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QUANTITATIVE GAS CHROMATOGRAPHY AND SINGLE-ION DETECTION OF ALIPHATIC α-KETO ACIDS FROM URINE AS THEIR O-TRIMETHYLSILYLQUINOXALINOL DERIVATIVES

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SUMMARY

A method is described for the quantitative determination of aliphatic α-keto acids in urine after derivatization with *o*-phenylenediamine and bis(trimethylsilyl)trifluoroacetamide. α-Ketovaleric acid and α-ketocaprylic acid are used as internal standards.

The chemical yield is 80—100%. At physiological concentrations, the coefficient of variation after repeated derivatizations is 4% for pyruvic acid and 14% for α-ketoglutaric acid.

With mass spectrometric single-ion detection at *m/e* = 217, 232 and 245, the biologically interesting aliphatic α-keto acids can be determined at very low levels in biological fluids.

INTRODUCTION

In a previous paper [1], we reported on the gas chromatography (GC) of eleven O-trimethylsilylquinoxalinols (TMS-quinoxalinols) which were synthesized from α-keto acids, *o*-phenylenediamine and bis(trimethylsilyl)trifluoroacetamide (BSTFA). On silicone phases, these derivatives have favourable chromatographic properties [1,2]. In addition, as a useful prerequisite to single-ion detection, their mass spectra have many abundant fragments in common with each other [3].

Owing to its inherent technical difficulties, the quantitative GC of α-keto acids in maple syrup urine disease, for example, has only rarely been reported [4—10]. It therefore seemed worthwile to develop a quantitative quinoxalinol procedure that could be reliably applied to the analysis of small urine samples

*Part of the methods described here were developed during medical thesis work of K.M. and H.-U.M.

of patients with hereditary ketoacidurias [11,12]. In this paper we report on our efforts to achieve that aim.

MATERIALS AND METHODS

The GC studies were carried out with a Hewlett-Packard Model 7611A gas chromatograph, equipped with a flame-ionization detector (FID) and a Model 7128A dual-pen recorder (1 and 10 mV full scale) from the same manufacturer. U-shaped glass columns, 180 cm × 3 mm I.D., were used. High-purity nitrogen at a flow-rate of 60 ml/min was used as the carrier gas.

A Finnigan Model 3000 quadrupole mass spectrometer was coupled to a Varian Model 1400 gas chromatograph by a heated glass jet Boehlke-type separator and a thermoconstant metal transfer line. The gas chromatograph was equipped with a spiral glass column, 240 cm × 1.5 mm I.D. High-purity helium was used as the carrier gas at 20 ml/min. The mass spectrometer was run at 70 eV with ion energies between 4 and 8 V and a multiplier voltage of 1.5—2.0 kV. A Servogor Model RE 511 recorder (Metrawatt) with multiple spans was used.

A Sartorius Model 4401 microbalance was used for weighing the keto acid standards.

The carrier gases were obtained from Messer Griesheim (Düsseldorf, G.F.R.).

Dexsil 300 GC, 3% on 100—120 mesh Supelcoport, was obtained from Supelco (Bellefonte, Pa., U.S.A.), OV-1, 3% on 100—120 mesh Gas-Chrom Q from Applied Science Labs. (State College, Pa., U.S.A.) through Serva (Heidelberg, G.F.R.) and BSTFA, pyridine (silylation grade) and reaction vials with PTFE-lined screw-caps from Pierce Eurochemie (Rotterdam, The Netherlands). Sodium pyruvate was obtained from Boehringer Mannheim (Tutzing, G.F.R.), α-ketoglutaric acid from E. Merck (Darmstadt, G.F.R.) and the remaining keto acids from Sigma (St. Louis, Mo., U.S.A.). All other chemicals and solvents were obtained from Merck.

