Journal of Chromatography, 224 (1981) 197—204
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 868

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF α-KETO ACIDS

TOKISHI HAYASHI*, HIDEMI TODORIKI and HIROSHI NARUSE

National Center for Nervous, Mental and Muscular Disorders, 2620, Ogawa-Higashi-Machi, Kodaira, Tokyo (Japan)

(First received November 5th, 1980; revised manuscript received February 18th, 1981)

SUMMARY

A high-performance liquid chromatographic method has been developed for the determination of eight kinds of α-keto acids. These acids were derivatized with *o*-phenylenediamine into 2-quinoxalinol derivatives, which were extracted into chloroform. The quinoxalinol derivatives were separated by reversed-phase high-performance liquid chromatography using a 250 mm × 2.1 mm I.D. column packed with LiChrosorb RP-8 (5 μm). This method could be satisfactorily applied to urine samples without any prepurification.

INTRODUCTION

Certain α-keto acids, metabolites of amino acids, carboxylic acids or sugars, show a change in concentration in urine or plasma not only in cases of some inborn errors of metabolism such as phenylketonuria, maple syrup urine disease and deficiencies of the pyruvate dehydrogenase complex, but also when there is some change in the balance of the biological system. Therefore, a reliable method for the determination of various kinds of α-keto acids may give some important diagnostic indications.

Several methods [1—6] have been reported for the determination of α-keto acids in biological samples. A colorimetric method [7] using 2,4-dinitrophenylhydrazine has been most widely used. The separation of these 2,4-dinitrophenylhydrazones has been also attempted with some success using paper chromatography [8], gas—liquid chromatography [9] and high-performance liquid chromatography (HPLC) [10, 11]. However, these chromatographic determinations are complicated since the derivative of each α-keto acid can exist as *syn—anti* isomers; moreover, the reaction is not specific for α-keto acids.

0378-4347/81/0000—0000/$02.50

Hinsberg [12] reported originally that α-keto acids react with *o*-phenylenediamine to form stable quinoxalinols in aqueous acidic media. These derivatives were separated by means of thin-layer and paper chromatographic systems [13] and O-trimethylsilyl derivatives were separated by means of gas—liquid chromatography [14—19].

Liao et al. [20] applied this derivatization to the HPLC determination of pyruvic and α-ketoglutaric acids in human urine, because no further derivatization is necessary and the quinoxalinol has high molar absorptivity in the ultraviolet region. We also reported an HPLC determination of phenylpyruvic acid using this kind of reaction [21].

The purpose of this present investigation was to develop an HPLC method utilizing quinoxalinol formation for the determination of pyruvic acid (PA), α-ketobutyric acid (KBA), *p*-hydroxyphenylpyruvic acid (PHPPA), α-ketovaleric acid (KVA), α-ketoisovaleric acid (KIVA), α-ketoisocaproic acid (KICA), phenylpyruvic acid (PPA) and α-keto-β-methylvaleric acid (KMVA), which might be related to some metabolic disorders.

EXPERIMENTAL

Apparatus

A Tri Rotar I high-performance liquid chromatograph equipped with a Model GP-A30 solvent programmer, a Uvidec 100 UV detector and a Model RC-225 strip chart recorder (Japan Spectroscopic Co., Tokyo, Japan) was used in these studies. The HPLC separation was carried out with a 250 mm × 2.1 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 and its temperature was kept constant by circulating water from a constant-temperature bath through the jacket. HPLC operating conditions are given in Fig. 1.

Reagents

Sodium pyruvate, sodium α-ketobutyrate, sodium α-ketovalerate, sodium α-ketoisovalerate, sodium α-ketoisocaproate, sodium α-keto-β-methylvalerate, phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid were purchased from Sigma (St. Louis, MO, U.S.A.). *o*-Phenylenediamine sulfate was purchased from Kanto Chemicals (Tokyo, Japan) and used after recrystallization from a mixture (1:1) of 1% aqueous sulfuric acid and ethanol. Flavone was purchased from Tokyo Organic Chemicals (Tokyo, Japan). The other reagents and solvents used were reagent grade.

