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Note

Gas chromatography of branched-chain keto acids analysed as their silylated oxime derivatives

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Questions raised by the investigation of branched-chain keto acid (BCKA) metabolism in burn patients [1] necessitated the measurement of the concentrations of these acids in venous blood. Several methods have been reported for the detection and quantification of BCKAs. Gas chromatographic (GC) methods using derivatives such as quinoxalinols [2, 3] and oximes [4–8], and high-performance liquid chromatography (HPLC) [9] are the current methods for BCKA analysis. These techniques have been applied to the diagnosis of hereditary metabolic disorders, such as maple syrup urine disease [8], characterized by the excretion of abnormal amounts of keto acids in the urine. However, few data on normal values of BCKA and the validation of the techniques used have been published because of difficulties with α-keto acid analysis.

This paper describes a GC technique based on oxime formation followed by trimethylsilylation. The method is validated and the normal range calculated.

EXPERIMENTAL

Reagents and chemicals

α-Ketoisovalerate (KIV), α-keto-β-methylvalerate (KMV) [60% L-isomer (L-KMV), 40% D-isomer (D-KMV)], α-ketoisocaproate (KIC), 2-phenylbutyrate (PB), α-ketocaproate (KC) and pyruvic acid (PYR) were obtained from Sigma (St. Louis, MO, U.S.A.). Solvents, hydroxylamine hydrochloride and inorganic reagents were purchased from Merck (Darmstadt, F.R.G.), and the silylation reagent from Supelco (Bellefonte, PA, U.S.A.). The GC studies were carried out with an Aerograph Varian Model 2700 gas chromatograph, equipped

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with a flame-ionization detector. High-purity nitrogen at a flow-rate at 20 ml/min was used as the carrier gas.

Sample preparation

For the quantitative derivatization of α-keto acids to the corresponding oximes, a modified version of the method of Lancaster et al. [8] was used as follows.

Isolation of keto acids from plasma. A 1-ml plasma sample was deproteinized by the addition of an equal volume of ethanol (95%, v/v). After centrifugation at 1500 *g* for 10 min, the supernatant was made strongly alkaline (pH 10) with sodium hydroxide (1 *M*), and 100 μl of a solution containing 0.25 mg KC per ml were added as an internal standard. The medium was then purified by three extractions with 2 ml of diethyl ether; these extracts were discarded. Hydroxylamine hydrochloride (10 mg) was added to the aqueous phase, which was then heated at 60°C for 30 min. A 100-μl volume of a solution containing 0.25 mg PB per ml was then added as an external standard, and the sample was acidified to pH 1 with 5 *M* hydrochloric acid and saturated with sodium chloride. Extraction was performed with 2 ml of ethyl acetate. The organic layer was removed and the remaining aqueous layer extracted twice more with ethyl acetate. The organic phases were combined over sodium sulphate and evaporated under reduced pressure (Rotavap) at room temperature. The dry residue was dissolved in 40 μl of pyridine, 20 μl of bis(trimethylsilyl)trifluoroacetamide were added, and the solution was left at room temperature for 30 min.

Gas chromatography of silylated oximes. The glass column (3 m×2 mm I.D.) was packed with 5% SE-52 on Chromosorb G, DMCS, 80–100 mesh (Spiral, Dijon, France). The gas chromatograph was operated under the following conditions: injector temperature, 260°C; isotherm temperature, 90°C for 2 min; temperature increase, 4°C/min from the initial temperature of 90 to 240°C; hydrogen flow-rate, 20 ml/min; air flow-rate 300 ml/min; detector flame ionization temperature, 260°C.

Quantification of keto acid oximes. Quantitative analysis of silylated oximes was carried out by the measurement of peak height. Under the conditions described, the peak height was directly proportional to the amount of keto acids in the sample and measurement of the area under the peak offered no advantage with respect to the accuracy of the assay. The plasma BCKA level was calculated with the internal standard method. However, KIC and D-KMV had the same retention time. Concerning the quantification of KIC, it was found necessary to use a correction. This correction depends on the molar response to KIC and D-KMV and on the percentage of D-KMV in commercially available KMV powder. The correction was subtracted from the peak height corresponding to the KIC in the standard mixture. It was not applied to GC traces from the serum of healthy adults because the D-isomer is not found in physiological fluids.

The external standard (PB) allows the two first steps of the preparation of oxime-trimethylsilyl (OTMS) derivatives to be checked. The ratio of the internal standard and external standard peak heights was found to be constant (0.90); in practice, when it was below 0.85 or above 0.95 the levels were measured again.