For the quantitative derivatization of α-keto acids to the corresponding quinoxalinols, we modified the method of Mowbray and Ottaway [13]. The procedure is as follows: 10 ml of urine are mixed with 500 μl each of acetic acid and toluene. The aliphatic urinary keto acids are then stable for about 5 days without refrigeration. To 2 ml of the acidified urine, 50 μl of internal standard solution [20 m*M* α-ketovaleric acid (KVA) and α-ketocaprylic acid (KCA)] in water plus acetic acid are added and well mixed. Then 0.5 ml of urine plus internal standard are heated for 1 h at 70° with 0.5 ml of 4 *N* hydrochloric acid and 1 ml of a 1% solution of *o*-phenylenediamine in 2 *N* hydrochloric acid. The incubation is carried out in vials with PTFE-lined screw-caps using a Reacti-Therm Heating Module (Pierce Eurochemie).

The samples are then saturated with solid ammonium sulphate and extracted twice with 5 ml of chloroform in vials as described by Mamer et al. [14]. Vigorous shaking for 1 min each time is performed with a VirTis Model Whirlmix shaker. The extract is dried for 2 h over anhydrous sodium sulphate and then filtered. After evaporation to dryness in a Heidolph-Elektro Model VV1 rotary evaporator at room temperature, the residue is taken up in 50 μl of pyridine and incubated for 30 min at 70° after addition of 50 μl of BSTFA. GC

is performed with a two-step linear temperature programme from 70° to 160° at 2°/min, then from 160° to 200° at 4°/min to purge the column. The injection port temperature is 200° and the FID 250°. About 1 μl of sample is injected, which corresponds to 5 μl of urine.

The molar response factors [15] are determined in normal urine samples in the following way. One sample is derivatized without any additional keto acids, and to a second sample of 2 ml only the internal standard solution is added. Five more samples of 2 ml each receive from 0.3 to 3 μmole of the natural α-keto acids in addition to a constant amount of about 1 μmole of internal standard.

Quantitation is made by peak height measurements. From these measurements, a straight line is obtained when the ratios of the peak heights of natural and internal standard keto acids are plotted against the amounts of natural keto acids added. The linear regression coefficient, B, was calculated on a Wang Laboratories Model 450 programmable desk-top calculator. The molar response factor (RF) is then obtained as

$$RF_1 = \frac{\text{Internal standard added } (\mu\text{mole})}{B}$$

The value of the correlation coefficient, r, reflects the quality of the analytical technique.

The molar chemical yield, A, of quinoxalinoles in the derivatization of keto acids can be calculated from the molar response factor, RF_2, of pure added quinoxalinoles (recrystallized from ethanol—water) versus the internal standard keto acids:

$$A(\%) = [RF_1/RF_2]100$$

With the molar response factor RF_1, differential chemical and extraction yields are fully taken into account.

RESULTS

Flame-ionization detection

The chromatograms of the derivatives of pyruvic acid, ketoglutaric acid and the internal standards KVA and KCA are shown in Fig. 1. On Dexsil 300, palmitic acid and carboxyethylquinoxalinol (from α-ketoglutaric acid) are well separated, whereas they are eluted as a single peak on OV-1. Palmitic acid may be excreted in considerable amounts. Therefore, chromatographic separation should be achieved in quantitative work with an FID.

Branched chain α-keto acids are determined on OV-1 because *n*-propylquinoxalinol (from KVA) and isobutyl-1-quinoxalinol (from α-keto-β-methylvaleric acid) are well separated on this phase (Fig. 2) but not on Dexsil 300 [1]. Hexylquinoxalinol (from KCA) overlaps with an unknown organic acid on OV-1 (methylene unit = 19, 23). However, the effect on quantitative determinations is negligible in most instances.

In Table I the molar response factors (RF_1) and the chemical yields for six aliphatic α-keto acids are given. In our method of determination of RF_1 we obtained correlation coefficients between 0.965 and 0.999 (mean 0.992). The

