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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF α-KETO ACIDS IN PLASMA WITH FLUOROMETRIC DETECTION

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SUMMARY

This paper describes a sensitive high performance liquid chromatographic method for the quantitative determination of α-keto acids in plasma using a fluorescence detector. This method is about ten times more sensitive than that reported in a previous paper. Only 50 μl of plasma are needed for the determination of α-keto acids. However, *p*-hydroxyphenylpyruvic acid could not be analysed because the quinoxalinol derived from it does not exhibit fluorescence.

INTRODUCTION

Sensitive and reliable methods are essential to biochemical and clinical investigations. Many methods [1—6] have been reported for the determination of α-keto acids using hydrazone formation. 2,4-Dinitrophenylhydrazine has been most widely used. Recently, Ohmori et al. [7] reported a sensitive gas chromatographic method to determine these substances using pentafluorophenylhydrazine. However, the derivatization reactions employed in these methods produced *syn—anti* isomers and then complicated the chromatographic separation.

Quinoxalinol formation, on the other hand, which has been used in gas chromatography [8, 9] or liquid chromatography [10—12], provides a single main product for each α-keto acid. However, these methods were restricted in their application because there was no effective clean-up method for α-keto acids.

In a previous paper [13] we reported a high-performance liquid chromatographic (HPLC) method for the determination of α-keto acids in biological samples. This method includes an effective new clean-up and derivatization technique using a hydrazide gel column.

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This paper describes a sensitive HPLC method for the determination of α-keto acids in plasma using fluorometric detection.

MATERIALS AND METHODS

Apparatus

A Tri Rotar I high-performance liquid chromatograph equipped with a Model GP-A30 solvent programmer, a Uvidec-100 UV detector, an FP-110 fluorescence detector and an RC-225 strip-chart recorder (Japan Spectroscopic Co., Tokyo, Japan) was used in this work. The HPLC separation was carried out with a 250 mm × 4 mm I.D. stainless-steel column packed with LiChrosorb RP-8 (5 μm) using a balanced density slurry packing method. The column was covered with a column jacket. The HPLC operating conditions are given in Figs. 1 and 2.

Fluorescence spectra were measured with a Model RF-510 spectrofluorophotometer (Shimadzu Seisakusho, Kyoto, Japan).

Reagents

Sodium α-ketoglutarate (KGA), sodium α-ketoadipate (KAA), sodium pyruvate (PA), sodium α-ketobutyrate (KBA), *p*-hydroxyphenylpyruvic acid (PHPPA), sodium α-ketovalerate (KVA), sodium α-ketoisovalerate (KIVA), sodium α-ketoisocaproate (KICA), phenylpyruvic acid (PPA), sodium α-keto-β-methylvalerate (KMVA) and α-ketooctanoic acid (KOA) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). *o*-Phenylenediamine sulfate was purchased from Tokyo Chemical Industry Co., (Tokyo, Japan) and used after recrystallization from a mixture (1:1) of 1% aqueous sulfuric acid and ethanol. Hydrazide gel was prepared according to the method reported in the previous paper [13].

Reagent preparation

A stock standard solution of each α-keto acid was prepared separately at a concentration of 2 μmol/ml in water or 10% aqueous ethanol (PPA, PHPPA). A standard mixture of α-keto acids was prepared weekly by mixing the stock standard solutions and diluting with redistilled water so that it contained 80 nmol/ml of each α-keto acid. The internal standard solution was prepared in a similar manner (80 nmol/ml). *o*-Phenylenediamine solution was prepared daily by dissolving 40 mg of *o*-phenylenediamine sulfate and 100 μl of mercaptoethanol in 40 ml of 2 *N* hydrochloric acid.

Procedure for the determination of α-keto acids in human plasma

Fifty microliters of human plasma were vigorously mixed with 100 μl of the internal standard solution and 400 μl of methanol. The mixture was centrifuged at 10,000 *g* for 10 min. The supernatant was concentrated to about 100 μl on a rotary evaporator. Three drops of 0.1 *M* aqueous acetic acid and 1 ml of 0.1 *M* sodium chloride solution were added. The mixture was poured into a 150 mm × 5 mm I.D. glass column containing 0.3 ml of hydrazide gel. After the column was drained, the gel was washed with 5 ml of 0.1 *M* sodium chloride solution. The gel was then transferred to a test tube. Two milliliters of

o-phenylenediamine solution were added to it and the test tube was warmed in a water bath at about 80°C for 2 h. The mixture was then diluted with 8 ml of saturated sodium sulfate solution. The derivatives of the α-keto acids were extracted into 10 ml of ethyl acetate by shaking for 5 min. The organic layer was dried on anhydrous sodium sulfate and evaporated to dryness. The residue was redissolved with two drops of N,N-dimethylformamide and the solution was diluted with five drops of water. About 40 μl of the resulting solution were subjected to HPLC.

