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GAS CHROMATOGRAPHIC DETERMINATION OF SERUM BRANCHED-CHAIN α-KETO ACIDS DERIVATIZED BY EXTRACTIVE ALKYLATION

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SUMMARY

The procedure presented for gas—liquid chromatographic analysis of α-keto acids is relatively simple, requiring only a few steps for the formation of derivatives suitable for measurement. The recoveries of the branched-chain α-keto acids varied from 92.7% to 106.7%, being sufficiently good especially when smaller amounts of the α-keto acids were added to serum. In addition, the coefficients of variation are satisfactorily small, also for biological samples. The measured values of branched-chain α-keto acids correspond well with those presented earlier by different methods. There exists a slight but insignificant difference between women and men, the values being lower in sera of women for the three branched-chain α-keto acids studied.

INTRODUCTION

Amino acid degradation in man proceeds through deamination yielding the corresponding α-keto acid [1]. Quantitative analysis of these metabolites is of interest from the point of view of metabolic defects as well as of parenteral feeding with nutrients containing amino acids [2]. Also, in the treatment of septic shock, a knowledge of blood levels of α-keto acids is important [3].

There are very few reports available concerning the concentrations of α-keto acids in the blood. The technical difficulties in measuring these compounds are probably the main reasons for this scarcity. α-Keto acid analysis has been performed by paper chromatography of the corresponding 2,4-dinitrophenylhydrazone derivatives [4,5]. Kallio and Linko [6] assayed α-keto acids by gas—liquid chromatography (GLC) of the esterified 2,4-dinitrophenylhydrazone derivatives. In another gas chromatographic procedure [7], derivatization to

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O-methyloxime methyl esters was found useful. Recently, the α-keto acids have been determined as their quinoxalinols by high-performance liquid chromatography [8]. Cree et al. [9] have successfully used silylated quinoxalinol derivatives for GLC.

Extractive alkylation is applicable for various types of organic molecules containing active hydrogen atoms [10]. Methods based on extractive alkylation of the carboxyl group for compounds other than α-keto acids [11,12] have been described earlier. The purpose of the present study has been to develop this technique for the determination of α-keto acids in blood, for clinical use.

MATERIALS AND METHODS

Materials

To determine branched-chain α-keto acids from biological samples, venous blood samples were used. Serum was separated as quickly as possible from cells and stored at 4°C before use unless otherwise stated. For serum values from apparently healthy people, blood samples from twenty women and twenty men were taken after overnight fasting and analysed during the day of sample collection.

α-Ketoisovaleric acid (α-ketovaline) sodium salt (Fluka, Buchs, Switzerland), α-keto-*n*-valeric acid (Sigma, St. Louis, MO, U.S.A.), α-keto-β-methyl-*n*-valeric acid (α-ketoisoleucine) sodium salt (Sigma) and α-ketoisocaproic acid (α-ketoleucine) (Fluka) were used as standards in procedural development tests and in calibration. Tetrabutylammonium hydrogen sulphate (TBA) (E. Merck, Darmstadt, F.R.G.), pentafluorobenzylbromide (PFBBr) (Fluka), dichloromethane (Merck) and petroleum ether 60—80°C (Merck) were used as reagents and solvents.

Sample preparation

The branched-chain α-keto acids were alkylated and extracted from serum samples for gas chromatographic analysis as follows. A 1-ml volume of 0.1 *M* TBA—phosphate buffer (pH 7—8) and 1 ml of 2% PFBBr in methylene chloride were added to 1 ml of serum sample in a stoppered test tube; 10 μl of a solution of internal standard (α-ketovaleric acid, 20 μg/ml) were added to each serum sample. The solution was carefully mixed for 2 h in a mixer and centrifuged for 10 min at 2000 *g*. The aqueous layer was removed and methylene chloride was evaporated in a flow of nitrogen. Then 20—50 μl of petroleum ether were added and the suspension was thoroughly mixed. Direct injection of the methylene chloride layer into the gas chromatograph is not feasible because of serious baseline disturbances caused by high reagent concentrations in this solvent.