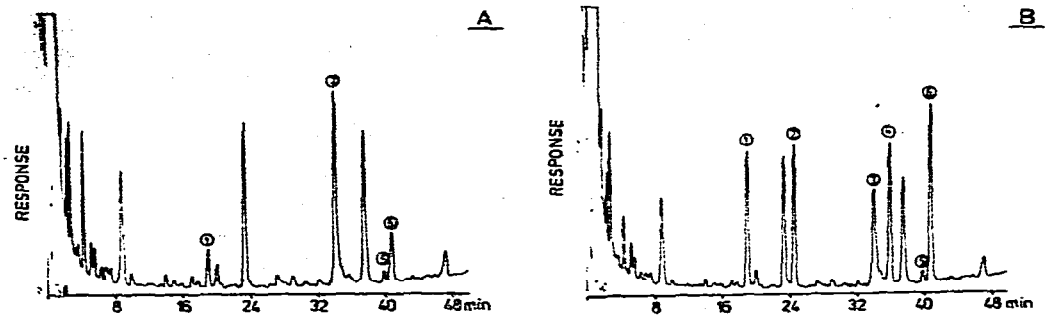

Fig. 1. Urinary extract of a normal male control. (A) No keto acids added; (B) Keto acids are added to urine: pyruvate 0.90; ketovaleric acid 1.04; ketocaprylic acid 1.03; ketoglutaric acid 1.33 μmole/ml. Methods of derivatization and extraction as described in the text. Conditions of chromatography: 3% Dexsil 300 GC; 50—160° at 2°/min; range 10^2; attenuation 2. The numbered peaks are the following trimethylsilylated compounds: 1, methylquinoxalinol (from pyruvic acid); 2, propylquinoxalinol (from α-ketovaleric acid); 3, hippuric acid; 4, hexylquinoxalinol (from α-ketocaprylic acid); 5, palmitic acid; 6, carboxyethylquinoxalinol (from α-ketoglutaric acid).

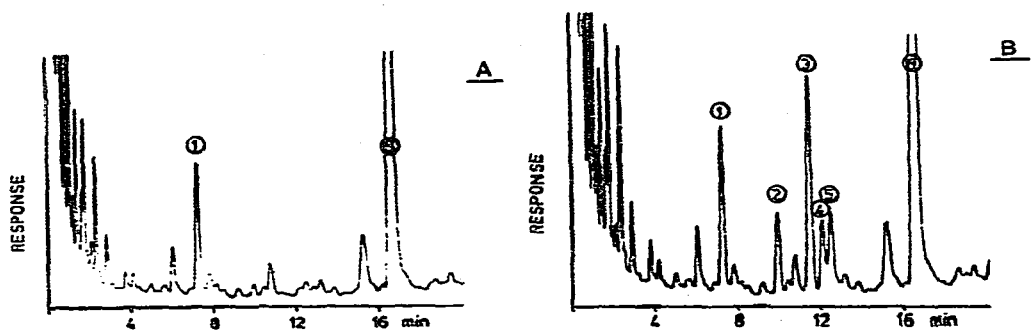

Fig. 2. Urinary extract of a normal female control. (A) No keto acids added; endogenous pyruvic acid (1) at a concentration of 356 μM; (B) keto acids added to give the following concentrations: 2, α-ketoisovaleric acid, 172 μM; 3, α-ketovaleric acid, 507 μM; 4, α-keto-β-methyl-n-valeric acid, 152 μM; 5, α-ketoisocaproic acid, 152 μM; 6, TMS-hippuric acid. Methods of derivatization and extraction as described in the text. Conditions of chromatography: 3% OV-1; 70—160° at 2°/min; range 10^2; attenuation 4.

recovery from extraction is about 50% for hexylquinoxalinol and about 30% for carboxyethylquinoxalinol. On repeated (n=6) derivatization and extraction of endogenous pyruvic and α-ketoglutaric acid from seven urines, the mean coefficients of variation were 4 and 14% for pyruvic and α-ketoglutaric acid, respectively.

In 11 urines from normal adults we determined the concentrations of pyruvic and α-ketoglutaric acids as 2.6 ± 0.8 mg-% and 4.8 ± 1.4 mg-%, respectively. The quantitative determination with either of the two internal standards agreed satisfactorily. On Dexsil 300, correlation coefficients of 0.995 and 0.997 were found for pyruvic and α-ketoglutaric acid, respectively (n = 21).

The GC samples could be stored for at least 2 weeks at room temperature without detectable (less than 5%) loss of the quinoxalinols with a carboxyalkyl side-chain. The other quinoxalinols are even more stable.

