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ESTIMATION OF BRANCHED-CHAIN α-KETO ACIDS IN BLOOD BY GAS CHROMATOGRAPHY

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SUMMARY

Plasma or whole blood is treated with *o*-phenylenediamine dihydrochloride in phosphoric acid under conditions found spectrophotometrically to give maximum yields of the quinoxalinols. The quinoxalinols are extracted and, after removing phosphoric acid, etc., silylated with bis-trimethylsilyltrifluoracetamide in acetonitrile. Other solvents caused instability of the trimethylsilyl(TMS)-quinoxalinols. Gas chromatography on a packed column of trifluoropropyl silicone gave good separation of the TMS-quinoxalinols from one another and from other substances derived from blood. Some representative values for normal arterial and venous human and canine plasma are reported.

INTRODUCTION

Questions posed by an investigation of amino-acid metabolism in septic shock [1] made it necessary to measure the concentrations of α-keto acids in arterial and venous blood from normal subjects, patients and experimental animals. This required a quantitative method of high sensitivity and considerable accuracy if the small arterial—venous differences in concentration were to be calculated with acceptable precision using appropriately small volumes of blood.

There are very few reports of the concentrations of α-keto acids in the blood. The technical difficulties in measuring these concentrations probably account for the paucity of data. There are reports in the older literature of paper or thin-layer chromatographic separation of 2,4-dinitrophenylhydrazones (or other derivatives) of the α-keto acids of blood [2—4], but paper and thin-layer chromatography do not lend themselves to accurate quantitation, *cis—trans*

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isomerism leads to multiple spots, and the identification of some spots has been questioned [5, 6]. Recently, the α-keto acids have been determined by high-performance liquid chromatography of their quinoxalinols [7]. Gas chromatography has been used relatively rarely.

Kallio and Linko [8] described a method for gas—liquid chromatography (GLC) of the esterified 2,4-dinitrophenylhydrazones of α-keto acids. Sternowksy et al. [9] analysed α-keto acids as their silylated oximes, using GLC. The O-methyloximes of the α-keto acids have been analysed by GLC of their trimethylsilyl esters [10, 11]. Liebich et al. [12], in the course of a thorough investigation of the organic acids of blood, separated the oxocarboxylic acids by GLC of their O-methyloxime methyl esters. In some cases *cis—trans* isomerism produces multiple peaks which may overlap.

The α-keto acids react with *o*-phenylenediamine to form stable quinoxalinols [13, 14] and these can be silylated to give derivatives suitable for GLC [15—17]. This technique has been used by Harper and associates [18, 19] to assay α-keto acids in animal tissues and fluids. A modification of the method, which in our hands gave more reproducible results, is described in the present report.

MATERIALS

Gas chromatograph

The instrument used was a Hewlett-Packard HP5840A reporting gas chromatograph equipped with flame ionization detectors. It was used in the single-column mode with SP-2401 stationary phase and in the dual-column mode with all other packings. The columns were nickel, 2.1 mm internal diameter and 1.8 m long, except as noted. Injection ports were glass-lined. The carrier gas was nitrogen flowing at 20 ml/min.

Column packings

The column packing finally adopted was 3% SP-2401 (trifluoropropyl silicone) on 100—120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). Other packings investigated included 3% SE-30 on 80—100 mesh Supelcoport, 5% SE-30 on 80—100 mesh Anakrom ABS (Analabs, North Haven, CN, U.S.A.), 3% OV-17 on 80—100 mesh Supelcoport, 10% DEGS on 80—100 mesh Anakrom ABS, 10% SP-216 PS (polyester) on 100—120 mesh Supelcoport, 10% SP-1000 (Carbowax + substituted terephthalic acid) on 100—120 mesh Chromosorb W AW (3.05-m column).

Standards and reagents

Except as noted below, all standards were purchased from Sigma (St. Louis, MO, U.S.A.). The purity of the different α-keto acids was determined spectrophotometrically (see below) and, where necessary, correction factors applied for calculating recoveries. The pure quinoxalinols corresponding to several α-keto acids were generous gifts from Dr. B.L. Goodwin, others were prepared by the method of Nielsen [6] and recrystallized from 70% ethanol in water.

Silylation grade dimethylsulphoxide, pyridine, acetonitrile, tetrahydro-

furan and dimethylformamide were purchased from Pierce, Rockford, IL, U.S.A., as were Tri-sil concentrate, bis-trimethylsilyltrifluoracetamide/trimethylchlorosilane (BSTFA/TMCS) and bis-trimethylsilylacetamide (BSA). BSTFA was purchased from Sigma. *o*-Phenylenediamine dihydrochloride (Sigma) was treated with alkali and the precipitated free base was recrystallized four times from chloroform. Final purification was by sublimation in vacuo.

