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QUANTITATIVE GAS CHROMATOGRAPHY—CHEMICAL IONIZATION MASS SPECTROMETRY OF 2-KETOGLUTARATE FROM URINE AS ITS O-TRIMETHYLSILYL-QUINOXALINOL DERIVATIVE

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SUMMARY

Quantitation of 2-ketoglutarate in urine as its O-trimethylsilyl-quinoxalinol derivative by gas chromatography—chemical ionization mass spectrometry is described. This technique, with ammonia as reactant gas, produces no fragmentation and allows only the detection of the protonated molecular ion. It gives the greatest known sensitivity, and could be applied to the determination of urinary 2-ketoglutarate in normal children and in various metabolic disorders, such as dihydrolipoyl dehydrogenase defect, pyruvate carboxylase deficiency and type I glutaric acidemia.

INTRODUCTION

2-Ketoglutaric acid is a normal metabolite of amino acids such as lysine, hydroxylysine and tryptophan. Its concentration in cells and serum results from a steady-state between three pathways: (1) in the citric acid cycle, production by the reaction of isocitrate dehydrogenase and degradation to succinyl-CoA by the reaction of 2-ketoglutarate dehydrogenase; (2) formation during the oxidative deamination of amino acids by the reaction of glutamate dehydrogenase; and (3) transamination from glutamate as part of the malate—aspartate cycle.

Changes of 2-ketoglutarate concentration in serum or urine may result from disorders involving those metabolic pathways, and especially increased values were observed in the blood of patients bearing tumors of various organs [1, 2] and in urines of patients presenting type I glycogenosis [3, 4] and lactic acidosis [5, 6].

Because of the unstable nature of the free acid, most analytical methods

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have utilized derivatization, with, for instance, 2,4-dinitrophenylhydrazine [7, 8], ethoxyamine [9] or *o*-phenylenediamine [10—13]. The most sensitive method, described by Langenbeck [13], consisted of gas chromatographic (GC) quantitation of the O-trimethylsilyl-quinoxalinol derivative with a nitrogen-selective detector; but this derivative showed considerable non-linearity if less than 250 pmol were injected on to the column.

Our purpose was to establish a more sensitive and reliable method of 2-ketoglutarate quantitation by *o*-phenylenediamine derivatization (Fig. 1) and analysis by gas chromatography—chemical ionization mass spectrometry.

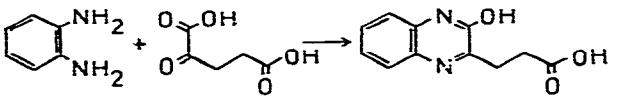

Fig. 1. Chemical structure of the quinoxalinol derivative of 2-ketoglutaric acid: 3-(2'-hydroxycarbonylethyl)-2-quinoxalinol.

EXPERIMENTAL

Reagents

All solvents and reagents were of analytical grade and used without further purification; 2-ketoglutaric acid (monosodium salt) and benzoylformic acid were purchased from Sigma (St. Louis, MO, U.S.A.). Ethyl acetate, chloroform, pyridine, diethyl ether, methylene chloride and *o*-phenylenediamine were obtained from Fluka (Buchs, Switzerland) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) from Pierce (Rockford, IL, U.S.A.). The OV-101 liquid phase was purchased from Varian (Palo Alto, CA, U.S.A.). Chromosorb W AW DMCS (80—100 mesh) from Merck (Darmstadt, G.F.R.) and the CPSil 5 capillary column from Chrompack (Middelburg, The Netherlands).

Derivatization

Urine samples were, whenever possible, 24-h collections, and were kept frozen at −80°C. Creatinine concentrations were determined by the automated Jaffé method [14].

For the derivatization of 2-ketoacids in standard solutions or in urine, we utilized our previously described method [15]: 0.1 ml of standard solution or urine and 0.1 ml of internal standard solution (1 μg ml^{-1} benzoylformic acid) was heated for 1 h at 90°C in a Reacti-Therm (Pierce) with 0.2 ml of a 1% solution of *o*-phenylenediamine in 4 *M* hydrochloric acid. The pH of the solution was adjusted to 1 ± 0.1 by addition of 5 *M* sodium hydroxide and extracted twice with 1.5 ml of ethyl acetate. The two extracts were collected and dried for 2 h over anhydrous sodium sulphate and evaporated to dryness at room temperature under a gentle stream of nitrogen. The residue was taken up in 0.1 ml of a (BSTFA + TMCS)—pyridine (1:1) mixture and heated at 90°C for 30 min. One microlitre of the solution was applied on the Ros injector.

