine centrifuged at ca. 1000 *g* for 5 min were added 5 μl of 40 nmol/ml KV and 90 μl of water. The resulting solution was used for the assay.

Apparatus and HPLC conditions

Uncorrected fluorescence excitation and emission spectra of the eluate were measured with a Hitachi 650-60 fluorescence spectrophotometer fitted with a 20-μl flow-cell; spectral bandwidths of 5 nm were employed for both the excitation and emission monochromators. The chromatograph and the HPLC conditions were as described previously [13]. The column was a Radial-Pak cartridge C_{18} (100 × 8 mm I.D., particle size 5 μm). The mobile phase was methanol—acetonitrile—40 m*M* phosphate buffer pH 7.0 (65:40:95), flow-rate 1.2 ml/min.

Fluorescence derivatization procedure

To 50 μl of the serum or urine sample solution placed in a screw-capped 1.5-ml vial, 50 μl of the DDB solution were added, and the vial was tightly closed and heated at 100°C for 2.5 h in the dark. The reaction mixture was cooled in ice—water to stop the reaction. A 10-μl aliquot of the resulting solution was injected into the chromatograph.

The calibration graph was prepared according to the derivatization procedure, except that 5 μl of the KV solution in the preparation of serum (or urine) sample solution was replaced with 5 μl of a standard solution containing 10—400 pmol per 5 μl of each of the α-keto acids. The net peak-height ratios of the individual α-keto acids and KV were plotted against the concentration of the acids spiked. The concentration of α-keto acid was read from the calibration graph.

RESULTS AND DISCUSSION

The conditions for the fluorescence derivatization and HPLC were almost identical with those described previously [13]. The efficiency of conversion of the α-keto acids into substituted quinoxalinones was examined by comparing the fluorescence peak heights obtained under the present conditions with those

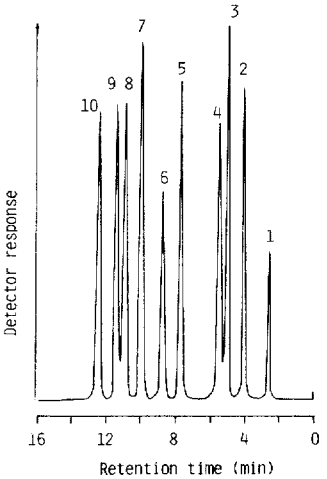

Fig. 2. Chromatogram of the DDB derivatives of α-keto acids. A portion (5 μl) of a mixture of ten α-keto acids was treated according to the described procedure. Peaks (pmol per 5 μl): 1 = KG (50); 2 = PY (100); 3 = HPP (7000); 4 = KB (200); 5 = KV (200); 6 = KIV (400); 7 = KIC (200); 8 = PP (600); 9 = KC (300); 10 = KMV (400).

given by the corresponding crystalline quinoxalinones [14]; the extents of conversion (%, mean ± S.D., n = 7) were 98.2 ± 1.8 for PY, 92.0 ± 1.2 for KG, 64.9 ± 0.8 for KB, 82.1 ± 1.4 for KV, 30.0 ± 2.1 for KIV, 72.4 ± 1.5 for KC, 100.5 ± 2.3 for KIC, 60.0 ± 0.8 for KMV, 18.1 ± 1.1 for PP and 44.6 ± 1.6 for HPP.

Fig. 2 shows a typical chromatogram obtained with a standard mixture of nine α-keto acids of biological importance and KV. The DDB derivatives of these α-keto acids could be completely separated within 14 min by isocratic elution.

Typical chromatograms obtained from a normal serum and a urine are shown in Figs. 3 and 4, respectively. The peaks for α-keto acids in the serum and urine could be identified on the basis of their retention times and the fluorescence excitation and emission spectra of the eluates in comparison with those of the standard compounds (Fig. 2), and also by co-chromatography of the standards and the serum and urine. The peak due to PP (Fig. 4, peak 8) could be observed in urine but not in serum, in agreement with results obtained previously [16]. Peak 11 in Figs. 3 and 4 could be ascribed to glyoxylic acid; when authentic glyoxylic acid was treated according to the described procedure, the peak due to the acid had exactly the same retention time as that for peak 11, and the fluorescence spectra of the DDB derivative of authentic glyoxylic acid were identical in shape and excitation and emission maxima (368 and 455 nm, respectively) with those of the eluate from peak 11. Some unidentified peaks (peak 12, Figs. 3 and 4) were observed in the chromatograms from both the serum and urine, though the DDB reaction is selective for α-keto acids. When serum or urine sample solution was replaced with water, no peak occurred except for that of the reagent DDB (Figs. 3c and 4c). Moreover, each eluate from peak 12 exhibited fluorescence excitation and emission maxima around 360 and 450 nm, almost identical with those of the