Stability of α-keto acids in serum at −20°C was measured by an external standard method using a Shimadzu CR 1A electronic integrator (Tokyo, Japan). By using known concentrations of derivatized α-keto acids in a 2-μl volume as external standards, it was possible to measure the concentrations of α-keto acids weekly from a serum pool stored in separate tubes up to one month.

Instrumentation

A Carlo Erba Fractovap 2350 gas chromatograph (Milan, Italy) fitted with a flame-ionization detector was used in combination with an SE-30 fused-silica capillary column, 24 m × 0.32 mm I.D. (Orion Analytica, Espoo, Finland). Helium was used as carrier gas at a flow-rate of 3 ml/min. Detector flow-rates for hydrogen and air were 30 ml/min and 240 ml/min, respectively. A Grob-type injector was operated in split mode with a split ratio of 1:5. The column oven was temperature programmed from 60°C to 110°C at a rate of 2.5°C/min. The GC electrometer was connected to a Shimadzu CR 1A electronic integrator. Peak identification was performed by gas chromatography—mass spectrometry (GC—MS) using a quadrupole instrument (gas—liquid chromatograph—mass spectrometer 5992A, Hewlett-Packard, Palo Alto, CA, U.S.A.). A representative chromatogram is shown in Fig. 1; the mass spectra of the α-keto acids are displayed in Fig. 2.

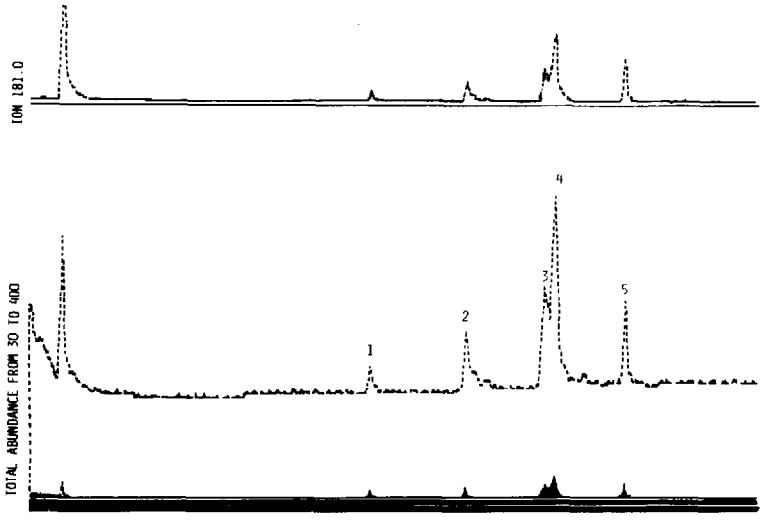

Fig. 1. Chromatogram of α-keto acids obtained using the Hewlett-Packard gas—liquid chromatograph—mass spectrometer with SE-30 silica capillary column, 25 m long. Splitless delay time was 80 sec. The instrument was programmed from 60°C to 110°C at a rate of 2.5°C/min, and operated at 2000 V and 70 eV. Peaks: 1 = α-ketoisovaleric acid, 2 = α-keto-valeric acid (internal standard), 3 = α-keto-β-methylvaleric acid, 4 = α-ketoisocaproic acid, 5 = unidentified acid.