Instruments

GC analyses were performed on a Varian Model 3700 gas chromatograph with 10% OV-101 as stationary phase, coupled with a CDS 111 integrator (Varian). The elution conditions for 2-ketoglutarate and benzoylformate derivatives were: 2 min isothermally at 220°C and linear temperature programme from 220°C to 270°C at 4°C/min, injector and flame ionization detector temperatures at 250°C, and carrier gas (nitrogen) at 26 ml min^{-1}.

Analyses by gas chromatography—mass spectrometry (GC—MS) were performed on a quadrupole Ribermag (Rueil-Malmaison, France) R 10-10 B mass spectrometer coupled with a Girdel (Suresnes, France) Model 31 gas chromatograph and a PDP 8a computer with a Sidar data system (Ribermag). The 2-ketoacid derivatives were resolved on a capillary glass column (25 m × 0.25 mm) coated with CPSil 5. The injector was an all-glass Ros type (Girdel). Injector and column temperatures were 250°C and 220°C, respectively, and the carrier gas was helium at a flow-rate of 1.5 ml min^{-1}. The mass spectrometer was run at 70 eV with an ion energy of 6V, a multiplier voltage of 1.8—2.5 kV and an emission current of 0.2 mA. Chemical ionization mass spectra were obtained using ammonia at 10^{-4} Torr.

Quantitation by Sidar data system

For automatic quantitation, the Sidar system programme previously described [15] was used by measuring the chromatographic peak areas at *m/z* 363.30 and 295.10 for the respective O-trimethylsilyl-quinoxalinol derivatives of 2-ketoglutarate and benzoylformate (internal standard).

RESULTS

Reinvestigation of 2-ketoglutarate derivative extraction

The literature was conflicting for the extraction of the quinoxalinol derivative of 2-ketoglutarate [11, 12] and, according to Hoffman and Haustein [11], ethyl acetate was found to be the best extraction solvent compared to chloroform, methylene chloride or diethyl ether (Table I). This extraction procedure

TABLE I

EXTRACTION OF 2-KETOGLUTARATE (KG) AND BENZOYLFORMATE (BF) DERIVATIVES BY DIFFERENT SOLVENTS

Recoveries of these two derivatives are expressed as percentage recovery in the best solvent (ethyl acetate).

	KG/C_{19}*	Recovery of KG (%)	BF/C_{19}**	Recovery of BF (%)
Diethyl ether	1.33 ± 0.05	83	1.48 ± 0.04	83
Ethyl acetate	1.60 ± 0.03	100	1.78 ± 0.03	100
Chloroform	0.43 ± 0.04	27	1.60 ± 0.03	90
Methylene chloride	0.30 ± 0.02	19	1.57 ± 0.06	88

*KG/C_{19} = ratio of peak area of 2-ketoglutarate derivative to that of nonadecane.
**BF/C_{19} = ratio of peak area of benzoylformate derivative to that of nonadecane.

was tested by analysing three duplicates for each solvent and measuring the chromatographic peak areas of 2-ketoglutarate and benzoylformate derivatives (at a concentration of 1 mg ml^{-1}) compared to the area of nonadecane added to the (BSTFA + TMCS)—pyridine mixture at a concentration of 750 $\mu g\ ml^{-1}$.

The extraction of 2-ketoglutarate derivative as a function of pH was also investigated, the optimal extraction pH appearing to be 1 ± 0.1 as shown in Fig. 2.

Typical chromatogram of standard solution

Fig. 3 shows a typical mass chromatogram of O-trimethylsilyl-quinoxalinol derivatives of 2-ketoglutarate and benzoylformate with single-ion monitoring (SIM) optimized, respectively, on the masses at *m/z* 363.30 and 295.10.