RESULTS AND DISCUSSION

The retention time of individual OTMS is critically dependent on the detailed assay conditions. Internal standardization techniques were used to verify the identity of each BCKA peak. This was achieved by assaying the sample alone and by comparing the chromatograms with those obtained by an injection of sample with each BCKA. The variation of the retention time was investigated. Fifteen repeated analyses of the sample gave coefficients of variation (C.V.) ranging from 1.2 to 2.5%. It was, therefore, easy to locate a peak corresponding to the BCKA.

Additionally, in another laboratory, the identity of each BCKA was verified. A gas chromatograph was used containing a capillary column (25 m × 0.22 mm I.D.) coated with SE-52, coupled to a mass spectrometer (Hewlett-Packard 5995) working under ionization by electron impact with an ionization energy of 70 eV. The analysis of the mass spectra of the various samples of normal human serum and the standard mixture showed that the peaks effectively corresponded to OTMS derivatives of α-keto acids eluted in the same order as on the column. We found, as with our experimental technique, only two compounds that were not α-keto acids, eluted on each side of pyruvic acid in certain samples of human serum. Since this technology was not available routinely, the width of each peak was measured at mid-height in each chromatogram in order to verify that the compounds were eluted singly.

In our method, there was a single peak for each of the keto acids investigated (Fig. 1) with the exception of KMV, which gave two peaks corresponding to the isomers. The peak attributed to the L-isomer was well separated from the other peaks. The D-isomer had the same retention time as KIC; however, the D-isomer is not known to occur in physiological fluids. All previous studies involving OTMS derivatives are obscure with regard to the quantification of D-KMV and KIC. This could explain some discrepancies in the values found for KMV and KIC in studies [8] using OTMS derivatives compared with those using trimethylsilyl-quinoxalinol (TMSQ) derivatives [9], in which the D- and L-isomers were co-chromatographed.

The precision of measurements is reported in Table I. The repeatability of the assay was determined from ten successive measurements of BCKA and pyruvate levels in a frozen serum pool. The C.V. values for the measurement of all three BCKAs and pyruvate ranged from 5.8 to 6.9%. The reproducibility test was carried out using an aliquot of the same serum pool in fifteen different analyses. The C.V. ranged from 6.9% to 9.0%. The limits of detection were measured by taking into account blank levels. The following formula was used: $x = x\,bl + 3\,Sbl$, x being the smallest detectable peak height, $x\,bl$ the mean of 30 measurements and 3 Sbl three gross mean relative standard deviations. Thus the limits of detection were 3.2 μM for PYR, 3 μM for KIV, 1.3 μM for KMV and 1.8 μM for KIC.

To test the linearity of the method, a set of calibration curves was generated by derivatizing aqueous samples of each BCKA and pyruvate at five different concentrations. Fig. 2 shows the linearity of this method up to 400 μM for KIC, 130 μM for KMV, 350 μM for KIV and 400 μM for PYR. The slopes of the regres-

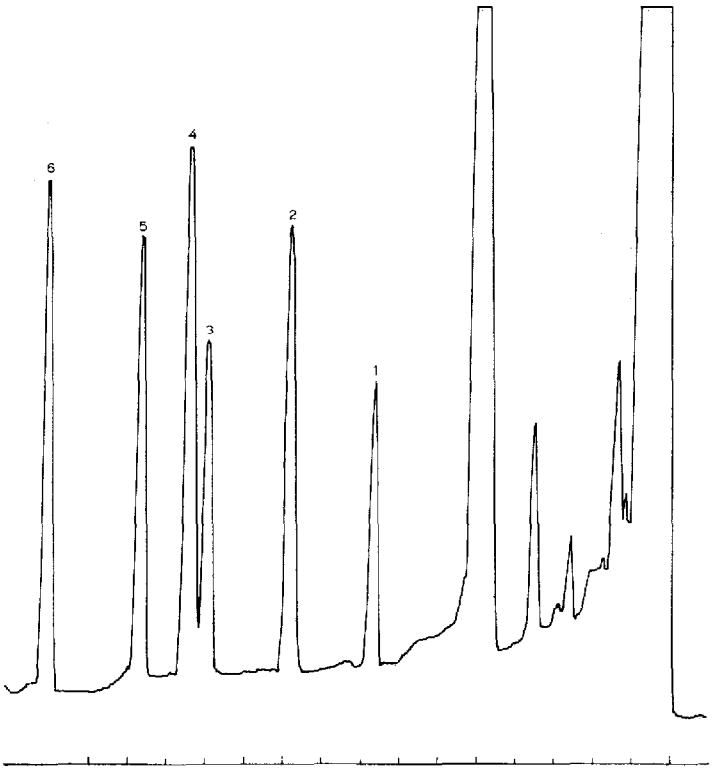

Fig. 1. Gas chromatogram of a calibration and standard mixture of six keto acids. Peaks: 1 = pyruvic acid; 2 = α-ketoisovalerate; 3 = α-keto-β-methylvalerate; 4 = α-ketoisocaproate; 5 = α-ketocaproate; 6 = 2-phenylbutyrate.

sion equations were measured in the linear section: $a = 0.91$ for PYR, 0.93 for KIC, 0.93 for KIV and 0.98 for KMV.