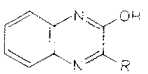
RESULTS AND DISCUSSION

In the previous paper [13] we reported on a HPLC determination of α-keto acids in human plasma using a new pretreatment method. This method was sensitive, selective and reproducible. Nevertheless, 500 μl of plasma were necessary for the exact determination. Therefore, it seemed worthwhile to develop a more sensitive method that could be applied to the analysis of α-keto acids in small plasma samples.

As fluorometry is known to increase the sensitivity of assay by several orders of magnitude, some investigators tried to develop a fluorometric method for the determination of α-keto acids. Spikner and Towne [14] developed a fluorometric method for the determination of α-keto acids based on the formation of quinoxalinol derivatives by a reaction between α-keto acid and *o*-phenylenediamine. Mizutani et al. [15] introduced 4′-hydrazino-2-stilbazole to the determination of α-keto acids. Takeda et al. [16] reported a fluorometric method based on the formation of fluorescent chelates with pyridoxamine and Zn(II) ion. However, HPLC methods using these reaction have not been reported as far as we know.

TABLE I

FLUORESCENCE SPECTRA OF QUINOXALINOL DERIVATIVES



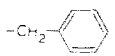
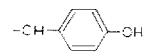
R	λ_{ex} (nm)	λ_{em} (nm)
$-CH_2CH_2COOH$	345	412
$-CH_2CH_2CH_2COOH$	355	413
$-CH_3$	350	412
$-CH_2CH_3$	352	412
$-CH_2CH_2CH_3$	356	412
$-CH(CH_3)_2$	354	410
$-CH_2CH(CH_3)_2$	359	411
$-CH(CH_3)CH_2CH_3$	353	410
	360	415
	—	—

Table I shows the fluorescence spectra of the quinoxalinol derivatives which were prepared according to the methods described in a previous paper [12]. These were measured after each quinoxalinol was dissolved in acetonitrile–0.1 *M* phosphate buffer (3:7). The quinoxalinol corresponding to PHPPA unfortunately did not show fluorescence under these conditions. For all the derivatives, the excitation (λ_{ex}) and emission maxima (λ_{em}) were observed at about 350 and 410 nm, respectively.

The upper chromatogram shown in Fig. 1 was obtained from a blank sample (redistilled water was used instead of human plasma and the internal standard solution) according to the procedure mentioned above using the fluorescence detector, and the lower one was obtained from a mixture of quinoxalinol derivatives. Chromatographic separation was carried out under the same conditions as those employed in the previous paper [13]. A strong peak was found on the chromatogram obtained from the blank sample. Its retention time was close to that of the derivative of KAA. The chromatographic separation of these compounds was further investigated. The results indicated a good separation, achieved merely by changing the concentration of tetrapropylammonium bromide in the first mobile phase down to 1/10.

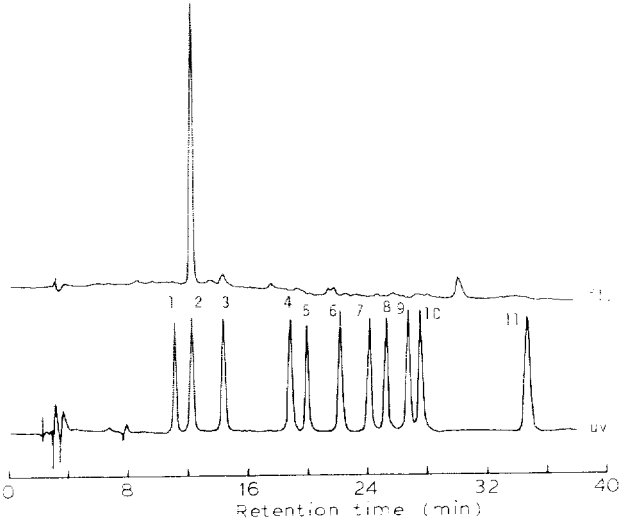


Fig. 1. High-performance liquid chromatograms obtained from blank sample (fluorescence detection) and standard mixture of quinoxalinol derivatives (UV detection). Operating conditions: column, 250 mm × 4 mm I.D., LiChrosorb RP-8 (5 μm); column temperature, 50°C. First solvent: acetonitrile—0.1 *M* aqueous tetrapropylammonium bromide solution—0.1 *M* sodium phosphate buffer (pH 7.0)—redistilled water (1:2:10:7). Second solvent: 80% aqueous acetonitrile. The gradient was prepared using a Model GP-A30 solvent programmer (convex 1, 64 min, 1 ml/min). Peaks: 1 = KGA, 2 = KAA, 3 = PA, 4 = KBA, 5 = PHPPA, 6 = KVA, 7 = KIVA, 8 = KICA, 9 = PPA, 10 = KMVA, 11 = KOA (internal standard).