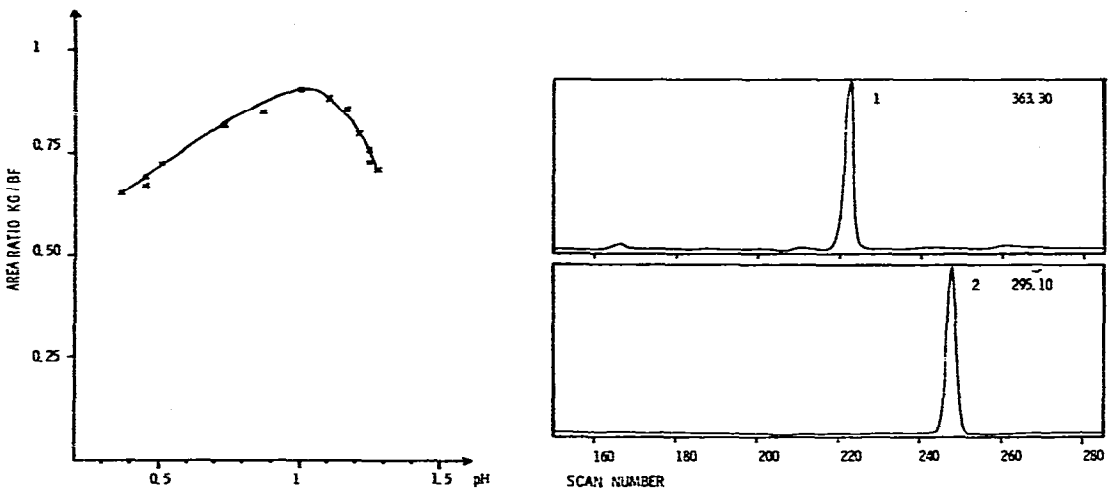

Fig. 2. Extraction of 2-ketoglutarate derivative (KG) with ethyl acetate as a function of pH. The extraction of benzoylformate derivative (BF) is independent of pH.

Fig. 3. Typical mass chromatogram of a standard solution at a concentration of 250 ng ml^{-1} for each product: 1 = derivative of 2-ketoglutarate; 2 = derivative of benzoylformate.

Mass spectra of O-trimethylsilyl-quinoxalinol derivatives

The two derivatives exhibit similar mass spectra with only the protonated molecular ion (MH^+) due to the great affinity of proton for the quinoxalinol structure (Fig. 4); the ammonium adducts were not detected. The electron impact mass spectrum of 2-ketoglutarate derivative was identical to the spectrum reported by Langenbeck et al. [16], the electron impact mass spectrum of the benzoylformate derivative being shown for comparison in Fig. 5. In the latter spectrum, the fragments at *m/z* 63, 90, 102 and 117 correspond to structures 1, 2, 3 and 4 (Fig. 6) proposed by Kovacik et al. [17], and the fragment at *m/z* 217 to structure 5 [16]. Structures 6, 7, 8 and 9 are proposed for the respective fragmentations at *m/z* 132, 203, 205 and 221 (Fig. 6).

Calibration curve

The calibration curve was automatically computed by the Sidar system after

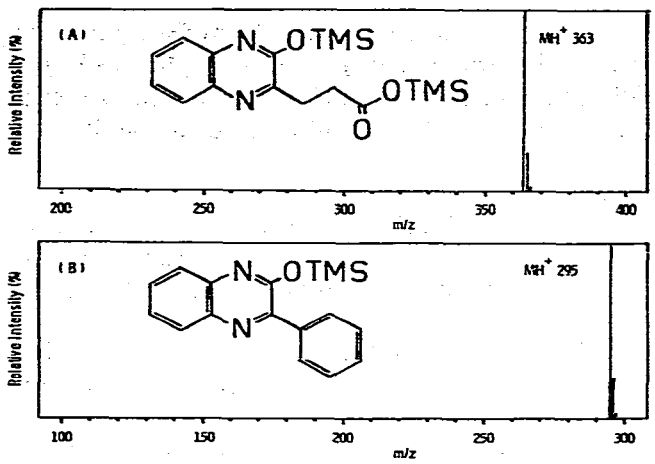

Fig. 4. Chemical ionization (ammonia) mass spectra of (A) 2-O-trimethylsilyl-3-(2'-O-trimethylsilyl-carbonylethyl)-quinoxalinol (derivative of 2-ketoglutarate), and (B) 2-O-trimethylsilyl-3-phenyl-quinoxalinol (derivative of benzoylformate).