TABLE I

MOLAR RESPONSE FACTORS (RF_t) OF ALIPHATIC α-KETO ACIDS IN FLAME-IONIZATION DETECTION WITH α-KETOVALERIC (KVA) AND α-KETOCAPRYLIC (KCA) ACIDS AS INTERNAL STANDARDS

For each keto acid, five experiments were performed.

Keto acid	RF_t with KVA	RF_t with KCA	Molar chemical yield (%)	GC column
Pyruvic	0.932 ± 0.058	0.894 ± 0.075	93 ± 11	Dexsil 300
α-Ketoisovaleric	0.845 ± 0.060	0.890 ± 0.077	81 ± 10	OV-1
α-Keto-β-methyl-*n*-valeric	0.850 ± 0.092	0.901 ± 0.166	N.D.*	OV-1
α-Ketoisocaproic	1.01 ± 0.077	1.09 ± 0.142	94 ± 12	OV-1
α-Ketoglutaric	0.781 ± 0.056	0.755 ± 0.065	115 ± 6	Dexsil 300
α-Ketoadipic	0.784 ± 0.139	0.784 ± 0.164	93 ± 4	Dexsil 300

*N.D. = not determined.

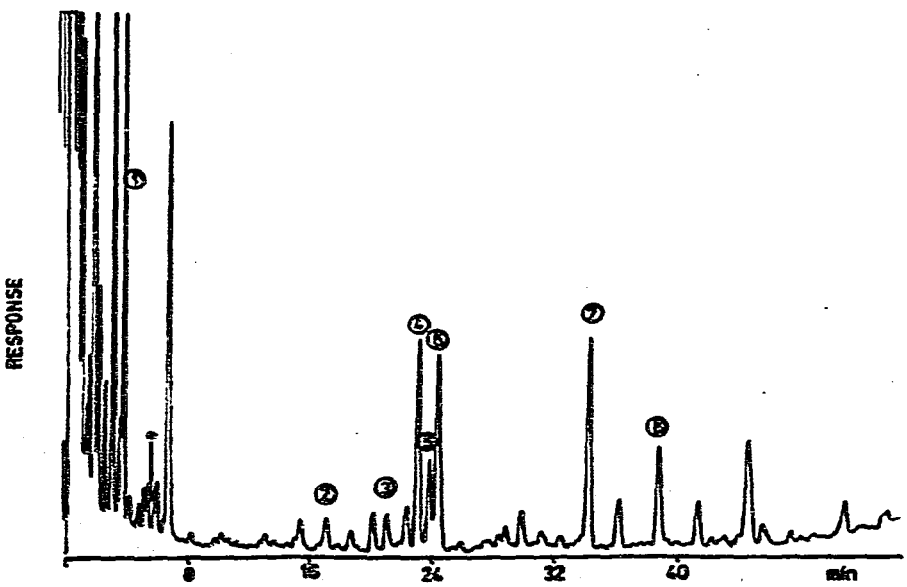

Fig. 3. Urinary extract of a 3-week-old Turkish girl (Tav.) with maple syrup urine disease. The urine was kept frozen from April 10th, 1974, to February, 1976. Methods of derivatization and extraction as described in the text. Conditions of chromatography: 3% OV-1; 50—60° at 1°/min, 60—160° at 2°/min then 160—200° at 4°/min; range 10^2; attenuation 2. Peaks: 1, α-hydroxyisovaleric acid (by methylene units); 2, methylquinoxalinol (from pyruvic acid); 3, isopropylquinoxalinol (from α-ketoisovaleric acid); 4, *n*-propylquinoxalinol (from α-keto-*n*-valeric acid, internal standard); 5, isobutyl-1-quinoxalinol (from α-keto-β-methyl-*n*-valeric acid); 6, isobutyl-2-quinoxalinol (from α-ketoisocaproic acid); 7, hexylquinoxalinol (from α-ketocaprylic acid, internal standard); 8, palmitic acid plus carboxyethylquinoxalinol (from α-ketoglutaric acid). * = Position of α-hydroxy-β-methyl-*n*-valeric acid and α-hydroxyisocaproic acid. The following concentrations (μM) were measured (The 24-h output in milligrams is given in parentheses): pyruvic acid, 69.0 (0.79); α-ketoglutaric acid, 187.3 (3.56) (both on Dexsil 300); α-ketoisovaleric acid, 103.1 (1.56); α-keto-β-methyl-*n*-valeric acid, 243.2 (4.20); α-ketoisocaproic acid, 476.0 (8.05).