RESULTS AND DISCUSSION

Pentafluorobenzylbromide is one of the best alkylating agents available for extractive alkylation. In the case of the compounds studied here, a complete reaction takes place in about 20—30 min at room temperature, provided that a sufficiently high concentration of PFBBr is present in the reaction mixture [13,14]. In extractive alkylation, the pH value of the aqueous phase should exceed by two units the pK_a values of the compounds to be alkylated [15,16]. The α-keto acids investigated here, with pK_a values ranging from 4.2 to 5.1 will thus easily undergo alkylation of the carboxyl group at the physiological pH

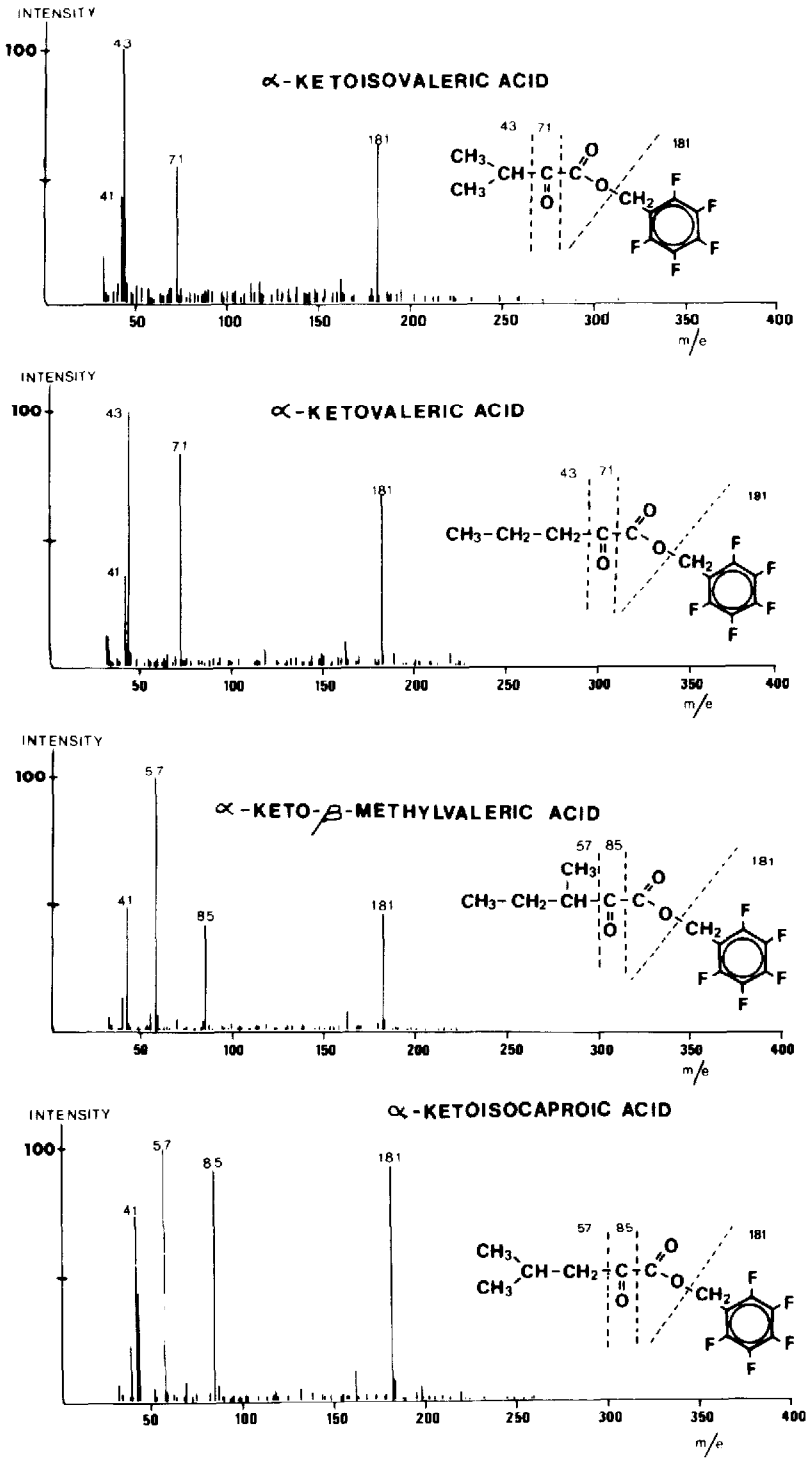

Fig. 2. Mass spectra of α-keto acids. For operating conditions, see Fig. 1.