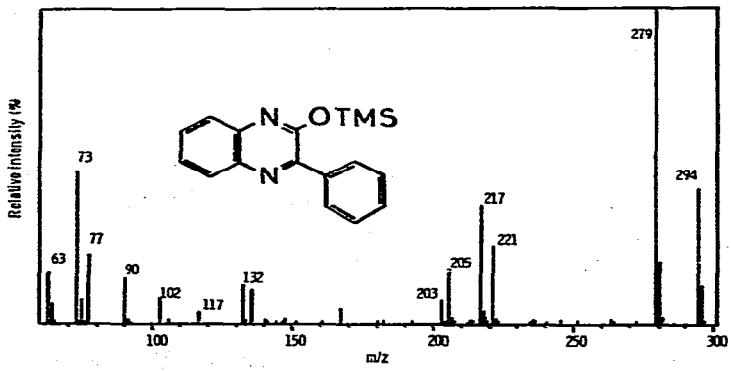

Fig. 5. Electron-impact mass spectrum of benzoylformate derivative.

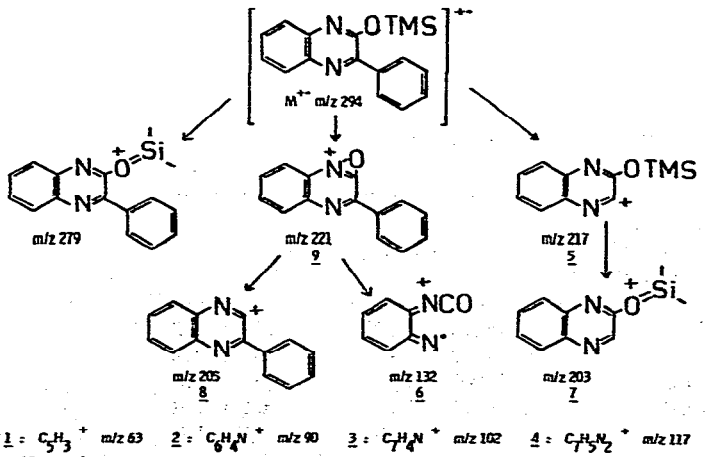

Fig. 6. Fragmentation pattern of benzoylformate derivative.

injection of six standard solutions in a concentration range from 50 ng ml^{-1} to 20 μg ml^{-1}. The obtained equation of the curve was $y = 0.26x + 0.1235$, and the correlation coefficient 0.99996. The limit of detection was in the order of 1 pg injected on to the column; in this case, the signal-to-noise ratio was 3.4.

Determination of urinary 2-ketoglutarate

The detection limit in urine as in standard solution was 7 pmol/ml (1 ng ml^{-1}). Forty urine samples of children from two months to ten years old were analysed by this method; the normal excretion was found to be 125 ± 100 μmol l^{-1} or 60 ± 55 mmol/mole of creatinine.

Twenty urines from a child (F.L.) who presented a dihydrolipoyl dehydrogenase defect were analysed, and 2-ketoglutarate excretion was found to be in the range of 820—2430 mmol/mole of creatinine.

An irregular accumulation of this product was also observed in five cases (T.K., D.K., S.P., C.E. and M.K.) of pyruvate carboxylase defect, and in this metabolic disorder the excretion was in the range 340—2200 mmol/mole of creatinine. Phenyllactic acid was also elevated in all the analysed urine samples of pyruvate carboxylase defects, but its concentration is not reported here.

Finally, 2-ketoglutarate was quantitated in different urine samples of a child (B.M.) who presented a type I glutaric acidemia [18]; the range was 220—930 mmol/mole of creatinine.

Accuracy and precision

The accuracy and precision of the method were determined by analysing standard solutions. Six samples from the same solution were analysed; the accuracy of the method was in the order of 2%. Each sample was analysed six times and the measured precision of the instrumentation was 3%.

Recovery of 2-ketoglutarate derivative from urine

The recovery of 2-ketoglutarate derivative was determined by analysing urines to which standard solutions of 2-ketoglutarate (in the range 200 pg to 20 ng) had been added. Each sample was analysed three times and the results obtained are shown in Table II.