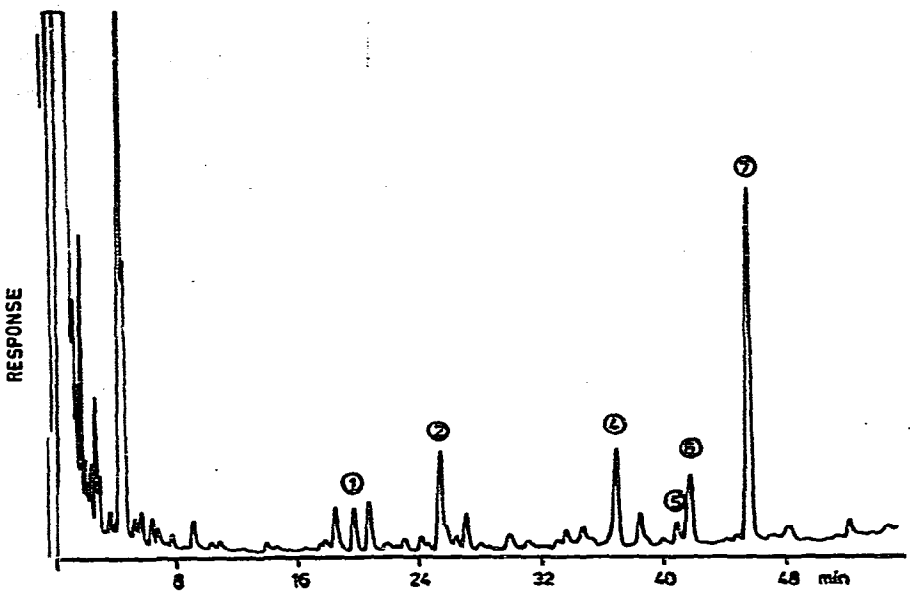

Fig. 4. Urinary extract of a patient with α-ketoadipic aciduria (S.G., described in ref. 17). The urine was kept frozen from September 11th, 1974, to February, 1976. See legend to Fig. 1 for explanation; range 10^2; attenuation 4. 7, Carboxypropylquinoxalinol (from α-ketoadipic acid). The following concentrations (μM) were measured (The 24-h output in milligrams is given in parentheses): pyruvic acid, 273.9 (3.13); α-ketoglutaric acid, 438.8 (8.33); α-ketoadipic acid, 2423 (50.39). The high concentration of α-ketoglutaric acid reported in ref. 17 could not be confirmed. The values for α-ketoadipic acid agree fairly well.

In Figs. 3 and 4, it is shown that our method can be applied, for example, to the study of maple syrup urine disease [16] and α-ketoadipic aciduria [17]. The GC of urine from a patient (T.) with pyruvic acid dehydrogenase deficiency was dominated by a large peak (6.0 mM) of O-TMS-methylquinoxalinol.

In a survey of ketoaciduria among patients with severe mental defects [18], we found a 14-year-old incontinent girl (A.Mü. 190262) with 106.7 μmole of pyruvic acid per mmole of creatinine and 262.5 μmole of ketoglutaric acid per mmole of creatinine, which are about 5 and 10 times the normal levels, respectively. The urine culture yielded various or no bacteria [19, 20].

We checked the method further on urine samples from two normal persons who were given orally 200 mg of L-leucine plus 400 mg of glucose per kilogram of body weight. No excretion of α-ketoisocaproic acid above 12 nmole/min could be detected within 10 h. The excretion rate of pyruvic and ketoglutaric acid (about 100 and 150 nmole/min, respectively) was never depressed [8].

