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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF α-KETO ACIDS IN HUMAN SERUM AND URINE USING 1,2-DIAMINO-4,5-METHYLENEDIOXYBENZENE AS A PRECOLUMN FLUORESCENCE DERIVATIZATION REAGENT

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SUMMARY

A simple and highly sensitive high-performance liquid chromatographic (HPLC) method for the determination of α-keto acids in human serum and urine is described. In an acidic solution, twelve species of α-keto acids examined were converted by reaction with 1,2-diamino-4,5-methylenedioxybenzene into highly fluorescent derivatives. The derivatives were separated isocratically by reversed-phase HPLC on a TSK gel ODS-80TM column and detected fluorimetrically. Eight α-keto acids in human serum and eleven α-keto acids in human urine can be determined simultaneously. The detection limits (signal-to-noise ratio = 5) are 6–44 fmol in an injection volume of 5 μl. The intra-assay relative standard deviations for both serum and urine sample analyses are usually ca. 5%.

INTRODUCTION

α-Keto acids are intermediates in a number of major biochemical pathways including glycolysis and amino acid and carbohydrate metabolism. There has been increasing interest in determining these compounds in biological samples in order to explain certain enzymic abnormalities associated with various pathological states, in both biochemical and clinical research [1–5]. Typical examples were shown in studies of hereditary metabolic diseases such as maple syrup urine disease, phenylketonuria, tyrosinosis, pyruvate dehydrogenase deficiency and hypermethioninaemia, where abnormal levels of certain α-keto acids in serum and urine were observed. Although several successful methods have been reported

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for the determination of α-keto acids in biological samples [6–10], a simple,rapid, sensitive and selective method is still required.

In a previous paper [11], 1,2-diamino-4,5-methylenedioxybenzene (DMB) was synthesized in this laboratory as a specific fluorogenic reagent for α-keto acids and was found to be the best precolumn derivatization reagent, in terms of selectivity and sensitivity, in the high-performance liquid chromatography (HPLC) of eight 1,2-diaminobenzene derivatives, including 1,2-diamino-4,5-dimethoxybenzene (DDB) [11], *o*-phenylenediamine (*o*-PDA) [10], 2,4-dinitrophenylhydrazine [12] and 4′-hydrazino-2-stilbazole [13]. In this work we applied DMB in the simultaneous HPLC determination of α-keto acids of biological importance in human serum and urine.

EXPERIMENTAL

Chemicals and solutions

Sodium salts of α-ketoglutaric acid (KG), pyruvic acid (PA), α-keto-γ-methylthiobutyric acid (KMB), α-ketobutyric acid (KB), α-ketovaleric acid (KV), α-ketoisovaleric acid (KIV), α-ketocaproic acid (KC), α-ketoisocaproic acid (KIC), D,L-α-keto-β-methyl-*n*-valeric acid (KMV), β-phenylpyruvic acid (PP), *p*-hydroxyphenylpyruvic acid (HPP) and glyoxylic acid (GA) were purchased from Sigma (St. Louis, MO, U.S.A.) and used without further purification. DMB dihydrochloride was provided by Dojindo Lab. (Kumamoto, Japan). Creatinine-Test was purchased from Wako (Osaka, Japan). The other reagents and solvents were of analytical-reagent grade.

Deionized water was passed through a Milli Q-II system (Japan Millipore, Tokyo, Japan). A stock standard solution of each α-keto acid (4 μmol/ml) was prepared in water, except for PP and HPP, which were dissolved in 10% (v/v) aqueous ethanol. These solutions were usable for at least three months when stored at −20°C. KG solution was prepared freshly, as it has been reported to be auto-decomposed with time in water [14]. Standard mixtures of α-keto acids were prepared from the individual stock standard solutions.

Commercial DMB dihydrochloride was purified as follows. The reagent (1 g) was dissolved in 28 ml of methanol, and 0.5 ml of 0.4 *M* hydrochloric acid containing 1.0 *M* 2-mercaptoethanol and 28 m*M* sodium hydrosulphite (both stabilizers for DMB) was added. The solution was mixed slowly with 120 ml of acetonitrile, and the resulting needles were washed twice with 2-ml portions of methanol–acetonitrile (1:5, v/v) and dried in vacuo overnight (fine colourless needles, 86% recovery).

