Journal of Chromatography, 115 (1975) 65–70
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CHROM. 8583

GAS CHROMATOGRAPHY OF α-KETO ACIDS AS THEIR O-TRIMETHYLSILYLQUINOXALINOL DERIVATIVES

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(Received May 29th, 1975)

SUMMARY

The O-trimethylsilyl (TMS) quinoxalinols are very useful derivatives for the gas chromatography of α-keto acids because of their high stability and the absence of stereoisomerism and because of the presence of specific, common and abundant fragments in electron impact mass spectra, which allows the low-level detection of whole groups of keto acids by single-ion detection.

In this paper, the chromatographic properties of eleven O-TMS-quinoxalinols on OV-1, OV-17 and Dexsil 300 are reported in terms of methylene units.

Also by use of methylene units, the chromatographic isotope effect is analyzed in detail for nine perdeutero-TMS derivatives. The effect is explained by the diminished interaction of the deuterated compounds with the unlabelled liquid phase.

INTRODUCTION

More than a dozen hereditary metabolic diseases[1,2] and also some cases of mental retardation in the adult[1] are characterized by the excretion of abnormal amounts of α-keto acids in the urine. Thus, techniques for qualitative and quantitative analyses of this class of compounds are of special interest to the human biochemical geneticist.

In the past, the thin-layer chromatography of 2,4-dinitrophenylhydrazones of keto acids has been used extensively for the analysis of ketoacidurias[2]. However, quantitative studies are difficult to perform by this method[3].

Gas chromatography (GC) should make possible the quantitative determination of keto acids in the biological fluids of patients. Such data are prerequisite, for example, to causal analysis of mental retardation in inborn errors of metabolism[4]. The great concern with keto acids and the analytical difficulties involved have resulted in the use of at least nine different derivatives for the GC of keto acids[5]. Of these nine types of derivatives, the trimethylsilyl (TMS) ethers of quinoxalinols [formed with *o*-phenylenediamine and bis(trimethylsilyl)acetamide (BSA)[6]] are, in our

* Part of the results described here was obtained during medical thesis work by H.-U. M. and K.-P. D.

opinion, the most stable compounds for use in the GC of α-keto acids. A few applications with these derivatives have been reported[7–9].

In this paper, we describe a systematic survey of the GC properties of TMS-quinoxalinol derivatives of eleven keto acids on three commonly used phases. In addition, we present results on the chromatographic isotope effect of deuterated TMS-quinoxalinols prepared from some of these keto acids.

MATERIALS AND METHODS

The studies were carried out with a Model 7611A gas chromatograph (Hewlett-Packard, Palo Alto, Calif., U.S.A.) equipped with a flame ionization detector and a Model 7128A dual-pen recorder from the same manufacturer. U-shaped glass columns, 6 ft. × 3 mm I.D., were used. High-purity nitrogen (Messer Griesheim, Düsseldorf, G.F.R.) was used as the carrier gas.

Dexsil 300 GC, 3% on 100–120-mesh Supelcoport, was obtained from Supelco (Bellefonte, Pa., U.S.A.); the 3% OV phases on 100–120-mesh Gas-Chrom Q and the even-numbered alkanes from Applied Science Labs. (State College, Pa., U.S.A.); and bis(trimethylsilyl)trifluoroacetamide (BSTFA), pyridine (silylation grade) and reaction vials with PTFE-lined screw-caps from Pierce Eurochemie (Rotterdam, The Netherlands). Perdeuterated BSA and trimethylchlorosilane (TMCS) were purchased from MSD through Sharp & Dohme (Munich, G.F.R.).

Pyruvate was obtained from Boehringer Mannheim (Tutzing, G.F.R.), α-ketoglutaric acid and oxaloacetic 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.

The quinoxalinols were prepared by the reaction of keto acids and *o*-phenylenediamine in methanol–acetic acid at 100° as described by Nielsen[10].

Silylation of 1–2-mg samples was carried out in 100 μl of pyridine with either 100 μl of BSTFA or with 100 μl of d_9-BSA plus 50 μl of d_9-TMCS at 70° for 30 min. Volumes of 1–2 μl of these samples were used for GC. The conditions of GC are given in the legend to Fig. 2.

The methylene units (MU) were determined with linear temperature programming as described by Dalgliesh *et al.*[11] and Horning *et al.*[12].

RESULTS

With a 5.6-fold excess of *o*-phenylenediamine and starting with 1.1 g of α-ketobutyric acid, the molar yield of ethylquinoxalinol was found to be 84%.

We, like Hoffman *et al.*[7], were unable to synthesize carboxymethylquinoxalinol from oxaloacetic acid, methylquinoxalinol being the only detectable product.

A sample of underivatized carboxyethylquinoxalinol, after storage for 1 year at room temperature, showed no sign of decarboxylation as evidenced by the absence in the chromatogram of O-TMS-ethylquinoxalinol in a proportion greater than 1:2000.