Fig. 2 shows a typical chromatogram obtained from the standard mixture of α-keto acids. The chromatogram represented by a full line was monitored by the fluorescence detector and the other (broken) was obtained by the UV detector (340 nm). A good separation was obtained in 35 min. It was found

that peaks corresponding to KGA and KAA shift to shorter retention times, but that the other peaks are hardly affected by this change. These results suggest that the peak observed on the chromatogram of the blank sample might correspond to non-anionic compounds.

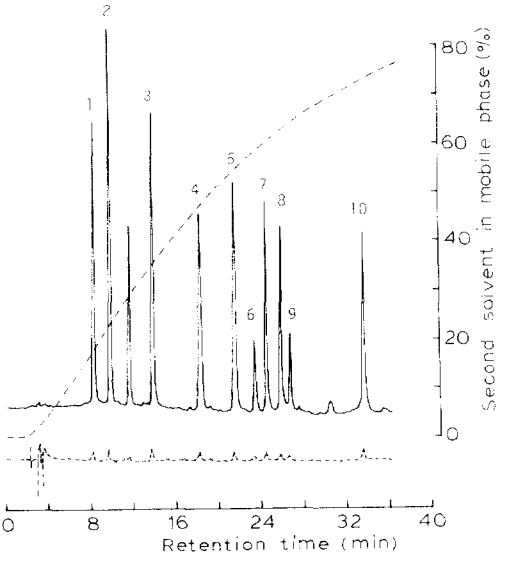


Fig. 2. High-performance liquid chromatograms obtained from a standard mixture of α-keto acids. Operating conditions: column, 250 mm × 4 mm I.D., LiChrosorb RP-8 (5 μm); column temperature, 50°C. First solvent: acetonitrile—0.01 *M* aqueous tetrapropylammonium bromide solution—(0.1 *M* sodium phosphate buffer—redistilled water (1:2:10:7). Second solvent: 80% aqueous acetonitrile. The gradient was prepared using a Model GP-A30 solvent programmer (convex 1, 64 min, 1 ml/min). (———), Fluorescence detection; (— — —), UV detection. Peaks: 1 = KGA, 2 = KAA, 3 = PA, 4 = KBA, 5 = KVA, 6 = KIVA, 7 = KICA, 8 = PPA, 9 = KMVA, 10 = KOA (internal standard).

The calibration graphs obtained using the recommended procedure are shown in Fig. 3. The ratios of each α-keto acid peak area to the internal standard peak area were plotted on the *y*-axis, and the amount of each α-keto acid on the *x*-axis. The curves were rectilinear for at least 0—4 nmol per sample.

Table II shows the percentage recoveries and the coefficients of variation obtained for eight replicate measurements according to the present method. These results are slightly inferior to those in the previous paper [13].

Fig. 4 shows the chromatograms obtained during the determination of α-keto acids in plasma from a classical maple syrup urine disease (MSUD) patient. Chromatograms A and B correspond to plasma samples before (at 14 days after birth) and during treatment, including an exchange transfusion and the use of low branched-chain amino acid milk (from 21 days after birth), respectively. The levels of α-keto acids in these plasma samples are shown with normal values in Table III.

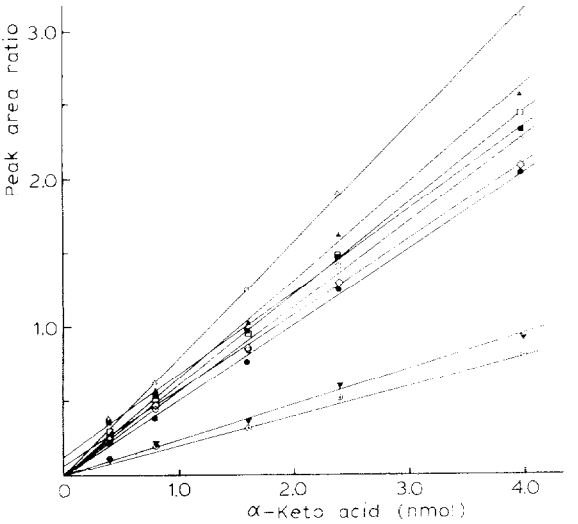


Fig. 3. Calibration graph for the determination of α-keto acids in human plasma. (△), KAA; (▲), KGA; (□), KVA; (■), PA; (○), KICA; (◇), PPA; (●), KBA; (▾), KMVA;((◉)), KIVA.