Mass spectrometric single-ion detection

With an FID, urinary keto acids can conveniently be quantitated down to a concentration of about 100—150 μM. At lower concentrations, other organic acids interfere in the analysis, as shown in Fig. 5A. *n*-Propyl- and isobutyl-2-quinoxalinols were added to urine to give a final concentration of 10 μM. Quantitation is no longer possible with an FID. Single-ion detection [21, 22] in prin-

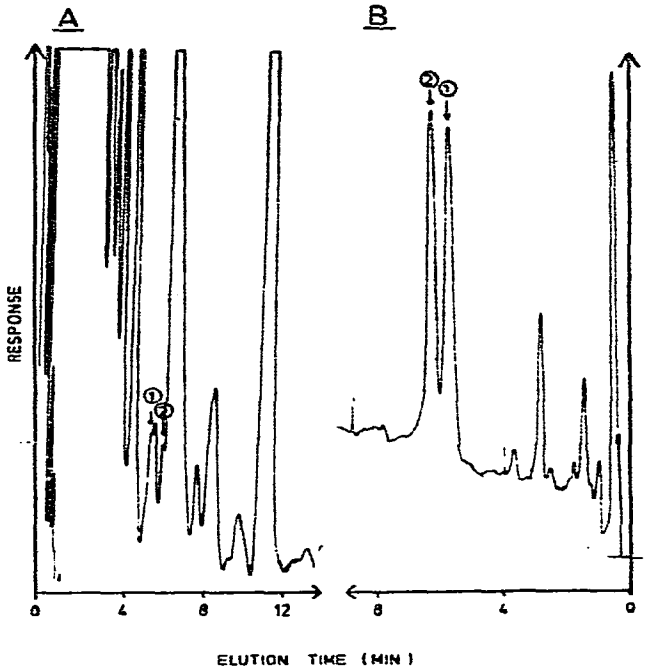

Fig. 5. Comparison of FID and single-ion detection of quinoxalinol standards in urine. To 5 ml urine 10 μl of a 5 m*M* standard solution of *n*-propyl- and isobutyl-2-quinoxalinol were added. After acidification, the urine was extracted three times with 4 ml of ethyl acetate and diethyl ether. Final sample volume, 250 μl. (A) Chromatography of 1 μl on a 180 cm × 3 mm I.D. Dexsil 300 GC column; 120° isothermal; FID; range 10^2; attenuation 4. (B) Chromatography of 1 μl on a 240 cm × 2 mm I.D. Dexsil 300 GC column; 180° isothermal; single-ion detection at *m/e* = 232; electron energy 70 eV; ion energy 6.0 V; recorder span 1 V. Peaks: 1, O-TMS-*n*-propylquinoxalinol; 2, O-TMS-isobutyl-2-quinoxalinol; 1 μl of final sample contained about 200 pmole of each compound from 20 μl of urine, assuming complete extraction.

ciple solves this problem, as shown in Fig. 5B. The mass spectrometer was focused at an *m/e* ratio of 232. Both quinoxalinols have their base peak at this *m/e* ratio owing to a McLafferty-type rearrangement [3]. The other urinary constituents were not detected because they have no major fragments in their mass spectra at *m/e* = 232. Thus, the quinoxalinols were easily determined at these very low concentrations.

In Fig. 6 it is shown that a linear and almost proportional response is obtained for *n*-propyl- and isobutyl-2-quinoxalinol when amounts from 30 to 800 pmole are injected. The molar response factor in 11 determinations carried out on the same day was 1.28 ± 0.02 (coefficient of variation: 1.5%).

In Table II the molar response factors of standard quinoxalinols in single-ion detection are given for three *m/e* ratios. (Interpretation of the mass spectra is the subject of a forthcoming paper [3].) In order to obtain the response factors in single-ion detection when keto acids are analyzed, the corresponding values in Tables I and II have to be multiplied: pyruvic acid, for example, measured at