DMB solution (5 mM). This was prepared in 0.4 *M* hydrochloric acid containing 1.0 *M* 2-mercaptoethanol and 28 m*M* sodium hydrosulphite. The solution was usable for at least two days.

Preparation of serum and urine sample solutions

Serum and urine samples were donated by healthy volunteers in this laboratory.

Serum sample solution. A 50-μl sample of serum was added to 0.45 ml of 0.8 *M* perchloric acid and 10 μl of 30 nmol/ml KC as an internal standard. The mixture

was allowed to stand at room temperature for 10 min and then centrifuged at ca. 1000 *g* for 10 min. The supernatant (0.25 ml) was diluted to 0.5 ml with water (perchloric acid concentration = 0.35 *M*) and used for the assay.

Urine sample solution. Urine (24 h) was collected in the presence of 10 ml of 6 *M* hydrochloric acid. A small portion of the sample was centrifuged at ca. 1000 *g* for 10 min and the supernatant was diluted ten-fold with water. To 0.1 ml of the solution was added 50 μl of 10 nmol/ml KC. The mixture was diluted with 0.9 ml of 0.5 *M* hydrochloric acid (acid concentration = 0.43 *M*), and a 0.5-ml portion was used for the assay. The creatinine concentration in urine was determined according to the instructions of the Creatinine-Test (Folin–Wu method).

Both serum and urine sample solutions were also prepared in the absence of internal standard by replacing the KC solution with water so as to test for the existence of KC in serum and urine. When a peak for KC was observed in the chromatograms, its peak height was subtracted.

Apparatus and HPLC conditions

HPLC was carried out on a TSK gel ODS-80TM column (250 mm × 4.6 mm I.D., particle size 5 μm) (Tosoh, Tokyo, Japan) with a Waters M-45 pump equipped with a Rheodyne 7125 syringe-loading sample injector valve (5-μl loop), a Hitachi F1000 fluorescence detector fitted with a 12-μl flow cell and a Hitachi 056 chart recorder. The separation was performed at ambient temperature with isocratic elution using acetonitrile–methanol–40 m*M* phosphate buffer (pH 7.0) (5:8:12, v/v/v) as the mobile phase. The flow-rate was 1.0 ml/min and the fluorescence of the eluate was monitored at an excitation wavelength of 365 nm and an emission wavelength of 445 nm.

Uncorrected fluorescence excitation and emission spectra of the eluate were measured with a Hitachi MPF-4 spectrofluorimeter in semimicro quartz cells (10 mm width parallel to the excitation beam, 3-mm length parallel to the emission beam, 1 ml); spectral band widths of 5 nm were used in both the excitation and emission monochromators.

Derivatization procedure

To 0.5 ml of serum (or urine) sample solution placed in a screw-capped 3-ml vial was added 0.5 ml of DMB solution, and the vial was tightly closed and heated at 100°C for 50 min. The reaction mixture was cooled in ice-water, and a 5-μl portion was injected into the chromatograph. The blank was prepared in the same way except that 0.5 ml of the sample solution was replaced with 0.5 ml of 0.35 *M* perchloric acid for serum blank or 0.5 ml of 0.43 *M* hydrochloric acid for urine blank.

RESULTS AND DISCUSSION

Derivatization conditions

Commercial DMB has some impurities and should be purified before use, otherwise there would be interferences with the determination of KG, PA and PP.

Both 2-mercaptoethanol and sodium hydrosulphite were necessary to prevent oxidation of the DMB solution.

The derivatization conditions were optimized using standard mixtures of α-keto acids (17–350 pmol per injection) and reconfirmed using a serum sample.

The derivatization process was examined at different temperatures (25–110°C) for different reaction periods (up to 150 min). Low temperatures seemed to be unfavourable for fluorescence development, and reaction at higher temperatures for prolonged times (100°C for 100 min or longer, 110°C for 30 min or longer) caused precipitation (unknown structure); heating at 100°C for 50 min was selected as the optimum.