Table II shows the recoveries of BCKA and pyruvate. Known and increasing amounts of keto acids were added to the above-mentioned serum pool. The recoveries of BCKA and pyruvate were generally good and better than for other methods that use silylated oximes [7, 10], in which known amounts of various

TABLE I

PRECISION OF RESULTS

Fifteen replicate determinations were carried out using a mixture of α-keto acids in a serum pool.

α-Keto acid	Repeatability		Reproducibility	
	Mean (μM)	C.V. (%)	Mean (μM)	C.V. (%)
KIC	55.7	6.6	57.2	7.8
KIV	38.4	6.7	37.7	9.0
KMV	13.9	5.8	13.0	6.9
PYR	150.1	6.9	152.1	8.0

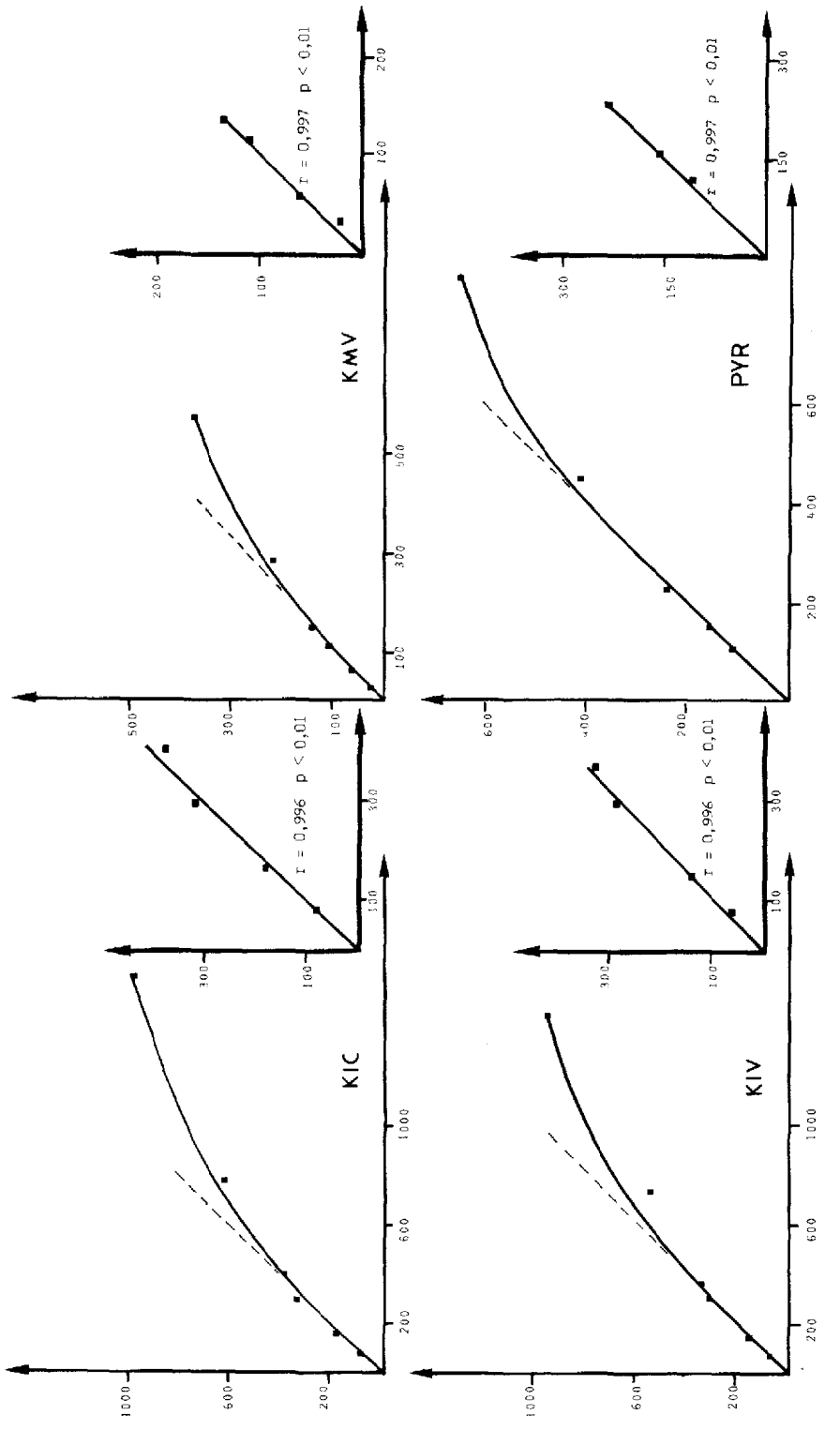

Fig. 2. Linearity test of the method. Plot of values obained versus theoretical values for KIC, KMV, KIV and PYR. On the left are represented the slopes of the regression equation as measured in the linear section.