Preparation of the quinoxalinol derivatives

The 2-quinoxalinol derivatives were prepared via interaction of α-keto acid (0.2 mmol) and *o*-phenylenediamine sulfate (0.25 mmol) in 2 *N* aqueous hydrochloric acid (5 ml) at about 80°C for 2 h. The reaction mixture was diluted with distilled water (20 ml) and the product was extracted with chloroform (20 ml). The organic layer was dried over sodium sulfate and evaporated to dryness. The product was recrystallized from a mixture of distilled water and methanol. The quinoxalinol derivatives prepared are listed in Table I together with melting points and UV spectrophotometric data.

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

To 200 μl of human urine, appropriately diluted with distilled water, were added 2 ml of 2 *N* aqueous hydrochloric acid containing 2 mg of *o*-phenylenediamine sulfate and 5 μl of 2-mercaptoethanol. The mixture was warmed in a water-bath at about 80°C for 2 h, then cooled and diluted with 8 ml of saturated aqueous sodium sulfate solution. The quinoxalinol derivatives were extracted into 10 ml of chloroform containing 50 nmoles of flavone. The organic phase was dried over sodium sulfate and evaporated to dryness. The residue was dissolved in 3 drops of N,N-dimethylformamide and the solution was diluted with 5 drops of water. About 50 μl of the resulting solution were subjected to HPLC under the conditions shown in Fig. 1.

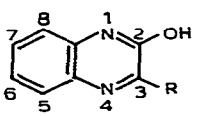
Calibration curves were prepared in a similar manner.

RESULTS AND DISCUSSION

Generally an α-keto acid does not have sufficient absorption in the UV region for sensitive monitoring with a spectrophotometric detector for HPLC. The quinoxalinol formation was used to provide sensitive determination of α-keto acids. Table I shows the melting points and the spectrophotometric data of the quinoxalinol derivatives. For all the derivatives, λ_{max} was observed at about 230, 280, 325 and 340 nm. Molar absorptivity was greatest at about 230 nm, but 340 nm was chosen as the monitoring wavelength because it seemed that a relatively high selectivity could be obtained using a longer wavelength.

TABLE I

MELTING POINTS AND SPECTROPHOTOMETRIC DATA OF QUINOXALINOL DERIVATIVES



R	m.p. (°C)	Spectrophotometric data [$\epsilon \times 10^{-4}$ (λ_{max}, nm)]			
CH_3	252—253	2.06(229)	0.56(279)	0.64(326)	0.67(338)
CH_2CH_3	198—199	2.15(229)	0.63(279)	0.71(326)	0.72(338)
p-CH_2-C_6H_4-OH	250—251	2.70(227)	0.74(280)	0.77(332)	0.78(342)
$CH_2CH_2CH_3$	188—189	2.13(229)	0.61(279)	0.69(326)	0.72(338)
$CH(CH_3)_2$	234—235	2.06(229)	0.59(270)	0.69(326)	0.71(338)
$CH_2CH(CH_3)_2$	192—193	2.32(229)	0.67(279)	0.74(326)	0.77(338)
CH_2-C_6H_5	205—206	2.59(230)	0.83(282)	0.86(326)	0.87(342)
$CH(CH_3)CH_2CH_3$	186—187	2.12(229)	0.62(279)	0.71(326)	0.73(338)

Quinoxalinol formation from α-keto acid is considered to result in a marked increase in its affinity for non-polar solvents, and various polar compounds present in biological samples. A reversed-phase chromatographic separation, therefore, offered a convenient method for the determination of α-keto acids in such samples. A LiChrosorb RP-8 column was used for this purpose. Table II shows the correlation between the capacity factors of the quinoxalinol derivatives and the acetonitrile percentage in the mobile phase. These data show that

TABLE II

CAPACITY FACTORS OF THE QUINOXALINOL DERIVATIVES DERIVED FROM α-KETO ACIDS

The values in parentheses are the percentage capacity factors compared with that at 15% acetonitrile in the mobile phase.