As described previously [11], α-keto acids react with DMB only under acidic conditions. The optimum hydrochloric acid concentration was found to be 0.35–0.5 *M* in the derivatization. Precipitation occurred at hydrochloric acid concentrations of 0.9 *M* or more in the derivatization and also at 0.3 *M* or more in the presence of perchloric acid (0.18 *M*). To prevent this undesirable precipitation, a mixture of hydrochloric acid (0.2 *M* in the derivatization) and perchloric acid (0.18 *M* in the derivatization) was used for serum samples and a hydrochloric acid concentration of 0.415 *M* in the derivatization was adopted for urine samples. The optimum acid concentrations are much lower than that used for derivatization with *o*-PDA [15].

The optimal concentration of DMB with the standard mixtures of α-keto acids

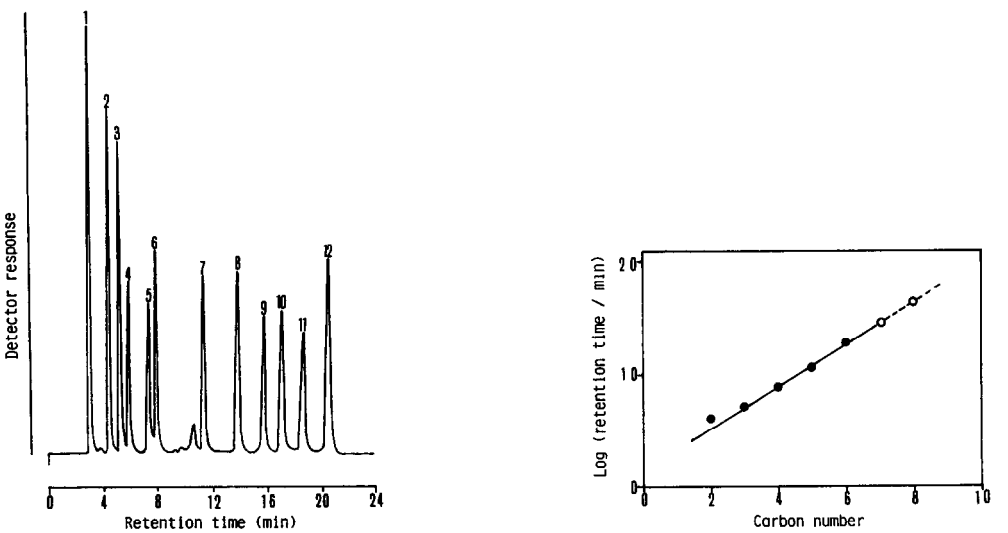

Fig. 1. Chromatogram of the DMB derivatives of α-keto acids. A standard mixture of twelve α-keto acids was treated as in the procedure for serum or urine. Peaks (pmol per 5 μl): 1=KG (1.0); 2=GA (1.2); 3=PA (0.8); 4=KMB (6.6); 5=HPP (225); 6=KB (2.2); 7=KV (1.9); 8=KIV (1.5); 9=KIC (1.7); 10=PP (1.3); 11=KC (1.8); 12=KMV (3.1). Small peaks between peaks 6 and 7 are impurities from KMB.

Fig. 2. Relationship between carbon numbers of straight-chain α-keto acids and the logarithm of their retention times. Open circles indicate extrapolated values for α-ketoheptanoic acid (C_7) and α-ketooctanoic acid (C_8).