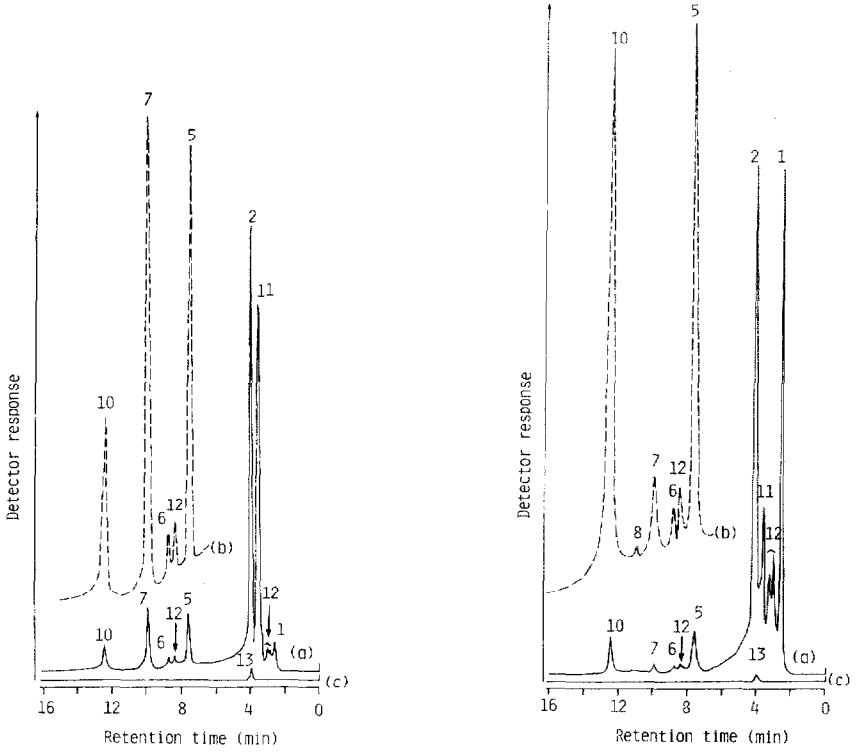

Fig. 3. Chromatograms of the DDB derivatives in normal serum (a and b) and the reagent blank (c). A portion (5 μl) of serum or water for the reagent blank was treated according to the described procedure. Peaks: 1—10, see Fig. 2; 11 = glyoxylic acid; 12 = unidentified; 13 = DDB. Detector sensitivity: a and c, 1; b, 8.

Fig. 4. Chromatograms of the DDB derivatives in normal urine (a and b) and the reagent blank (c). A portion (5 μl) of urine or water for the reagent blank was treated according to the described procedure. Peaks as in Figs. 2 and 3. Detector sensitivity: a and c, 1; b, 16.

DDB derivatives of α-keto acids. These observations suggest that the peaks may be caused by unknown endogenous 1,2-diketo compounds in serum and urine. Their peaks did not interfere with the determination of KG, PY and KIV in normal serum and urine, and so further clean-up of the sample solutions was not necessary. The peak of DDB (peak 13, Figs. 3c and 4c) was overlapped with that from PY. However, since the peak height for DDB was 1/50—1/150 that for PY in the chromatograms from normal serum and urine, the peak of DDB was practically negligible in the determination of PY.

Linear relationships were observed between the ratios of the peak heights of α-keto acids to that of KV and the amounts of α-keto acids each spiked in the range of 0.01—4 nmol to 5 μl of serum and urine, and no change in the slopes of these relationships was observed depending on the serum or urine used. These facts indicate that the present internal standard method permits the determination of α-keto acids in urine and serum over wide ranges of their concentrations.

The recoveries (%, mean ± S.D.; each n = 8) of α-keto acids (each 0.4 nmol per 5 μl) added to pooled normal serum were 74.1 ± 2.0 (PY), 74.5 ± 3.2 (KG),

68.4 ± 0.9 (KB), 66.3 ± 1.8 (KIV), 74.9 ± 1.0 (KIC), 73.2 ± 1.6 (PP), 73.0 ± 0.5 (KC) and 67.3 ± 1.6 (KMV). When these α-keto acids (each 0.4 nmol) were added to 5 μl of pooled normal urine the mean recoveries were in the range 94—106% and the S.D. did not exceed 4% (each $n = 8$).