α-Keto acid	Acetonitrile in mobile phase				
	15%	20%	25%	30%	35%
PA	2.3(100)	1.2(52)	0.7(30)	0.5(22)	0.3(13)
KBA	7.3(100)	3.8(52)	2.5(34)	1.6(22)	0.8(11)
PHPPA	12.1(100)	5.1(42)	2.9(24)	1.6(13)	0.7(6)
KVA	16.9(100)	8.2(49)	5.1(30)	3.0(18)	1.5(9)
KIVA	25.1(100)	12.2(49)	7.5(30)	4.2(17)	2.1(8)
KICA	35.6(100)	16.2(46)	9.6(27)	5.2(15)	2.6(7)
PPA	56.4(100)	23.9(42)	13.5(24)	6.8(12)	3.1(5)
KMVA	56.4(100)	25.4(45)	14.6(26)	7.7(14)	3.7(7)

the capacity factors of the quinoxalinol derivatives which contain an aromatic ring in the substituent group at the 3-position change more sensitively than those of the others. The phenomenon is assumed to be the result of the difference in strength of the π-electron—π-electron interaction between the acetonitrile in the mobile phase and the quinoxalinol derivatives. And the lower concentration of acetonitrile in the mobile phase is more favorable for the separation of 3-ethyl-2-quinoxalinol and 3-(*p*-hydroxybenzyl)-2-quinoxalinol, while the higher concentration is more favorable for the separation of 3-benzyl-2-quinoxalinol and 3-(1-methylpropyl)-2-quinoxalinol in the acetonitrile concentration range investigated. Therefore, a gradient elution technique was adapted for the HPLC separation of the quinoxalinol derivatives. Fig. 1 shows a typical chromatogram obtained from a standard mixture of quinoxalinol derivatives and flavone (internal standard). The broken line in Fig. 1 shows a gradient curve of acetonitrile in the mobile phase measured at the top of the column. Good separation is obtained in 25 min.

The quinoxalinol derivative is formed when the α-keto acid reacts with *o*-phenylenediamine. On the basis of experiments concerning the reaction conditions that may affect the yields of the quinoxalinol derivatives, those described in the procedure were adopted as recommended reaction conditions. The addition of mercaptoethanol did not affect the reaction in the case of standard solutions of α-keto acids, but it depressed the appearance of interfering peaks in the chromatogram for the determination of α-keto acids in human urine sample. Table III shows the yields of the quinoxalinol derivatives, which were calculated by comparing the peak height ratios to those of a standard mixture of the authentic quinoxalinol derivatives and flavone after correction for the percentage extraction.

The extraction conditions of the quinoxalinol derivatives were then studied. The quinoxalinol derivatives could be extracted from the aqueous layer with chloroform and ethyl acetate; chloroform was chosen as the extracting solvent as it separated from the aqueous phase as a lower layer, which was convenient