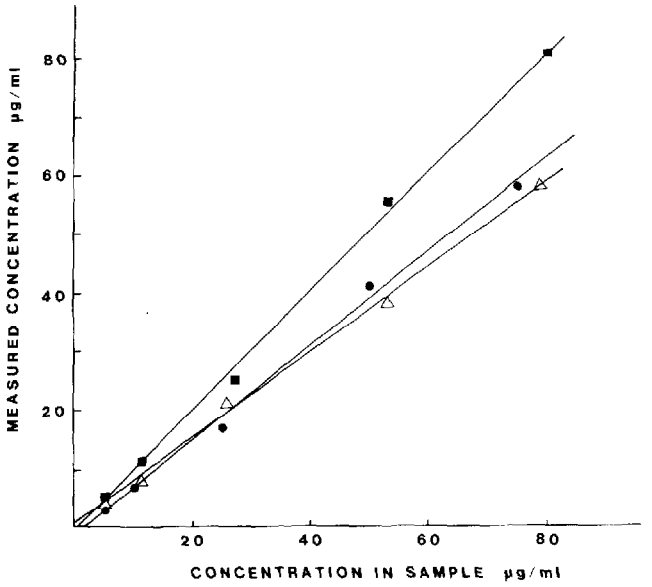

Fig. 3. Linearity curves of α-keto acids: (■) α-ketoisovaleric acid; (●) α-keto-β-methyl-*n*-valeric acid; (△) α-ketoisocaproic acid.

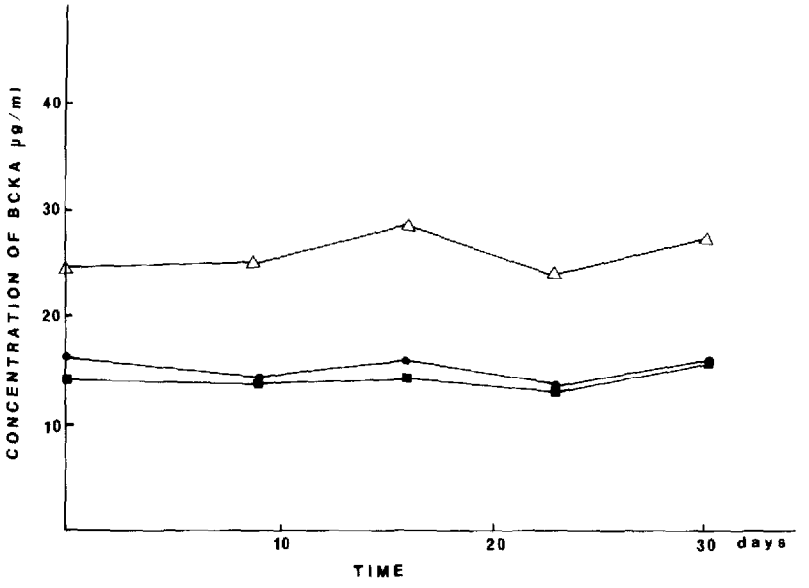

Fig. 4. Stability of branched-chain α-keto acids (BCKA) in serum at −20°C. Results are measured by the external standard method. (■) α-ketoisovaleric acid; (●) α-keto-β-methyl-*n*-valeric acid; (△) α-ketoisocaproic acid.

values of serum samples. The tetrabutylammonium counter-ion was added to the samples in a solution of phosphate buffer (pH 7.5), the pH range 7—8 yielding best values. Linearity of all compounds measured is good up to 80 µg/ml (Fig. 3). Serum samples can be analysed even after one month after derivatization when stored at temperatures of −20°C or below (Fig. 4).