The precision of the method for the determination of the biogenic α-keto acids was also examined by performing eight replicate analyses on normal serum and urine. The coefficients of variation [concentration (nmol/ml) in parentheses] in serum were 3.95 (KG, 4.7), 2.55 (PY, 53.0), 2.22 (KIV, 19.4), 1.31 (KIC, 27.8) and 2.04% (KMV, 20.7), and in urine were 4.26 (KG, 314.6), 2.27 (PY, 316.7), 4.81 (KIV, 30.7), 2.05 (KIC, 3.4), 4.26 (PP, 0.8) and 2.02% (KMV, 54.8). The limits of detection (pmol/ml) for the α-keto acids were 40 (KG), 1200 (PY), 380 (KIV), 80 (KIC) and 270 (KMV) in serum and urine, and 170 (PP) in urine, at a signal-to-noise ratio of 2. These values correspond to amounts of 10—300 fmol in an injection volume of 10 μl. This sensitivity permits the precise determination of PP, which occurs at extremely low concentrations in normal urine.

The amounts of α-keto acids in sera and 24-h urines from healthy volunteers were determined by the described method (Tables I and II). The mean values for the individual α-keto acids in serum were in good agreement with published data [10, 12]. The amounts of α-keto acids, except for PP, in 24-h urine were determined for the first time in this study. When the mean values were calculated per mg creatinine, the values were not very different from those obtained by the other workers [10, 11]. The amount of PP in 24-h urine was the same as found previously [16]. Peaks ascribable to HPP, KB and KC could not be observed in the chromatograms obtained with the sera and the urines, probably because they occur at extremely low concentrations (below the limits of detection) in these samples.

TABLE I

CONCENTRATIONS OF α-KETO ACIDS IN SERA FROM HEALTHY PERSONS

Age	Sex*	Concentration (nmol/ml)				
		KG	PY	KIV	KIC	KMV
21	M	4.7	53.0	19.4	27.8	20.7
21	F	6.1	96.3	16.5	29.3	19.3
21	F	6.1	67.7	15.8	42.1	26.1
22	M	5.4	46.1	24.5	43.5	27.2
22	M	5.9	107.7	18.9	44.0	25.0
22	M	4.3	74.4	22.9	27.8	27.2
23	M	5.9	104.7	35.5	61.9	50.0
23	M	5.9	89.6	16.0	33.4	19.3
23	F	9.7	53.4	29.0	27.8	25.0
24	F	7.7	92.2	19.4	35.4	19.6
26	M	6.6	66.0	32.3	53.8	38.0
34	M	5.4	39.8	22.6	28.7	21.7
Mean		6.1	74.2	22.7	38.0	26.6
S.D.		1.4	22.5	6.2	10.8	8.7

*M = Male; F = female.

TABLE II

URINARY EXCRETION (24 h) OF α-KETO ACIDS FROM HEALTHY PERSONS

Age	Sex*	Excretion (μmol per day)					
		KG	PY	KIV	KIC	PP	KMV
21	F	269.5	208.1	26.0	2.7	0.6	31.7
21	F	264.1	167.1	19.6	2.5	1.0	36.5
21	F	435.9	203.9	47.9	4.7	0.8	64.2
21	F	235.9	283.7	9.9	4.0	0.8	17.7
22	M	287.9	363.5	91.3	7.7	2.3	99.9
22	M	288.4	337.5	63.5	6.3	1.0	59.1
22	M	236.5	279.2	71.8	3.4	1.2	105.3
26	M	273.7	275.5	26.7	2.9	0.7	47.7
34	M	235.8	353.0	27.2	8.2	2.8	24.5
Mean		280.9	274.6	42.7	4.7	1.2	54.1
S.D.		58.3	65.9	25.9	2.1	0.7	29.6

*M = Male; F = female.

This study provides the first HPLC method for the simultaneous assay of α-keto acids including PP. The present method does not require a complicated clean-up procedure compared with other HPLC methods [10, 11], and the DDB derivatives can be separated isocratically within 14 min. Thus, the method is rapid enough to assay ten samples within 3 h. It is highly sensitive, permitting the assay of various α-keto acids using minute amounts of serum or urine. In particular, PP could be detected even in normal urine. The method should be useful for biological and biomedical investigations of some hereditary metabolic diseases.

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