TABLE II

RECOVERY OF α-KETO ACID ADDED TO PLASMA SAMPLES

α-Keto acid	Amount added (nmol)	Amount measured (μM)	Amount recovered (μM)	Recovery (%)
PYR	90	237	240	99
	112	270	262	103
	150	282	300	94
	225	337	375	90
KIV	74	105	112	93
	90	123	128	96
	150	170	188	91
	295	315	343	92
KMV	28	40	41	97
	42	51	55	91
	56	70	69	101
	112	110	125	88
KIC	80	125	135	92
	100	135	155	87
	160	222	215	103
	319	325	374	87

α-keto acids were added to whole blood but showed large losses, probably at the evaporation stage.

Fig. 3 shows a chromatogram from a normal human plasma: the peaks corresponding to the three BCKA and pyruvate are clearly separated from each other and from the background, giving peak heights suitable for the accuracy required.

The concentrations of BCKA and pyruvate in 30 normal fasting men were determined by this method; the α-keto acid concentrations (mean $\pm$ S.D.) were: KIV, 14.4 ± 4.0 μM; KMV, 12.1 ± 3.6 μM; KIC, 35.3 ± 10.1 μM; PYR, 63.3 ± 8.6 μM. These concentrations are in agreement with the average values reported by other authors [8, 11]. However, they are much lower than those obtained using quinoxalinol derivatives [12] and much higher than those obtained using capillary GC [13]. It is difficult to consider as representative of normal values the results of the majority of these studies because they concern a very small number of subjects, apart from the work of Koike and Koike [9].

The current methods for BCKA analysis have several advantages and disadvantages: those using 2,4-dinitrophenylhydrazone derivatives do not separate KMV from KIC [14]; silylquinoxalinol derivatives do not always give a satisfactory separation of KMV and KIC [2]. Reversed-phase HPLC of α-keto acids after derivatization to fluorescent quinoxalinones by reaction with *o*-phenylenediamine [9] gives better separation than GC methods but is expensive. Capillary column separation must still be applied [13] to determine the relative concentrations of D- and L-KMV, but in our case the patients studied did not present pathologies such as the maple syrup urine disease.

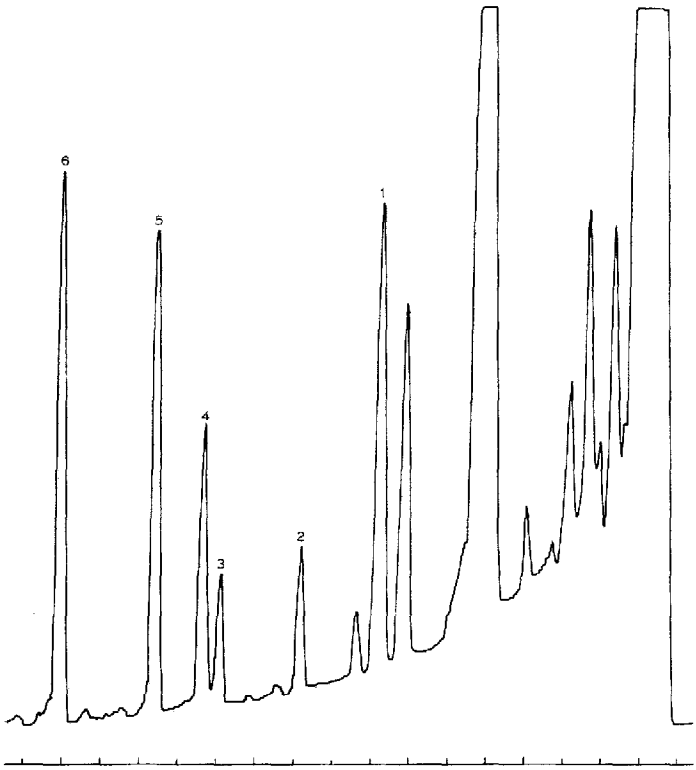

Fig. 3. Gas chromatogram from the serum of a healthy adult. Peaks: 1 = pyruvic acid; 2 = α-ketoisovalerate; 3 = α-keto-β-methylvalerate; 4 = α-ketoisocaproate; 5 = α-ketocaproate; 6 = 2-phenylbutyrate.

Finally, the present method for the isolation of BCKAs as oximes prior to quantitative analysis offers several advantages: better extraction into ethyl acetate before silylation, good baseline, reproducible separation and quantitative determination of derivatives. It could thus be applied to studies of the BCKA metabolism in humans [1].

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