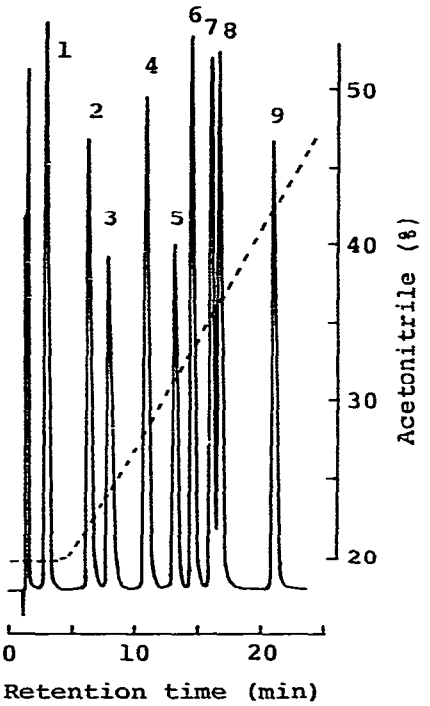


Fig. 1. High-performance liquid chromatogram of a standard mixture of the quinoxalinol derivatives of α-keto acids. The dashed line indicates the gradient curve. Operating conditions: column, 250 mm × 2.1 mm I.D. LiChrosorb RP-8 (5 μm); column temperature, 50°C; mobile phase, aqueous acetonitrile solution; the gradient was prepared using a Model GP-A30 solvent programmer (convex 1, 64 min); flow-rate, 0.6 ml/min; detector, UV spectrophotometer (340 nm). Peaks: 1 = PPA, 2 = KBA, 3 = HPPA, 4 = KVA, 5 = KIVA, 6 = KICA, 7 = PPA, 8 = KMVA, 9 = flavone (IS).

TABLE III

YIELDS OF THE QUINOXALINOL DERIVATIVES DERIVED FROM α-KETO ACIDS UNDER ANALYTICAL CONDITIONS

α-Keto acid	Yield (%)	α-Keto acid	Yield (%)
PA	100	KIVA	54
KBA	76	KICA	75
HPPA	70	PPA	75
KVA	81	KMVA	74

for the separation procedure. The relationship between the extractability of the quinoxalinol derivatives with chloroform and the hydrochloric acid concentration in the aqueous layer is shown in Fig. 2. The extractability of the quinoxalinol derivatives that have a relatively low hydrophobic substituent in 3-position, such as the methyl, ethyl or *p*-hydroxybenzyl group, decreases with the increase in hydrochloric acid concentration in the aqueous phase. Table IV shows the percentage extraction for the quinoxalinol derivatives in the presence of various kinds of salts. The addition of sodium sulfate gives satisfactory

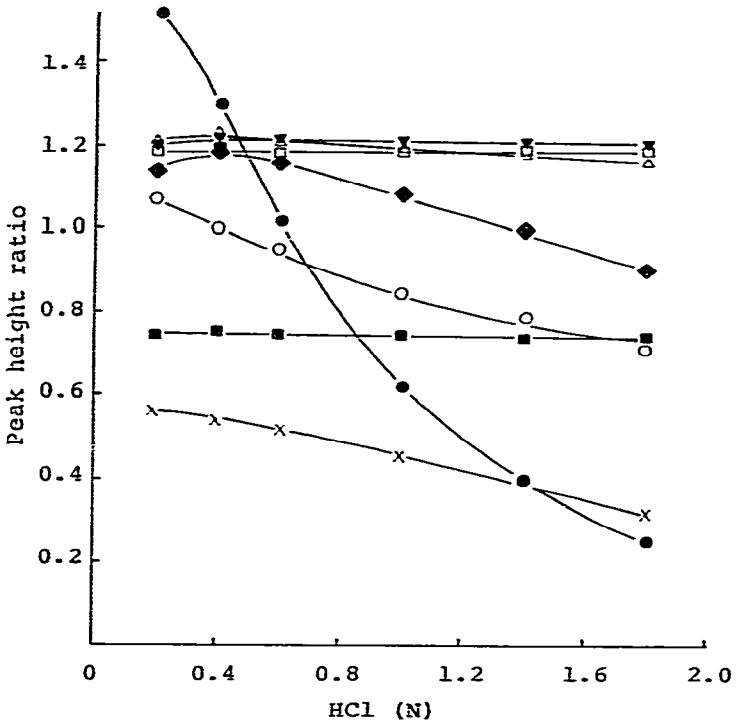


Fig. 2. Relationship between the extractability of the quinoxalinol derivatives and hydrochloric acid concentration in the aqueous layer. The quinoxalinol derivatives of the α-keto acids were extracted from 10 ml of the aqueous phase with 10 ml of chloroform. (●), PA; (○), KBA; (×), HPPA; (◆), KVA; (■), KIVA; (△), KICA; (□), PPA; (▼), KMVA.