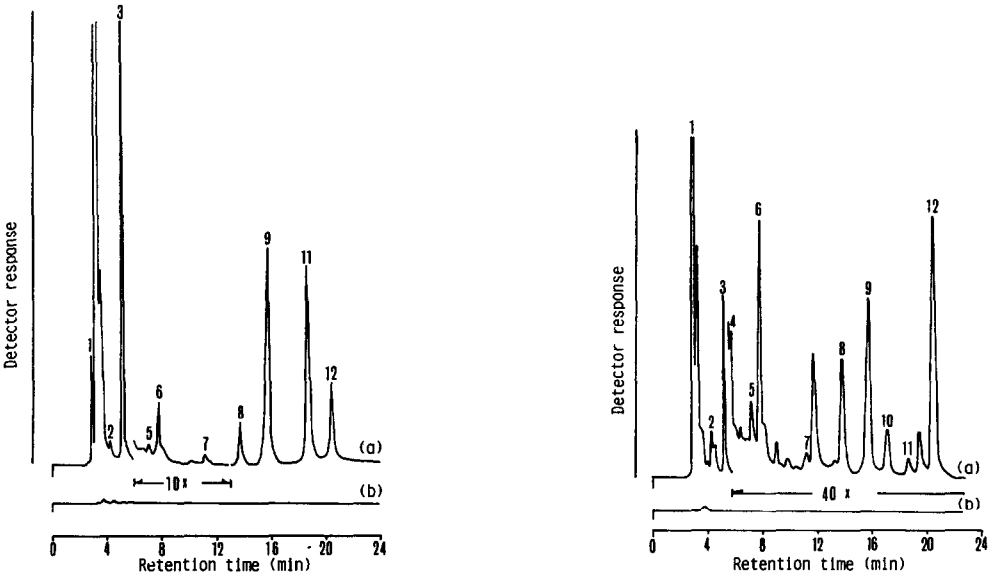

Fig. 3. Chromatograms of the DMB derivatives (a) in a normal serum sample and (b) in the reagent blank. Serum or 0.35 *M* perchloric acid for the reagent blank was treated according to the procedure (in the presence of KC as an internal standard). Peaks as in Fig. 1; others, unidentified. The detector sensitivity was increased ten-fold between elution for 6 and 13 min.

Fig. 4. Chromatograms of the DMB derivatives (a) in normal urine and (b) in the reagent blank. Urine or 0.43 *M* hydrochloric acid for the reagent blank was treated according to the procedure (in the absence of KC as an internal standard). Peaks as in Fig. 1; others, unidentified. The detector sensitivity was increased 40-fold after elution for 6 min.

was obtained with a molar ratio of DMB to the sum of α-keto acids (0.189 m*M* in the derivatization) higher than 8; the DMB concentrations corresponded to 1.5 m*M* or more in the derivatization. When normal sera were used, the highest peak heights for the individual α-keto acids were obtained at DMB concentrations above 2.0 m*M* in the derivatization; 5 m*M* DMB solution (2.5 m*M* in the derivatization) was chosen for the derivatization of α-keto acids in serum. This DMB solution could also be used in the derivatization of urinary α-keto acids.

HPLC separation

The DMB derivatives of α-keto acids could be successfully separated on a reversed-phase column (TSK gel ODS-80TM) with isocratic elution.

Fig. 1 shows a typical chromatogram obtained with a standard mixture of twelve α-keto acids. Each of the standards was also subjected to the same procedure and confirmed to give single peaks, except KMB, which gave several small peaks between the peaks for KB and KV. These peaks are due to impurities present in standard KMB. A plot of the logarithm of the retention times of straight-chain α-keto acids versus carbon number was reasonably linear (Fig. 2). This suggests, by extrapolation, that α-ketoheptanoic acid (C_7; predicted retention time 27 min)

TABLE I

RELATIVE PEAK HEIGHTS, DETECTION LIMITS AND RECOVERIES OF α-KETO ACIDS AND REPEATABILITY OF THE METHOD

Compound	Relative peak height[a]	Detection limit (S/N=5) (fmol per 5 μl)	Recovery[b] (%)		Repeatability[c] (R.S.D.[d], %)	
			Serum	Urine	Serum	Urine
KG	107	6	106	102	7	4
GA	96	7	–[e]	–[e]	–[e]	–[e]
PA	100	7	93	99	5	4
KMB	14	44	–[e]	–[e]	–[f]	–[f]
HPP	0.7	900	93	98	–[f]	6
KB	54	12	86	99	5	6
KV	64	11	103	100	20	–[g]
KIV	36	20	97	105	5	10
KIC	82	8	100	98	5	6
PP	36	18	75	101	–[g]	50
KC	54	13	96	98	–[h]	–[h]
KMV	25	29	98	102	5	8

[a]The peak height of PA (1 pmol) was taken as 100.
[b]Recoveries were obtained from serum ($n=4$) or urine samples ($n=4$) spiked with a standard mixture (see text).
[c]Replicate determinations ($n=8$) of α-keto acids in serum or urine samples were carried out.
[d]Relative standard deviation.
[e]Not examined.
[f]Difficult to determine.
[g]Not detected.
[h]Internal standard.

and α-ketooctanoic acid (C_8; predicted retention time 44 min) should be detectable if present in both serum and urine samples (see below).