Orthophosphoric acid, "Transistor" grade, 85% H_3PO_4 (Mallinckrodt, Paris, KY, U.S.A.) was diluted to 8 *M* before use. Triethylamine and 2,4-dinitrophenylhydrazine were from Eastman, Rochester, NY, U.S.A., and hydroxylamine hydrochloride from Allied Chemical, Vancouver, Canada. Silicic acid ("Sil-A-200", 60—200 mesh) was from Sigma, and ethyl acetate and methylene chloride ("distilled in glass") were from Burdick & Jackson Labs., Muskegon, MI, U.S.A.

o-Phenylenediamine reagent

Method A: 0.362 g of *o*-phenylenediamine dihydrochloride, as purchased, was dissolved in 15 ml of 8 *M* H_3PO_4.

Method B: 0.215 g of purified *o*-phenylenediamine was dissolved in 15 ml of 8 *M* H_3PO_4 plus 0.67 ml of 6 *N* HCl.

The two methods were found to be equally satisfactory and method A was routinely used in later work. The reagent was prepared immediately before use.

METHOD

To whole blood (2.5 ml plus 2.5 ml of water), plasma (5 ml) or aqueous solution of α-keto acids (5 ml), was added 0.25 μmole α-ketocaproic acid (sodium salt) as internal standard. The *o*-phenylenediamine reagent, 5 ml, was added and the mixture incubated for 90 min at 40°C. The solution was cooled and extracted three times with 20 ml of ethyl acetate each time. To the combined ethyl acetate extracts was added 1 g of silicic acid; the mixture was well shaken and centrifuged. To the clear supernatant were added 2 ml of triethylamine and the mixture was centrifuged. The clear supernatant was evaporated to dryness in vacuo at 40°C in a Buchler Rotary Evapo-Mix. Methylene chloride, 3 ml, was added and the solution evaporated to dryness as before. The addition and evaporation of methylene chloride was repeated twice more.

Acetonitrile, 0.1 ml, and BSTFA, 0.3 ml, were added to the tube which was rotated to dissolve and mix all the dried deposits on the walls. The stoppered tube stood overnight at room temperature.

Between 1 and 2 μl of the mixture, depending on expected peak heights, were injected onto the GLC column, using a glass-lined injection port at 250°C. The column was programmed to rise at 4°C/min from 60°C to 240°C. An attenuation of 2^6 was appropriate for the concentrations of the branched-chain α-keto acids found in normal or pathological plasma, but pyruvic acid usually required a higher attenuation. Known amounts of pure quinoxalinols were silylated with acetonitrile and BSTFA as above and examined by GLC. α-Keto acids were added to water and to plasma to give known concentra-

tions, then the solutions were taken through the whole derivatisation, extraction and GLC as described above to check recoveries.

RESULTS AND DISCUSSION

Organic acids, as well as the quinoxalinols of α-keto acids, were extracted from plasma, blood or aqueous solution and formed trimethylsilyl (TMS) derivatives which gave a multitude of peaks on the gas chromatogram. No attempt was made to identify or quantitate most of these substances.

A 1.8 m column of 3% SP-2401 on 100—200 mesh Supelcoport gave clear separation of TMS-3-isobutylquinoxalinol and TMS-3-(1-methylpropyl)-quinoxalinol (from α-ketoisocaproic acid and α-keto-β-methyl-*n*-valeric acid, respectively), a separation difficult to achieve on other packed columns. Fig. 1 shows the results when a mixture of quinoxalinols and α-hydroxy acids were silylated and chromatographed. When known amounts of branched-chain α-hydroxy and α-keto acids were added to plasma which was then taken through the procedure, recoveries were usually quantitative for the α-keto acids (Table I). The recoveries were calculated, using an internal or external standard, from the GLC peak areas as percentages of the peak areas obtained