TABLE II

RECOVERY OF 2-KETOGLUTARATE (KG) FROM URINE

Urinary concentration of KG (pg per 100 μl)	Added KG in 100 μl (pg)	Urinary theoretical concentration (pg per 100 μl)	Urinary measured concentration (pg per 100 μl)	Recovery (%)
7500	200	7700	7620 ± 110*	99 ± 1.4
7500	500	8000	7760 ± 180	97 ± 2.2
7500	1000	8500	8840 ± 150	104 ± 1.8
7500	5000	12,500	12,630 ± 230	101 ± 1.8
7500	20,000	27,500	26,670 ± 510	97 ± 1.8

*Mean ± S.D.

DISCUSSION

Various internal standards have been used for 2-ketoglutaric acid quantitation: 2,6-dimethylphenol, *p*-propylphenol [10] and trimellitic acid [8] in high-performance liquid chromatography (HPLC), undecyl-, tetradecyl- and hexadecylcyanides [11], *p*-nitrophenylphenyl ether [19] or 2-ketovaleric and 2-ketocaproic acids [12] in GC. In our opinion, only a structure analogous to 2-ketoglutarate such as 2-ketovalerate should be retained. However, this product is not suitable for 2-ketoglutarate quantitation because of its very different retention time. Thus, another 2-ketoacid, benzoylformic acid, was preferred since this compound has a suitable retention time, and it is not physiological: duplicate runs were performed in which no internal standard was added to six urine samples, and no benzoylformate was detected.

The normal excretion of 2-ketoglutarate in urine found by our technique was intermediate between the values of Liao et al. [10] and those of Langenbeck et al. [12]. However, the former authors did not utilize a structural analogue, and the latter reported values for adults.

The deficiency of dihydrolipoyl dehydrogenase (E_3 component of the pyruvate dehydrogenase complex) is also characterized by a decreased activity of 2-ketoglutarate dehydrogenase in all tissues [20], as a result of the probable identity of the E_3 components of pyruvate and 2-ketoglutarate dehydrogenase complexes [21]. However, other mechanisms may be involved in the secondary deficiency of 2-ketoglutarate dehydrogenase activity during metabolic defects. Thus, glyoxylic acid [22] and branched-chain 2-ketoacids [23, 24] inhibit 2-ketoglutarate dehydrogenase with a K_i of around 1.5 mmol l^{-1}, and their serum concentrations may exceed this value for K_i as, for instance, during maple syrup urine disease [25]. Such a mechanism might apply to pyruvate carboxylase deficiencies, which present a urine accumulation of glyoxylic and branched-chain 2-ketoacids [15].

The explanation of 2-ketoglutarate elevation in type I glutaric acidemia is more difficult; however, it is possible to relate this increase to a disturbance in the metabolism of glutamate and 4-aminobutyric acid. Leibel et al. [26] have observed a decreased activity of glutamate decarboxylase and low levels of 4-aminobutyrate in the brain of a child presenting this disease. Glutaric acid inhibits glutamate decarboxylase [26, 27], and is also a strong inhibitor of glutamate dehydrogenase [28]. In this hypothesis, the 2-ketoglutarate accumulation might be secondary to an elevation of glutamate by inhibition of glutamate decarboxylase and of glutamate dehydrogenase.

CONCLUSION

The utilization of the gas chromatography—chemical ionization (ammonia) mass spectrometry procedure applied to the O-trimethylsilyl-quinoxalinol derivative of 2-ketoglutarate allows this product to be quantitated in biological fluids such as urine with the greatest known sensitivity. This method is very specific and reliable, as compared to detection by a nitrogen-selective detector in GC analysis [13], or HPLC [10]. Thus, it is possible to direct the diagnosis of some metabolic disorders by quantitation of 2-ketoglutarate as well

as of pyruvate and other aliphatic 2-ketoacids [15], and to make these two measurements in the same urine extract by utilising a mixture of 2-ketovalerate and benzoylformate as internal standards, and proceeding at two different elution temperatures. The derivatization technique described here may be applied to determinations of 2-ketoglutarate and its homologue, 2-ketoadipate, in deproteinized plasma; this work is now in progress.

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