The contribution of sample treatment operations to the variation of the results is reasonably small as determined by ten parallel analyses of a serum sample (Table I). The coefficient of variation is less than 13% for all compounds investigated. Similarly, the instrumental error is quite small, the

TABLE I

MEAN CONCENTRATION, COEFFICIENT OF VARIATION (C.V.) AND VARIATION RANGE OF α-KETO ACIDS OBTAINED BY ANALYSIS OF A SINGLE SERUM SAMPLE

n = 10.

	Concentration (μmol/l, mean ± S.D.)	C.V. (%)	Range (μmol/l)
α-Ketoisovaleric acid	13.9 ± 0.9	6.5	12.6—14.8
α-Keto-β-methyl-*n*-valeric acid	25.6 ± 3.1	12.1	20.9—30.2
α-Ketoisocaproic acid	36.9 ± 4.6	12.5	29.2—43.8

TABLE II

MEAN CONCENTRATION, COEFFICIENT OF VARIATION AND VARIATION RANGE OF α-KETO ACIDS OBTAINED BY ANALYSIS OF AN ARTIFICIAL SAMPLE

n = 10. Concentrations in artificial sample are: 173.7 μmol/l ketoisovaleric acid, 158.8 μmol/l keto-β-methyl-*n*-valeric acid, 161.4 μmol/l ketoisocaproic acid.

	Concentration (μmol/l, mean ± S.D.)	C.V. (%)	Range (μmol/l)
α-Ketoisovaleric acid	171.1 ± 5.2	3.0%	163.3—181.6
α-Keto-β-methyl-*n*-valeric acid	146.4 ± 3.9	2.7%	139.4—152.6
α-Ketoisocaproic acid	153.9 ± 8.5	5.5%	140.0—163.7

TABLE III

RECOVERY TEST

Serum concentrations of α-keto acids were measured with the external standard method after addition of the corresponding α-keto acids.

Acid	Concentration (μmol/l)			Percentage recovery
	In serum	Added	Found	
α-Ketoisovaleric acid	9	0	9	100
	9	87	96	100
	9	347	330	93
	9	695	678	96
α-Keto-β-methyl-*n*-valeric acid	8	0	8	100
	8	77	85	100
	8	318	302	93
	8	635	604	94
α-Ketoisocaproic acid	31	0	31	100
	31	85	123	106
	31	323	361	102
	31	645	661	98

coefficient of variation being less than 6% for all keto acids, as calculated from ten GC runs of a petroleum ether extract of a serum sample (Table II). The recovery of all compounds studied is quite satisfactory (when a sufficiently long alkylation time is used) (Table III).

The peaks of α-keto-β-methylvaleric and α-ketoisocaproic acids are quite near each other (Fig. 5). When an intelligent integrator is used, reproducible results are obtained with a 24-m capillary column. If a longer column is used, the separation is more complete but the running time is markedly increased.

The results of an investigation of blood levels of α-keto acids in a group of healthy females and males, performed by the method here described, are presented in Table IV. The concentrations of the compounds studied are slightly higher in males. This may be due to higher protein mass found in males. Penttilä [4] found an α-ketoisovaleric acid concentration of 10 ±1 μmol/l (mean ± S.D.) and an α-ketoisocaproic acid + α-keto-β-methylvaleric acid concentration of 49 ± 2 μmol/l, which is in good agreement with our results. In addition, Nissen et al. [8], using high-performance liquid chromatography, reported 28, 18 and 17 μmol/l in venous blood for α-ketoisocaproic, α-keto-β-methylvaleric and α-ketoisovaleric acids, correspondingly.