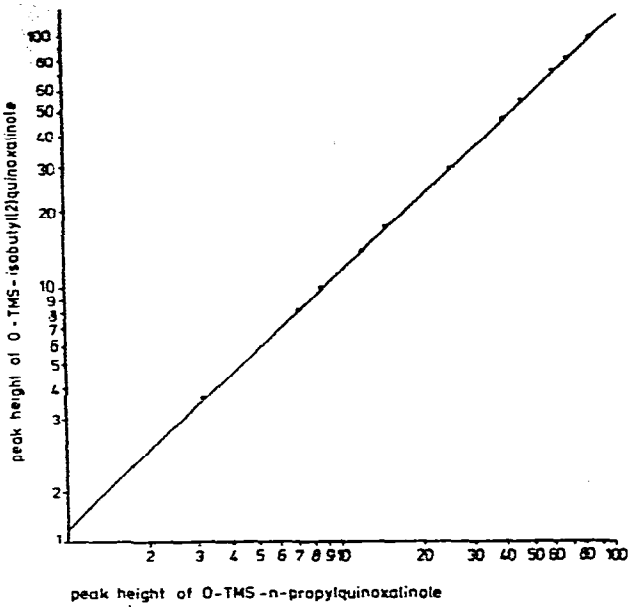

Fig. 6. Single-ion detection of O-TMS-*n*-propyl- and isobutyl-2-quinoxalinol standards at *m/e* = 232. From 30 to 800 pmole of each compound were injected on to 3% Dexsil 300 at 180°. Electron energy 70 eV; ion energy 6.0 V; recorder span 0.5—5 V.

TABLE II

MOLAR RESPONSE FACTORS (RF) OF O-TMS-QUINOXALINOL STANDARD COMPOUNDS IN SINGLE-ION DETECTION WITH O-TMS-HEXYLQUINOXALINOL AS INTERNAL STANDARD

Similar data are to be obtained with propylquinoxalinol as internal standard (cf., Table III). The conditions of operation were: 240 cm × 2 mm I.D. glass column; 100—230° at 4°/min; electron energy 70 eV; ion energy 4.0 V; recorder span 1 V; 0.5—1 nmole of each compound per injection yielded peak heights of up to 25 cm.

Substituent in position 3 in quinoxalinol	Parent α-keto acid	RF		
		m/e = 217	*m/e* = 232	*m/e* = 245
Methyl	Pyruvic	2.37	0.17	0
Ethyl	Ketobutyric	0.40	0.14	0
Isopropyl	Ketoisovaleric	0.25	0.06	3.30
Isobutyl-1	Keto-β-methylvaleric	0.33	0.43	0.42
Isobutyl-2	Ketoisocaproic	1.05	0.95	0
2-(Methylthio)ethyl	Keto-γ-(methylthio)butyric	0.11	0.04	3.90
Carboxyethyl	Ketoglutaric	0.20	0	8.66

m/e = 217 with α-ketocaprylic acid as the internal standard would give a molar response factor of 0.894 × 2.37 = 2.12. The response factors in Table II are directly dependent on the percentage portion of the total ion current that is present in these fragments ($\%\Sigma_{35}$). These values are given in Table III.

TABLE III

PERCENTAGE PORTION OF THE TOTAL ION CURRENT FROM *m/e* = 35 TO $M^{+}+2$ ($\%\Sigma_{35}$) FOUND IN THE THREE MAJOR FRAGMENTS OF O-TMS-QUINOXALINOLS AT 70 eV

Substituent in position 3	*m/e*		
	217	232	245
Methyl	32.6	4.1	0
Ethyl	5.1	3.1	0
Isopropyl	2.6	0.9	11.3
Propyl	9.8	19.5	7.8
Isobutyl-1	2.4	5.2	0.8
Isobutyl-2	11.9	18.3	<0.1
Hexyl	7.2	12.6	2.2
2-(Methylthio)ethyl	0.8	0.4	11.3
Carboxyethyl	0.7	0	11.2
Carboxypropyl	4.3	4.6	18.6

The data in Tables II and III were collected at different times, and a close examination reveals that they are not entirely compatible and can serve only as a guide. Because of day-to-day variations in performance of the mass spectrometer for single-ion detection, standards must be run every day. Re-focusing of the fragments had to be carried out every 3—4 h. The good results of determination of isobutyl-2-quinoxalinol with *n*-propylquinoxalinol as the internal standard (Figs. 5B and 6) could not be duplicated with hexylquinoxalinol. This internal standard was eluted 6 min after isobutyl-2-quinoxalinol, leaving the system enough time to change. Fluctuations in the beam current seem to be the major source of this analytical error.