KC has rarely been detected in normal human serum and urine samples [7, 9, 16], so it was used as an internal standard. Fig. 3 shows a typical chromatogram obtained with normal serum. Nine of the eleven α-keto acids under test could be found. PP and KC could not be traced, as previously reported [9]. No peaks were observed at retention times between 22 and 50 min.

A chromatogram obtained with a urine sample (without KC, internal standard) is shown in Fig. 4. Although the peaks for all twelve α-keto acids are present, the KC peak was barely detectable in other samples examined. No peaks were found in the retention time range 22–50 min.

The peaks for α-keto acids in serum and urine could be identified by their retention times and by co-chromatography of the standard compounds and a serum or urine sample. Peaks of PA, KB, KV, KIV, KIC and KMV in the samples were also characterized fluorimetrically by comparing the fluorescence excitation and emission spectra of the eluates with those of the standard compounds. Small peaks or peaks suffering from strong interference (KG, GA, KMB and PP) could not be examined fluorimetrically.

TABLE II

SERUM LEVELS OF α-KETO ACIDS IN HEALTHY SUBJECTS

Compound	Concentration (nmol/ml)				
	Proposed method		Published methods		
	Males (n=9), mean ± S.D. (range)	Females (n=8), mean ± S.D. (range)	o-PDA [16] (n=10), mean ± 2S.D.	o-PDA [7] (n=100), mean ± S.D.	DDB [9] (n=12), mean ± S.D.
KG	6.5 ±1.4 (4.7-8.8)	7.6 ±1.1 (6.4-9.4)	6.0± 2.9	4.6± 2.5	6.1± 1.4
GA	0.3 ±0.2 (0.0-0.6)	0.4 ±0.4 (0.0-1.2)	–[a]	1.5± 1.3	–
PA	82.4 ±32.2 (38-131)	66.9 ±28.3 (30-115)	58.8±28.6	75.0±19.2	74.2±22.5
HPP	0.0[b] (<0.1)	0.0 (<0.1)	–	–	–
KB	1.9 ±0.8 (0.9-3.4)	1.4 ±0.5 (0.4-1.9)	–	1.5± 1.1	–
KV	0.06±0.03 (0.00-0.12)	0.06±0.02 (0.00-0.08)	–	–	–
KIV	13.2 ±4.3 (8.6-21)	12.0 ±2.2 (9.4-15)	14.6± 5.6	13.0± 2.9	22.7± 6.2
KIC	40.8 ±9.3 (32-59)	33.0 ±9.9 (24-51)	38.8±15.8	35.5± 7.7	38.0±10.8
KMV	24.3 ±7.0 (16-40)	19.9 ±5.9 (15-30)	26.2± 8.5	22.5± 5.4	26.6± 8.7

[a]No data available.

[b]A small peak was observed but the amount could not be determined precisely.

Validation of the method

The detection limits for α-keto acids were 6–44 fmol, except for HPP (900 fmol), at a signal-to-noise ratio (S/N) of 5 (Table I). The sensitivity of this method was at least six times higher than that of the DDB method and 160 times higher than that of the o-PDA method. Linearity was observed for both standard calibration and internal calibration between the peak height and amount added up to 10 pmol per injection volume (correlation coefficient = 0.997–0.999).

Serum and urine were spiked with a mixture of α-keto acids at the concentrations of 3.24 (KG), 4.40 (PA), 69.0 (HPP), 0.60 (KB), 0.58 (KV), 3.00 (KIV), 5.50 (KIC), 1.00 (PP), 0.7 (KC) and 6.00 (KMV) pmol per injection volume for serum and of 101 (KG), 101 (PA), 178 (HPP), 3.00 (KB), 2.90 (KV), 5.00 (KIV), 2.50 (KIC), 5.00 (PP), 4.00 (KC) and 5.00 (KMV) pmol per injection volume for urine. Average recoveries of 94.7 ± 8.4% (mean ± S.D., n=4) from serum and 100.2 ± 2.2% (mean ± S.D., n=4) from urine were obtained (Table I).