TABLE IV

PERCENTAGE EXTRACTION OF THE QUINOXALINOL DERIVATIVES OF α-KETO ACIDS IN THE PRESENCE OF VARIOUS SALTS

α-Keto acid	Percentage extraction				
	—	KCl	NaCl	NH_4Cl	Na_2SO_4
PA	81.9	68.5	50.9	45.9	98.9
KBA	100.6	106.3	89.9	89.4	99.2
PHPPA	64.2	78.9	80.4	65.7	90.8
KVA	98.7	99.8	98.0	97.9	101.3
KIVA	98.7	100.5	98.0	101.2	98.5
KICA	101.5	101.2	102.0	101.5	100.2
PPA	99.2	100.4	98.7	100.3	98.1
KMVA	99.2	100.1	95.6	100.1	99.5

TABLE V

RECOVERY OF α-KETO ACIDS FROM HUMAN URINE

All values are expressed in nmol except where indicated.

Sample No.	PA	KBA	PHPPA	KVA	KIVA	KICA	PPA	PMVA
1	14.8	15.0	16.0	15.0	15.4	14.7	15.2	15.2
2	15.1	15.1	13.9	14.9	15.2	14.7	15.3	15.2
3	15.8	15.7	13.3	15.3	16.6	15.2	14.7	15.5
4	15.6	15.3	16.4	15.2	16.2	14.9	15.5	15.6
5	15.3	14.5	16.4	14.4	15.7	14.5	15.3	15.5
6	15.2	14.5	15.0	14.7	15.3	14.5	14.7	14.9
7	15.3	15.5	15.3	14.8	15.0	14.5	14.8	15.1
8	16.1	15.1	16.3	15.0	15.6	14.8	14.9	15.3
9	15.0	15.1	17.5	14.3	16.0	14.7	14.8	15.4
10	16.3	15.4	15.1	15.1	15.2	14.8	14.3	15.0
Average	15.5	15.1	15.5	14.9	15.6	14.7	15.0	15.3
C.V. (%)	3.1	2.6	8.4	2.2	3.4	1.4	2.4	1.0
Added	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Recovery (%)	103	101	103	99	104	98	100	102

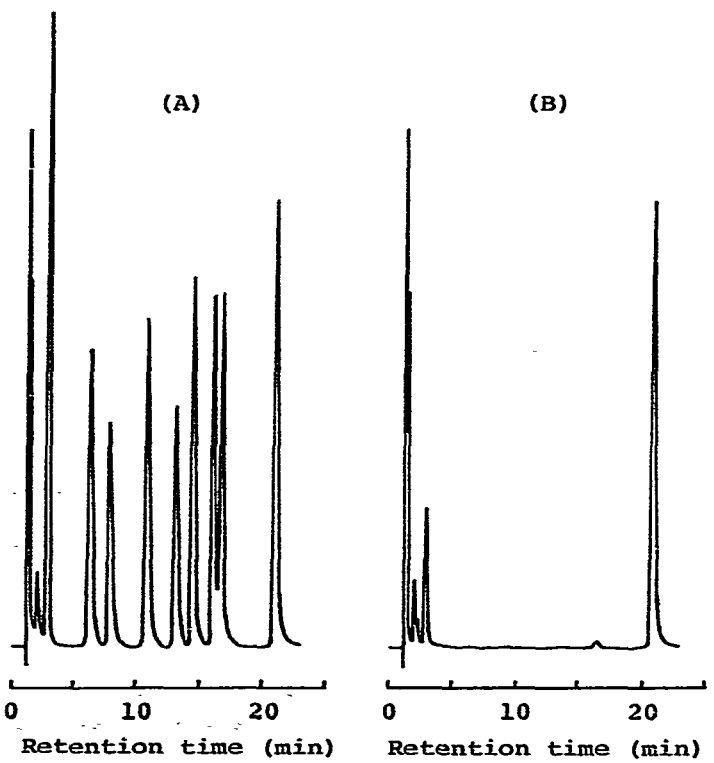


Fig. 3. High-performance liquid chromatograms obtained in the recovery experiments. (A) Spiked urine sample. (B) Urine sample.