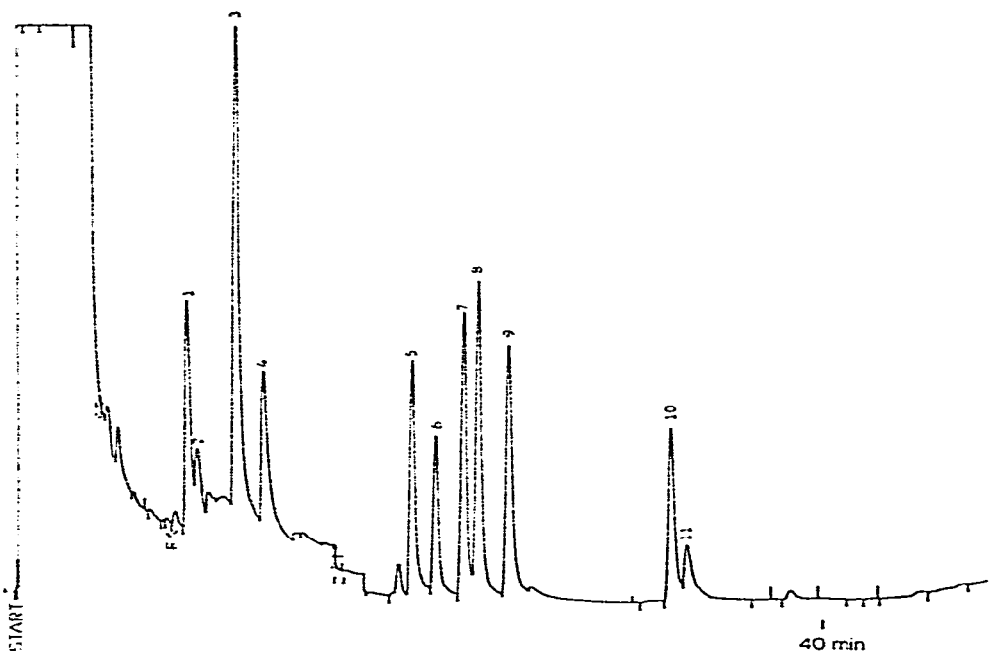

Fig. 1. Chromatogram of trimethylsilyl derivatives of hydroxy acids and of quinoxalinols. 1 = α-hydroxyisovaleric acid; 2 = β-hydroxybutyric acid; 3 = α-hydroxyisocaproic acid + α-hydroxy-β-methyl-*n*-valeric acid; 4 = α-hydroxycaproic acid; 5 = pyruvic acid; 6 = α-keto-isovaleric acid; 7 = α-keto-β-methyl-*n*-valeric acid; 8 = α-ketoisocaproic acid; 9 = α-keto-caproic acid; 10 = *p*-hydroxyphenylpyruvic acid; 11 = phenylpyruvic acid. Column: 1.8 m × 2.1 mm of 3% SP-2401 on 100—120 mesh Supelcoport. Carrier gas: nitrogen at 20 ml/min. Initial temperature 60°C rising at 4°C/min to 240°C. Flame ionization detector.

TABLE I

RECOVERY OF KETO AND HYDROXY ACIDS ADDED TO PLASMA OR WATER

Volume of plasma or water = 5 ml. The results have been corrected for the small amounts of these acids present in the plasma used. The purity of each α-keto acid was determined spectrophotometrically.

Acid	Amount added (μmole)	Amount found (μmole)	Recovery (%)
Pyruvic	0.50	0.55	110
α-Ketoisovaleric	0.22	0.22	100
α-Keto-β-methyl-*n*-valeric	0.20	0.204	102
α-Ketoisocaproic	0.32	0.33	103
α-Ketocaproic	0.306	0.306	100
α-Ketoglutaric	0.50	0.505	101
α-Hydroxyisovaleric	0.25	0.232	93
α-Hydroxyisocaproic	0.225	0.200	89

when equivalent amounts of the appropriate α-hydroxy acids and crystalline quinoxalinols were directly silylated with BSTFA and acetonitrile for GLC. Reproducibility was high (Table II). The concentrations of the branched-chain α-keto acids in arterial, femoral-venous and hepatic venous blood plasma from fasting normal dogs was determined by this method; its sensitivity and accuracy permitted calculation of the arterial—venous differences in concentration in each dog and the biological variation (Table III). Results obtained in fasting normal men are shown in Table IV. Fig. 2 shows a chromatogram of a specimen of normal human plasma: the peaks corresponding to the three branched-chain α-keto acids are clearly separated from each other and from the background, giving area counts adequate for the required precision. The peaks in the positions corresponding to the branched-chain α-hydroxy acids (expected retention times: α-hydroxyisovaleric 7.60, α-hy-

TABLE II

REPRODUCIBILITY OF RESULTS

Five replicate determinations were made using a mixture of α-keto acids in aqueous solution.

Acid	Amount per tube (μmoles)	Relative area*	
		Mean ± S.D.	Coefficient of variation (%)
Pyruvic	0.636	0.893 ± 0.045	5.04
α-Ketoisovaleric	0.724	0.700 ± 0.033	4.71
α-Keto-β-methylvaleric	0.533	0.909 ± 0.018	1.98
α-Ketoisocaproic	0.569	1.176 ± 0.026	2.21
α-Ketocaproic	0.572	—	—

*Area under the GLC peak expressed as a ratio to the area under the peak corresponding to α-ketocaproic acid.

