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O-TRIMETHYLSILYLQUINOXALINOL DERIVATIVES OF AROMATIC α-KETO ACIDS

MASS SPECTRA AND QUANTITATIVE GAS CHROMATOGRAPHY

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SUMMARY

As an extension of earlier work on aliphatic α-keto acids, a method is described for the quantitative gas chromatographic determination of urinary aromatic α-keto acids. The keto acids are derivatized with *o*-phenylenediamine to yield the quinoxalinols. These compounds are chromatographed after trimethylsilylation.

The aromatic keto acids are stabilized by sodium dithionite (4 mg/ml urine) and storage below 0°. The final derivatives are stable for weeks at room temperature.

Low resolution mass spectra are reported. The fragmentation mechanisms are elucidated by analysis of O-trimethylsilyl-(TMS)-quinoxalinols, O-(TMS-d_9)-quinoxalinols and O-TMS-6(7)-chloroquinoxalinols.

INTRODUCTION

α-Keto acids are of considerable biomedical interest, as a number of hereditary metabolic defects are known to disturb the metabolism of these compounds [1] . They are also a challenge to the analytical chemist because of dimerization, enolization and decarboxylation.

In a series of papers [2—4] we showed that aliphatic α-keto acids are conveniently determined by gas chromatography (GC) of the O-trimethylsilyl-(TMS)quinoxalinols, either with conventional flame ionization detection, or the most sensitive selective ion monitoring [5] , in a gas chromatograph—mass spectrometer combination. In the present paper we discuss our experience with the quantitative GC determination of urinary β-phenylpyruvic acid (PPA) and *p*-hydroxy-β-phenylpyruvic acid (PHPPA) using the same analytical principle. In addition, the mass spectra of the O-TMS-quinoxalinols are presented.

Some of the results we have reported at two recent conferences [6, 7].

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

While our own work was in progress a report appeared [8] which documented the value of another bifunctional reagent, naphthalene-2,3-diamine, in the analysis of PPA by high-speed liquid chromatography.

MATERIALS AND METHODS

The gas chromatograph and other equipment and the sources of most chemicals have been described in a previous publication [3]. The sodium salt of PPA was obtained from Fluka (Buchs, Switzerland). Free PHPPA and 4-chlorophenylene-1,2-diamine [9] were from EGA-Chemie (Steinheim, G.F.R.). The PPA sodium salt was stored at 5° and PHPPA at −20° . Both compounds were used without further purification. Sodium dithionite ($Na_2S_2O_4$) was from Merck (Darmstadt, G.F.R.) and L-phenylalanine (pharmaceutical grade) from Serva (Heidelberg, G.F.R.).

Our GC method, as well as the determination of molar response factors (using peak heights), has also been described in detail in the same publication [3]. We have standardized two more steps in the procedure since then. Extraction of the quinoxalinols is performed after the addition of exactly 1.5 g ammonium sulphate to the reaction mixture. After evaporation of the chloroform extract the quinoxalinols are taken up with exactly 1 ml ether after shaking for 1 min. (This modification somewhat improves the precision of the ketoglutaric acid assay.)

We have meanwhile abandoned the use of free α-ketocaprylic acid (Sigma) as a second internal standard because of problems encountered with the long-term stability of this hygroscopic acid. For good results the sodium salt must be prepared also for this internal standard.

In our standard procedure for aromatic α-keto acids, $Na_2S_2O_4$ (4 mg/ml) is added to the urine, immediately after voiding, instead of toluene and acetic acid. All other steps, as well as the preparation of O-TMS-6(7)-chloroquinoxalinols [9], were the same as described before [3]. The deuterated derivatives were prepared as described previously [2].

Urinary creatinine was measured using the picric acid—NaOH kit supplied by Boehringer (Mannheim, G.F.R.). Quality control of these measurements was carried out with Monitrol II (Merz & Dade, München, G.F.R.).