results. The extraction was therefore carried out after dilution of the reaction mixture (2 ml) with saturated sodium sulfate solution (8 ml). The internal standard flavone was also almost completely extracted under these conditions.

The calibration graphs obtained using the recommended procedure were rectilinear for at least 0—20 nmoles of α-keto acids.

Table V shows the percentage recoveries and the coefficients of variation obtained for ten replicate measurements made on an identical ten-times diluted sample of human urine (200 μl) to which 15 nmoles of each α-keto acid were added. Fig. 3 shows the chromatograms obtained in the recovery experiments. No interfering peaks were observed. Then, the determination of α-keto acids in normal human urine was carried out. The pyruvic acid/creatinine ratios for normal samples were almost in accord with published values [20]. The other α-keto monocarboxylic acids were not found in normal urine samples.

We are now developing a further method for determining α-keto dicarboxylic acids that might be related to some metabolic disorders.

CONCLUSION

A method for the HPLC determination of eight α-keto acids in human urine has been developed. This method is sensitive, selective and reproducible.

REFERENCES

1 T.E. Friedman and G.E. Haugen, J. Biol. Chem., 147 (1943) 415.
2 Z. Horii, M. Makita and Y. Tamura, Chem. Ind., (1965) 1494.
3 P. Greengard, Nature (London), 178 (1952) 632.
4 J.E. Spinkner and J.C. Town, Anal. Chem., 34 (1962) 1468.
5 S. Mizutani, T. Nakajima, A. Matsumoto and Z. Tamura, Chem. Pharm. Bull., 12 (1964) 850.
6 M. Takeda, T. Kinoshita and A. Tsuji, Anal. Biochem., 72 (1976) 184.
7 D. Caballini, N. Frontali and G. Toschi, Nature (London), 163 (1949) 568.
8 F.A. Isherwood and D.H. Cruickshank, Nature (London), 173 (1949) 568.
9 H. Kallio and R.P. Linko, J. Chromatogr., 76 (1973) 229.
10 H. Terada, T. Hayashi, S. Kawai and T. Ohno, J. Chromatogr., 130 (1977) 281.
11 B.C. Hemming and C.J. Gubler, Anal. Biochem., 12 (1979) 31.
12 O. Hinsberg, Ann. Chem., 237 (1887) 327.
13 J. Mowbray and J.H. Attaway, Biochem. J., 120 (1970) 171.
14 N.E. Hoffman and T.A. Killinger, Anal. Chem., 41 (1969) 162.
15 N.E. Hoffman, K.M. Gooding, K.M. Sheahan and C.A. Tylanda, Res. Commun. Chem. Pathol. Pharmacol., 2 (1971) 87.
16 U. Langenbeck, H.-U. Möhring and K.P. Dieckmann, J. Chromatogr., 115 (1975) 65.
17 U. Langenbeck, A. Hoinowski, K. Mantel and H.-U. Möhring, J. Chromatogr., 143 (1977) 39.
18 U. Langenbeck, H.U. Möhring, B. Hinney and M. Spiteller, Biomed. Mass Spectrom., 4 (1977) 197.
19 T.C. Cree, S.M. Hutson and A.E. Harper, Anal. Biochem., 92 (1979) 156.
20 J.C. Liao, N.E. Hoffman, J.J. Barboriak and D.A. Roth, Clin. Chem., 23 (1977) 802.
21 T. Hayashi, T. Sugiura, H. Terada, S. Kawai and T. Ohno, J. Chromatogr., 118 (1976) 403.