TABLE III

ARTERIAL PLASMA CONCENTRATIONS OF THE BRANCHED-CHAIN α-KETO ACIDS AND THEIR ARTERIAL—FEMORAL VENOUS AND ARTERIAL—HEPATIC VENOUS DIFFERENCES IN CONCENTRATION IN FASTING DOGS (n = 10)

Acid	Concentration (μmoles/l; mean ± S.D.)		
	Arterial	Arterial—femoral venous difference	Arterial—hepatic venous difference
α-Ketoisovaleric	8.4 ± 3.8	—4.1 ± 3.2	0.52 ± 5.1
α-Keto-β-methylvaleric	20.1 ± 8.2	—3.8 ± 5.6	1.14 ± 8.3
α-Ketoisocaproic	25.6 ± 8.5	—6.9 ± 6.8	3.54 ± 6.8

TABLE IV

ARTERIAL PLASMA CONCENTRATIONS OF THE BRANCHED-CHAIN α-KETO ACIDS AND THEIR ARTERIAL—FEMORAL VENOUS DIFFERENCES IN CONCENTRATION IN FASTING NORMAL MEN (n = 10)

Acid	Concentration (μmoles/l; mean ± S.D.)	
	Arterial	Arterial—femoral venous difference
α-Ketoisovaleric	9.36 ± 6.0	1.53 ± 3.8
α-Keto-β-methylvaleric	16.2 ± 9.5	—1.16 ± 7.1
α-Ketoisocaproic	24.29 ± 9.3	—1.10 ± 4.8

droxy-β-methylvaleric 9.42, α-hydroxyisocaproic 9.42) are plainly too small to be distinguished from the background in this specimen. In some specimens it was possible to distinguish small peaks in the positions expected for α-hydroxyisovaleric, α-hydroxyisocaproic and/or α-hydroxy-β-methylvaleric acid, but the areas under these peaks were always much smaller than under the peaks corresponding to the α-keto acids, suggesting that equilibrium in each oxido-reduction was far over towards the branched-chain α-keto acids.

The method of Kallio and Linko [8] was used with mixtures of pure α-keto acids. Using a 1.8-m column of SE-30 it was found that esterified 2,4-dinitrophenylhydrazones of the α-keto acids were well separated except for those of α-ketoisocaproic acid (corresponding to leucine) and α-keto-β-methyl-*n*-valeric acid (corresponding to isoleucine) which ran as a single peak. Altering the conditions of running did not produce any separation, and since the branched-chain α-keto acids are of particular importance in septic shock the method was abandoned.

Separation of α-keto acids of blood as silylated oximes has been reported [9]. The method involves precipitation of the blood proteins with three volumes of ethanol and evaporation of the ethanolic supernatant before conversion to the oximes. Recovery experiments in which known amounts of various α-keto acids were added to whole blood revealed unacceptably large and variable losses, probably at the evaporation stage. This method was therefore abandoned.