The repeatability of the methods was established by performing eight replicate analyses on normal serum and urine. The relative standard deviation (R.S.D.) was about 5% for α-keto acids giving strong signals and 10–50% for those giving weak signals in the chromatogram (Table I).

TABLE III

URINARY LEVELS (24 h) OF α-KETO ACIDS IN HEALTHY SUBJECTS

Compound	Males[a] ($n=12$), mean ± S.D. (range)		Females[b] ($n=9$), mean ± S.D. (range)	
	μmol/day	μmol/g of creatinine	μmol/day	μmol/g of creatinine
KG	130 ± 29 (78-198)	89 ± 20 (61-126)	202 ± 54 (126-309)	219 ± 50 (123-274)
GA	50 ± 20 (24-93)	34 ± 14 (19-68)	45 ± 26 (12-98)	50 ± 30 (24-104)
PA	182 ± 41 (110-250)	124 ± 20 (86-136)	142 ± 35 (90-201)	152 ± 20 (126-192)
KMB	2.4 ± 1.4 (0.0-5.9)	1.7 ± 1.0 (0.0-4.1)	N.D.[c]	N.D.
HPP	26.6 ± 7.7 (9.5-31)	14.4 ± 6.0 (5.9-26)	23.3 ± 8.0 (12.3-34)	25.3 ± 8.0 (12.3-37)
KB	4.4 ± 1.3 (2.8-6.9)	3.0 ± 0.7 (1.7-4.3)	2.8 ± 0.7 (1.8-3.7)	3.0 ± 0.5 (2.5-4.0)
KV	0.2 ± 0.1 (0.0-0.4)	0.1 ± 0.1 (0.0-0.2)	0.1 ± 0.1 (0.0-0.3)	0.1 ± 0.1 (0.0-0.3)
KIV	3.8 ± 1.2 (2.3-6.1)	2.6 ± 0.7 (1.6-3.9)	3.2 ± 0.9 (2.1-4.8)	3.5 ± 1.3 (2.1-6.0)
KIC	2.7 ± 0.6 (1.7-3.6)	1.9 ± 0.3 (1.3-2.2)	2.2 ± 0.4 (1.6-2.8)	2.4 ± 0.8 (1.5-3.5)
PP	0.5 ± 0.2 (0.0-1.1)	0.3 ± 0.2 (0.0-0.7)	0.5 ± 0.1 (0.0-0.7)	0.5 ± 0.1 (0.0-0.7)
KMV	7.1 ± 2.4 (4.3-11)	4.9 ± 1.6 (2.7-6.9)	7.8 ± 3.1 (3.3-12)	8.9 ± 4.1 (2.6-14)

[a]23-41 years old.
[b]22-28 years old.
[c]Not detected.

α-Keto acids in healthy human serum and urine

Serum and urine levels of α-keto acids in healthy subjects were determined by the proposed method, and the results are summarized in Tables II and III, respectively.

Eight α-keto acids in serum could be determined (Table II). Slight differences in α-keto acid concentrations between the male and the female groups could be observed, i.e., the concentrations of branched-chain α-keto acids in the female group were lower than those in the male group. The determined values for KG, PA, KB, KIV, KIC and KMV are in agreement with published data (Table II).

The 24-h urinary levels of α-keto acids were expressed in two conventional ways (μmol/day and μmol/g of creatinine) (Table III). It is interesting that the excretion ratio of KG to PA in the male group was 0.73 ± 0.16 (mean ± S.D.) whereas in the female group it was 1.48 ± 0.45 (mean ± S.D.); almost twice the amount of KG is excreted by the female than by the male subjects. Whereas KMB was detectable in most of the urine samples from the male subjects, it was not detected in the samples from the female group. The mean values for the individ-

ual α-keto acids given in Table III were not very different from those obtained by the other methods [16].

The proposed method is the most sensitive of the HPLC methods so far reported and permits the simple simultaneous determination of a variety of α-keto acids in human serum and urine. The method should be useful in biological and biochemical research on hereditary metabolic deseases.

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