The sensitivity of the single-ion detection method could not be increased further by lowering the electron energy. The increase in the percentage portion of the fragments at *m/e* 217, 232 and 245 is more than compensated for by the decrease in the total ion current.

DISCUSSION

Disorders that are characterized by the accumulation of α-keto acids in the body fluids are phenylketonuria [23], maple syrup urine disease [4—10, 16], pyruvic acid dehydrogenase deficiency [24—26], α-ketoadipic aciduria [17, 27], ketotic hypoglycaemia with dwarfism and congenital cataract [28], methionine malabsorption syndrome [29], hereditary tyrosinemia [30] and cystinosis [31]. The association of most of these disorders with severe mental deficiency deserves much interest and justifies efforts to improve techniques for the analysis of α-keto acids.

The GC of α-keto acids has been reported for the free acids [32] as well as for the methyl esters of the free acids [6, 33], methoximes [34, 48] and 2,4-dinitrophenylhydrazones [35]. Furthermore, trimethylsilyl esters of the free acids [35], the oximes [8, 9, 36, 37], methoximes [38—40], ethoximes [40]

and benzoximes [39, 40] have been used in GC. This large number of analytical methods indicates the inherent technical difficulties.

The O-TMS-quinoxalinol method described here and elsewhere [1, 2, 41—43] offers some useful advantages which make it worthy of consideration: (1) the derivatives have a low volatility; (2) multiple derivatives do not occur; (3) derivatives of isomeric α-keto acids are completely separated; (4) complete separation from the corresponding α-hydroxy acids is accomplished; (5) the chemical yield is close to 100%; (6) the derivatives are stable for several weeks; (7) chromatographic losses are negligible even at $3 \cdot 10^{-11}$ mole per injection; (8) with only three fragments at relatively high mass numbers (m/e = 217, 232 and 245), all α-keto acids can be measured by combined GC—MS.

The method described here also offers advantages over the original quantitative O-TMS-quinoxalinol method [41—43]: (1) we take 2 ml of urine instead of 50 ml for addition of the internal standard, and only 0.5 ml or even 0.1 ml is used for derivatization; (2) use of α-keto acids instead of decyl cyanides as internal standards gives a greater specificity to the assay of natural α-keto acids; (3) extraction with chloroform instead of ethyl acetate leaves most of the by-products in the aqueous phase. The extraction recovery of quinoxalinols with chloroform is not quantitative, but this does not have much influence on the quantitative determination of keto acids because the internal standards belong to the same chemical class. The response factors indicate that all steps in the procedure, including extraction, are reproducible fairly well.

Only two α-keto acids cannot be measured with the quinoxalinol method: (1) oxaloacetic acid decarboxylates to yield pyruvic acid [1, 42, 44]; also the quinoxalinol is unstable [13, 44]; (2) *o*-hydroxyphenylpyruvic acid at low pH forms a δ-lactone which does not react with *o*-phenylenediamine [45].

Our quantitative results with single-ion detection were not as good as expected. More modern instrumentation (ours dates from 1969) with stable electronic parts [46] might not pose such problems. We have no experience yet with the use of a selective nitrogen-sensitive FID as described by Hoffman and co-workers [41—43]. High selectivity will also eventually be obtained with electron-capture detection of fluoroquinoxalinoles: it has been reported [13] that 1,2-diamino-4-fluorobenzene reacts with α-keto acids to the same extent as *o*-phenylenediamine. 1,2-Diamino-3,4,5,6-tetrafluorobenzene can also be used [47].

In urine extracts from four untreated adult patients with phenylketonuria [18] we consistently found a large peak of benzylquinoxalinol (from β-phenylpyruvic acid) together with a similarly large peak of phenyllactic acid. Our quantitative GC—FID results on aromatic keto acids will be published later. Our further work is aimed at extending the quinoxalinol method to the GC study of α-keto acids in blood [44] and tissues.

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