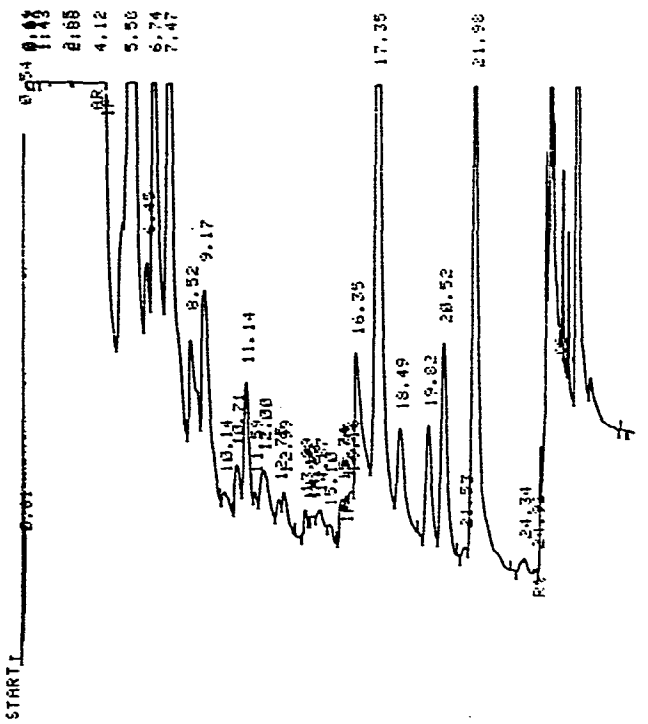

Fig. 2. Chromatogram from normal human plasma. Retention times of the TMS-quinoxalinols from the α-keto acids are: 17.35 min, pyruvic acid (217,400 area count); 18.49 min, α-ketoisovaleric acid (32,050 area count); 19.82 min, α-keto-β-methylvaleric acid (29,640 area count); 20.52 min, α-ketoisocaproic acid (56,140 area count); 21.98 min, α-ketocaproic acid (internal standard, 162,900 area count). Column: 1.8 m × 2.1 mm of 3% SP-2401 on 100—120 mesh Supelcoport. Carrier gas: nitrogen at 20 ml/min. Initial temperature 60°C rising at 4°C/min for 25 min, then at 30°C/min to 240°C. Flame ionization detector.

Quinoxalinols are stable compounds formed by reacting the unstable α-keto acids with *o*-phenylenediamine in acidic aqueous or non-aqueous solution. The absorbance of 3-isobutylquinoxalinol (corresponding to α-ketoisocaproic acid) dissolved in ethyl acetate was found to have a maximum at 330 nm. Quinoxalinols prepared from other aliphatic α-keto acids gave an absorption peak at the same wavelength in ethyl acetate or aqueous solution.

By measuring the absorbance of aqueous solutions at 330 nm, it was found that quinoxalinol formation was maximum at about pH 1.4 with a very large excess of *o*-phenylenediamine dihydrochloride (molar ratio from 100:1 at high concentrations of α-keto acid to 5000:1 at very low concentrations). High concentrations of phosphoric acid were found to increase the yield of quinoxalinols. The method described was found to give optimum, nearly quantitative yields of the quinoxalinols. The quinoxalinols formed were quantitatively extracted by ethyl acetate.

The silicic acid removed pigments extracted by ethyl acetate when whole blood was used. It also served to remove small droplets of aqueous phase carried over with the ethyl acetate. It did not affect recovery of either quinoxalinols or hydroxy acids.

Triethylamine converted the somewhat volatile α-hydroxy (and other) acids to their much less volatile triethylammonium salts which, however, remained soluble in ethyl acetate. Triethylamine also precipitated a small amount of phosphoric acid which was extracted from aqueous solution by ethyl acetate and which would otherwise have interfered at the evaporation stage. Triethylamine did not adversely affect the recovery of any quinoxalinol.

3-Alkylquinoxalinols were rapidly silylated at room temperature in BSTFA alone or in mixtures of pyridine and BSTFA. However, with purely aliphatic substituents at the 3-position, the silylated products were unstable — decomposition was appreciable within 30 min at room temperature and complete within 48 h — making it impossible to repeat GLC on the mixture. Heating the quinoxalinols with pyridine and BSTFA accelerated the decomposition. Each silylated 3-alkylquinoxalinol appeared to be converted (more rapidly on heating) into a compound giving a sharp peak of longer retention time on GLC, but this second compound was itself unstable. It is possible that the first change was a tautomeric transformation from O-trimethylsilyl-3-alkylquinoxalinol to N-trimethylsilyl-3-alkylquinoxalinol [20]. It made no difference to stability whether the *o*-phenylenediamine reagent had been prepared from purified *o*-phenylenediamine or from the dihydrochloride as purchased; pure crystalline 3-alkylquinoxalinols treated with pyridine and BSTFA showed the same instability. (In contrast to the quinoxalinols with straight- or branched-chain purely aliphatic substituents at the 3-position, 3-benzylquinoxalinol gave a stable TMS derivative with both BSTFA—pyridine and BSTFA—acetonitrile.) Of the solvents investigated, only mixtures of acetonitrile and BSTFA avoided this problem. Frigerio et al. [20] also used mixtures of BSTFA and acetonitrile in preparing TMS derivatives of quinoxalinols; these workers reported that use of pyridine caused difficulties in isolation of the derivative. Silylation was much slower in acetonitrile than in pyridine, but the silylated 3-alkylquinoxalinols were stable for at least 48 h; after 96 h some decomposition was apparent. Since as little as 10% of acetonitrile had an appreciable effect in stabilising the TMS-quinoxalinols, it seems likely that the BSTFA contains an impurity that catalyses both the silylation reaction and the subsequent decomposition of the TMS-quinoxalinols and that acetonitrile poisons this catalyst, perhaps by forming a complex with the impurity.

Solid 3-alkylquinoxalinols were found to be stable for over 17 years on the laboratory bench, but solutions in pyridine showed considerable decomposition after standing overnight.

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