Reports from the literature[6,13,14] and our as yet unpublished GC–MS and GC–IR data suggest a reaction sequence for the formation of O-TMS-quinoxalinols as indicated in Fig. 1.

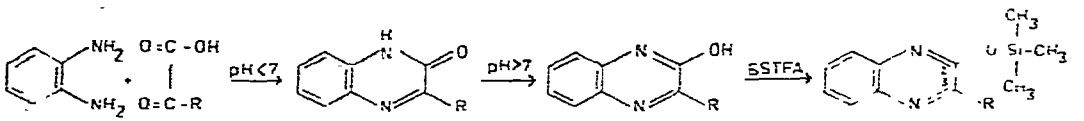

Fig. 1. Pathway of formation of O-TMS-quinoxalinol derivatives of α-keto acids. This scheme explains previous discrepancies in IR and MS data[6,13,14].

The pattern of separation of 11 quinoxalinol-TMS ethers on OV-1 is shown in Fig. 2. Besides oxaloacetic acid, of the more interesting α-keto acids only imidazolepyruvic, *p*-hydroxyphenylpyruvic and glyoxylic acids are missing in this study. α-Ketovaleric and α-ketooctanoic acids are included in order to serve as internal standards in quantitative studies. The derivatives of all keto acids show single, symmetrical peaks and the separations on OV-1 are complete for most derivatives. The

The chromatographic behaviour of the quinoxalinol-TMS ethers is given in terms of methylene units in Table I. With regard to polarity, as indicated in Table I by the Δ $MU_{OV\text{-}17-OV\text{-}1}$ values, three classes can be distinguished: aliphatic substituents (Δ MU = 1.24–1.45), substituents with a carboxyl group (derived from dicarboxylic α-keto acids) (Δ MU = 1.80–1.86) and the highly polar sulphur-containing and aromatic substituents (Δ MU = 2.35–2.61).

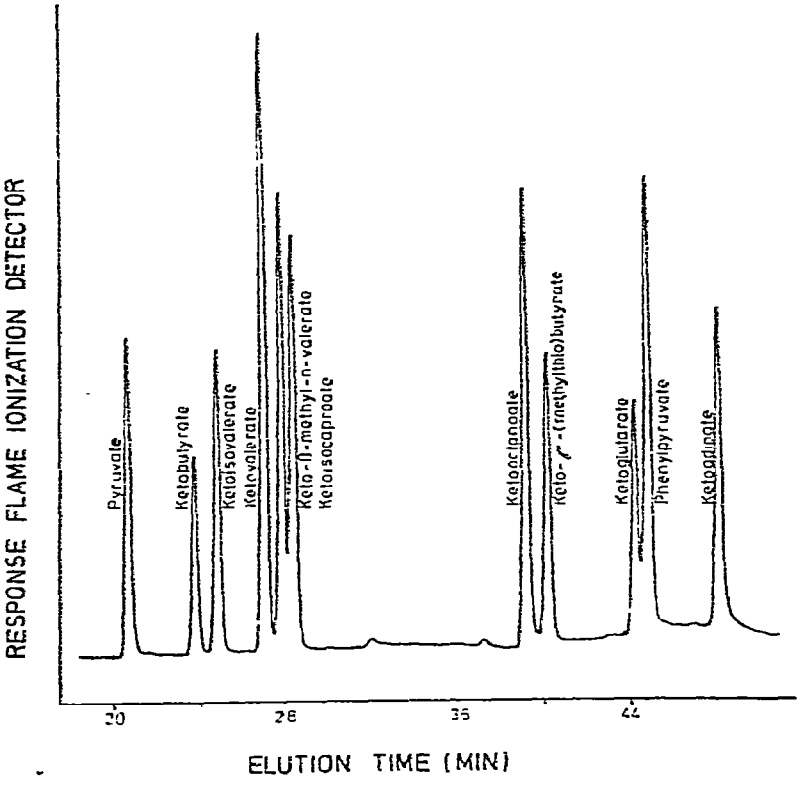

Fig. 2. Chromatographic separation of O-TMS-quinoxalinols on the silicone phase OV-1 (3% on 100–120-mesh Gas-Chrom Q). A linear temperature programme from 50 to 180° at 2°/min was applied. Nitrogen was used as the carrier gas at 60 ml/min.

TABLE I

MU VALUES OF TRIMETHYLSILYLQUINOXALIN-2-OLS SUBSTITUTED IN POSITION 3
Conditions of operation as detailed in legend to Fig. 2.