TABLE IV

NORMAL VALUES OF α-KETO ACIDS IN HUMANS

Acid	Concentration (μmol/l, mean ± S.D.)	
	Female (n = 20)	Male (n = 20)
α-Ketoisovaleric acid	11.3 ± 3.0	13.3 ± 3.3
α-Keto-β-methyl-n-valeric acid	19.0 ± 7.4	23.3 ± 9.5
α-Ketoisocaproic acid	24.5 ± 8.9	37.4 ± 11.5

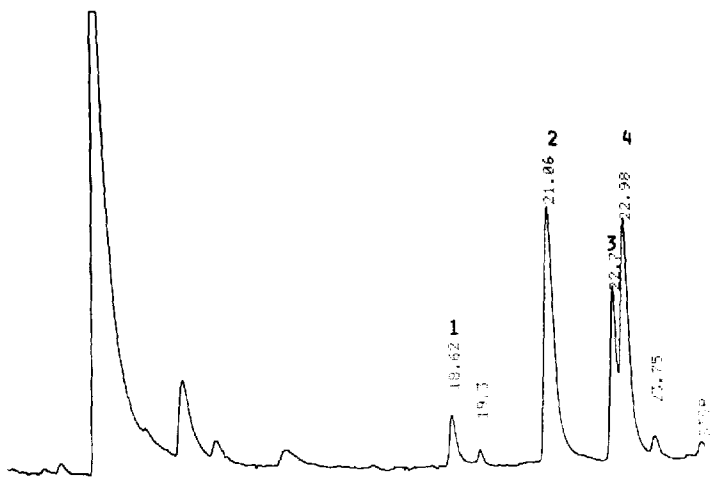

Fig. 5. Chromatogram of α-keto acids. Carlo Erba Fractovap 2350 gas chromatograph with a SE-30 fused-silica capillary column, 24 m long, with flame-ionization detector. Peaks: 1 = α-ketoisovaleric acid, 2 = α-ketovaleric acid (internal standard), 3 = α-keto-β-methylvaleric acid, 4 = α-ketoisocaproic acid.

The present method is very suitable for clinical application. It is easy to handle large numbers of samples, because the extraction and derivatization of the α-keto acids can be performed in the same tube. The combined procedure improves analytical precision by reducing the number of steps associated with sample treatment. It is also interesting to keep in mind that the present method can be used to measure α-keto acids from tissue samples by extractive alkylation to pentafluorobenzyl derivatives for GLC analysis.

REFERENCES

1 A.L. Lehninger, Biochemistry, Worth Publishers, New York, 1976, p. 699.
2 A. Kari, K. Hersiö, M. Laitinen, I. Penttilä and M. Pöyhönen, Käytännön Lääkäri (Helsinki), 26 (1983) 34.
3 L.I. Woolf, A.C. Groves and J.H. Duff, Surgery, 85 (1979) 212.
4 I.M. Penttilä, Ann. Med. Exp. Biol. Fenn., 44, Suppl. 11 (1966) 1.
5 T.A. Miettinen and I.M. Penttilä, Acta Med. Scand., 184 (1968) 159.
6 H. Kallio and R.R. Linko, J. Chromatogr., 76 (1973) 229.
7 H.M. Liebich, A. Pickert and J. Wöll, J. Chromatogr., 217 (1981) 255.
8 S.L. Nissen, C.V. Huysen and M.W. Haymond, J. Chromatogr., 232 (1982) 170.
9 T.C. Cree, S.M. Hutson and A.E. Harper, Anal. Biochem., 92 (1979) 159.
10 E. Fogelqvist, B. Josefsson and C. Roos, J. High Resolut. Chrom. Chrom. Commun., 3 (1980) 568.
11 J. Rosenfield, A. Phatak, T.L. Ting and W. Lawrence, Anal. Lett., 13 (1980) 1373.
12 P. Hartvig, C. Fagerlund and B.-M. Emanuelsson, J. Chromatogr., 228 (1982) 340.
13 F.K. Kawahara, Anal. Chem., 40 (1968) 1009.
14 O. Gyllenhaal, H. Brötell and B. Sandgren, J. Chromatogr., 122 (1976) 471.
15 O. Gyllenhaal, Academic Dissertation, Institutionen för Analytis Farmaceutisk Kemi, Farmaceutiska Fakulteten, Uppsala, 1978.
16 H. Brötell, H. Ehrsson and O. Gyllenhaal, J. Chromatogr., 78 (1973) 293.