Low resolution mass spectra of O-TMS-benzylquinoxalinol, O-(TMS-d_9)-benzylquinoxalinol and O-$(TMS)_2$-*p*-hydroxybenzylquinoxalinol were obtained on a Finnigan Model 3000 quadrupole mass spectrometer. Samples were introduced via a Varian Model 1400 gas chromatograph equipped with a 3% Dexsil 300 GC column (180 cm) and a glass jet separator kept at 250°. The injection port temperature was 200°. GC was performed isothermally at 230° . The electron energy was 70 eV and the ion energy between 4 and 6 V.

Low resolution mass spectra of the other derivatives were obtained on a Varian Model CH 7 magnetic mass spectrometer. Samples were introduced via a Varian Model 1700 gas chromatograph equipped with a 3% SE-30 column (150 cm) and a Biemann-Watson separator at 250°. The injection port was kept at 275°. GC was performed isothermally at 240°. The electron energy was 70 eV, and the accelerating voltage was 3 kV. All mass spectra were recorded on an oscillograph and evaluated manually.

RESULTS

Mass spectra

The quadrupole mass spectra of the O-TMS-quinoxalinols derived from PPA and PHPPA are presented in Fig. 1. For the sake of brevity the spectra of perdeutero and chloro derivatives are not shown. They may be obtained on request. In the quadrupole mass spectrometer *m/e* 73 (TMS) is the base peak. In contrast, the spectra obtained with the magnetic instrument have the molecular ion as the base peak. These spectra of O-TMS-quinoxalinols with an aromatic substituent in position 3 have few details only (cf. refs. 4 and 9).

The PPA derivative loses a methyl radical from the TMS group. There is also a distinctive (M—17) ion in the normal as well as in the chloro derivative. This ion has 19% relative abundance (R.A.) and is composed of two even electron species. One has lost two hydrogens, most probably from the phenyl ring (M—20 ion in the deuterated derivative, with 14% R.A.). The other has lost one hydrogen from the phenyl ring and one from the TMS group (M—21 ion in the deuterated derivative, with 7% R.A.). Migration of a TMS-related methylene group to the phenyl ring may have occurred in the latter ion.

The ion *m/e* 217 carries nine TMS-derived hydrogens as do all TMS-quinoxalinols which are not able to perform a McLafferty type rearrangement [4]. This ion can be used for single ion monitoring [5] of PPA using α-ketovaleric acid or α-ketocaprylic acid as internal standard [3, 4].

The ion *m/e* 219 carries the quinoxalinol moiety but no TMS-derived hydrogen. It is formed by the loss of $(CH_3)_2SiO$ from the M—15 ion. Most interesting is the ion *m/e* 146 (isotope peak at *m/e* 146.5). In the deutero and chloro derivatives it shows up as *m/e* 149 (isotope peak at *m/e* 149.5) and