TABLE II

RECOVERY OF α-KETO ACIDS* FROM HUMAN PLASMA

Concentrations are given in nmol per 50 μl.

Sample	KGA	KAA	PA	KBA	KVA	KIVA	KICA	PPA	KMVA
Spiked plasma									
1	4.00	3.75	7.28	3.69	3.41	4.20	5.12	3.46	4.83
2	3.92	3.68	7.06	3.37	3.28	4.45	4.60	3.60	5.43
3	3.80	3.42	7.18	3.61	3.52	4.35	5.01	3.75	5.09
4	4.07	3.89	8.26	4.00	3.74	4.25	5.44	3.90	4.87
5	4.51	3.88	7.47	3.86	3.52	5.35	5.76	3.79	5.30
6	4.33	3.88	6.76	3.71	3.52	4.60	5.23	3.73	4.96
7	4.31	3.81	7.20	3.84	3.54	5.05	5.65	4.00	5.78
8	4.42	3.76	7.23	3.55	3.36	4.70	5.10	3.81	5.04
Mean	4.17	3.76	7.31	3.70	3.49	4.62	5.24	3.76	5.16
C.V. (%)	6.2	4.1	6.0	5.4	4.0	8.7	7.1	4.5	6.3
Plasma blank	0.33	—	3.90	0.27	—	1.05	1.85	0.21	1.65
Amount added	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Recovery (%)	96	94	85	86	87	89	85	89	88

*For abbreviations see text.

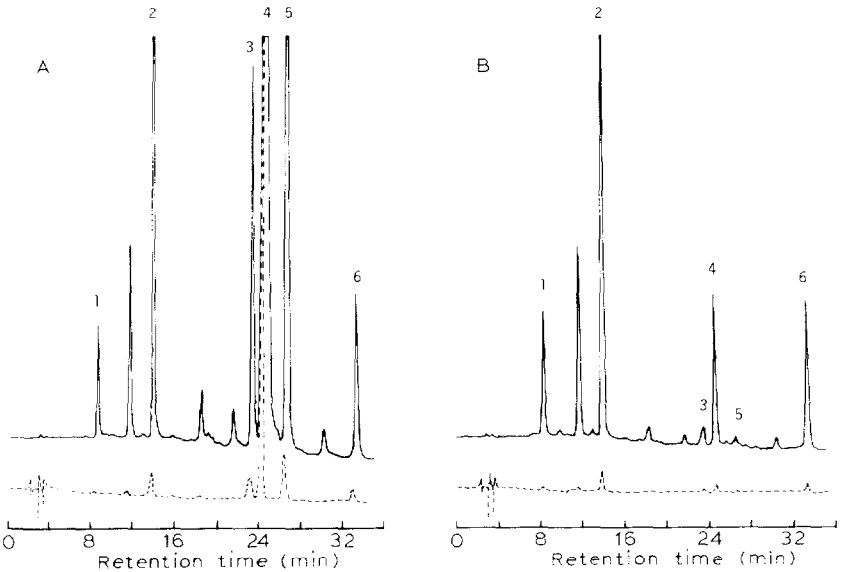


Fig. 4. High-performance liquid chromatograms obtained from plasma samples of an MSUD patient: (A) before treatment (14 days old); (B) during treatment (21 days old). (——), Fluorescence detection: (— — —), UV detection. Peaks: 1 = KGA, 2 = PA, 3 = KIVA, 4 = KICA, 5 = KMVA, 6 = KOA (internal standard).

TABLE III

α-KETO ACIDS* IN HUMAN PLASMA

Values are expressed in nmol/ml.

Sample (No. of samples)	KGA	PA	KIVA	KICA	KMVA
Normal (10)	6.0 ± 2.9**	58.8 ± 28.6	14.6 ± 5.6	38.8 ± 15.8	26.2 ± 8.5
Classical MSUD					
14 days	16.1	109	164	1230	327
21 days	18.2	96.2	10.6	31.2	3.6

*For abbreviations see text.
**± 2 S.D.

CONCLUSION

A sensitive method for the HPLC determination of α-keto acids in plasma has been developed. This method is about ten times more sensitive than that reported in the previous paper [13]. Only 50 μl of plasma are needed for the present method. However, PHPPA could not be analyzed using this method, and the coefficients of variation are slightly inferior to those of the previous method. Therefore, it is recommended that one of the two methods be selected depending upon the situation of the samples.

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