Substituent in position 3	*Parent α-keto acid*	*OV-1*	*OV-17*	*Dexsil 300*	$\Delta MU_{OV-17-OV-1}$
Methyl	Pyruvic	15.13	16.57	15.41	1.44
Ethyl	Ketobutyric	15.78	17.17	16.06	1.39
Isopropyl	Ketoisovaleric	16.00	17.24	16.20	1.24
Propyl	Ketovaleric	16.46	17.84	16.73	1.38
Isobutyl (1)	Keto-β-methyl-*n*-valeric	16.65	17.90	16.81	1.25
Isobutyl (2)	Ketoisocaproic	16.77	18.07	16.95	1.30
Hexyl	Ketooctanoic	19.23	20.68	19.50	1.45
2-(Methylthio)ethyl	Keto-γ-(methylthio)butyric	19.48	21.83	20.11	2.35
Carboxyethyl	Ketoglutaric	20.48	22.28	20.76	1.80
Benzyl	Phenylpyruvic	20.63	23.24	21.26	2.61
Carboxypropyl	Ketoadipic	21.48	23.34	21.81	1.86

The chromatographic isotope effect of the perdeutero-TMS derivatives is shown in Table II. On OV-1, compounds with a single d_9-TMS group elute by 0.053 ± 0.006 MU earlier than the unlabelled molecules. On this phase, the effect is exactly additive for compounds with two d_9-TMS groups. On OV-17, a larger isotope effect is observed for a single d_9-TMS group: 0.076 ± 0.004. Additivity seems not to be as perfect as on OV-1.

On OV-1, 0.053 MU corresponds to an elution time of 14 sec. The standard deviation of the methylene unit of a given peak measured repeatedly on a few successive days is about ± 0.006, corresponding to 1.5 sec.

The $\Delta MU_{OV-17-OV-1}$ values for the d_9-TMS compounds are smaller by 0.027 ± 0.010 compared with the corresponding data for the unlabelled compounds. The highest difference (0.047) was found for carboxyethylquinoxalinol the smallest (0.012) for hexylquinoxalinol.

TABLE II

CHROMATOGRAPHIC ISOTOPE EFFECT OF d_9-TMS-QUINOXALIN-2-OLS SUBSTITUTED IN POSITION 3

The numbers indicate the fraction of a methylene unit (MU) by which the species elute earlier than the unlabelled ones; 0.05 MU correspond to about 14 sec. N.D. = not determined.

Substituent in position 3	*OV-1*	*OV-17*
Methyl	0.048	0.075
Ethyl	0.052	0.079
Isopropyl	0.057	N.D.
Propyl	0.041	0.072
Isobutyl (1)	0.053	0.079
Isobutyl (2)	0.057	N.D.
Hexyl	0.059	0.071
2-(Methylthio)ethyl	0.059	0.082
Carboxyethyl	0.108	0.155
Benzyl	0.053	0.073
Carboxypropyl	0.103	0.133

DISCUSSION

Hinsberg, who bears the distinction of having detected in 1883 the reaction of aromatic diamines with *o*-dicarbonyl compounds, remarked in 1887 (ref. 15) (translation), "the quinoxaline reaction... eventually will prove advantageous also for the analysis of α-keto acids". We share his optimism: of the ten or so methods at present available for the derivatization of α-keto acids, some yield multiple derivatives or show multiple peaks due to *syn–anti* isomerism[5]. The methoxime and benzoxime TMS esters, in addition, show chromatographic losses in the lower nanomole range. The quinoxalinol-TMS derivatives, in contrast, give perfect qualitative and quantitative chromatographic behaviour down to at least 100 pmoles[16]. There is no possibility of obtaining multiple derivatives or isomers (Fig. 1).

The mass spectra[14,16], because of common, specific and abundant fragments, allow quantitative single-ion detection of keto acids, rarely occurring keto acids being used as internal standards[16]. The mass spectra of other derivatives[18,19] do not offer such a possibility, at least not at higher mass numbers.

The difficulties encountered in the quantitation of oxaloacetic acid by chromatographic means pertain to all derivatives (see, for example, ref. 20). Oxaloacetic acid behaves as a β-keto acid, thus decarboxylating very easily to pyruvic acid. Interestingly, Mowbray and Ottaway[13] observed decarboxylation on prolonged storage of carboxymethylquinoxalinol itself, which they were able to obtain from oxaloacetic acid. As shown above, the next higher homologues, ketoglutaric acid and carboxyethylquinoxalinol, do not pose such problems.

Chromatographic isotope effects have been observed earlier in gas and liquid chromatography[17,21]. As indicated by our results, deuterium seems to diminish the interaction of the sample molecules with the unlabelled liquid phases, thus leading to reduced elution times and smaller $\Delta MU_{OV\text{-}17-OV\text{-}1}$ values. More thorough physical chemical explanations are not at our disposal.

We do not know whether the additivity of the chromatographic isotope effect described above extends to more than two d_9-TMS groups per molecule. Nevertheless, at least in principle, this phenomenon suggests the possibility of determining the number of reactive groups in a molecule without recourse to mass spectrometry.

ACKNOWLEDGEMENT

One of us (U.L.) acknowledges the support by the Deutsche Forschungsgemeinschaft, Bad Godesberg, G.F.R. (SFB 33).

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