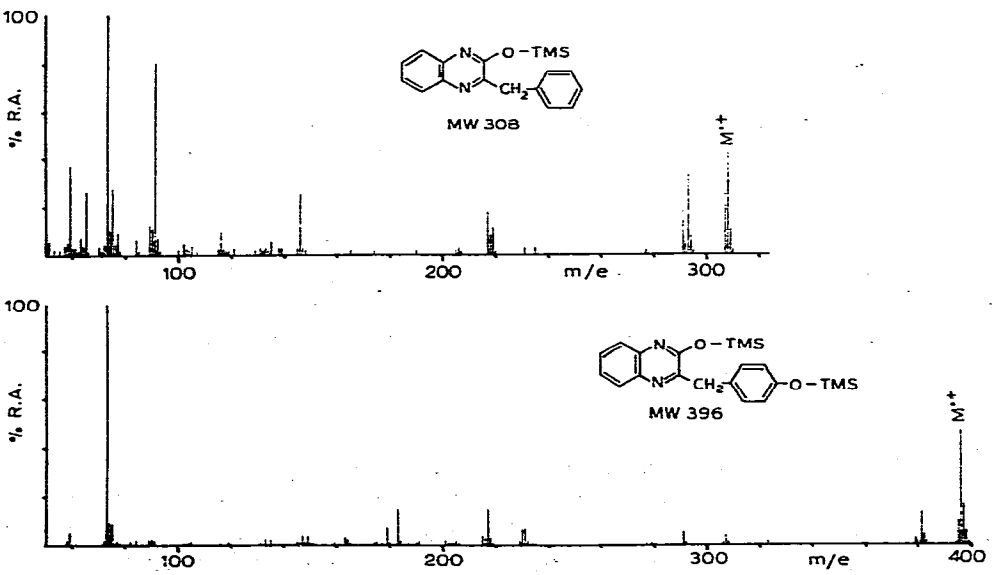

Fig. 1. Quadrupole mass spectra (70 eV) of O-TMS-quinoxalinols derived from β-phenylpyruvic acid (top) and from *p*-hydroxy-β-phenylpyruvic acid (bottom). The spectra of the deutero and chloro derivatives may be obtained on request.

m/e 163 (isotope peak at *m/e* 163.5 and 164), respectively. It thus represents an $(M-15-1)^{2+}$ ion where the single hydrogen most probably is abstracted from the phenyl ring. The ion *m/e* 135 carries six TMS-related hydrogens but no chlorine in the respective derivative. The structure $[C_6H_5-Si(CH_3)_2]^+$ is suggested. The ion *m/e* 116 carries neither a TMS-related hydrogen nor a chlorine in the respective derivative. Its origin is uncertain.

The most prominent ion in the lower mass range, expectedly, is the tropylium ion *m/e* 91. The trivial TMS-related ions in the lowest mass range need not be discussed here [4].

In the mass spectrum of the PHPPA derivative (Fig. 1, lower mass spectrum) M—15 and M—17 are found as described above for the PPA derivative. Three major fragments in the upper mass range of the PHPPA derivative contain six TMS-related hydrogens and the chlorine from the quinoxaline moiety, respectively (*m/e* 291, 307, 323). The ion *m/e* 291 is therefore formed by loss of trimethylsilanol from the $(M-15)^+$ ion, a loss similarly observed in sterol-TMS ethers [10]. The ion *m/e* 217 is observed also in the PHPPA derivative (see above).

The ion *m/e* 183 (15% R.A.) is an $(M-15-15)^{2+}$ ion as evidenced by an isotope peak at *m/e* 183.5, a shift of 6 a.m.u. in the deuterated derivative and a shift of 17 a.m.u. in the chlorinated derivative. Most probably each silicon atom carries one of each charge. The ion $(M-30)^+$ has only a very low intensity (less than 0.3% R.A.). Intense $(M-30)^{2+}$ ions have also been observed by VandenHeuvel et al. [11] in di-TMS derivatives of dihydroxydiphenyls and related compounds. The situation with the PHPPA derivative is evidently analogous.

All ions below *m/e* 180 are free of chlorine in the respective derivative. Most prominent in this range is ion *m/e* 179 with nine TMS-related hydrogens. Its composition is therefore $[CH_2{\cdots}C_6H_4{\cdots}O{\cdots}TMS]^+$.

Gas chromatographic properties

In Table I we present the methylene units (MU) [12] of the PPA and PHPPA derivatives for three commonly used phases. The O-TMS-6(7)-chloroquinoxalinols yield symmetrical peaks on OV-17 only. It was not possible to deduce which structural isomer (6-chloro or 7-chloro) it is that elutes first on the less-polar phases.

Interestingly, O-TMS-3-(*p*-hydroxybenzyl)quinoxalin-2-ol eluted with two peaks on OV-101 (not shown). On OV-1, as well as on OV-17, this derivative yields perfectly symmetrical peaks, as does the respective derivative of PPA. Due to the presence of a benzyl moiety all derivatives are highly polar, as evidenced by the large ΔMU values [2].

Quantitative parameters

One of us has introduced $Na_2S_2O_4$ as a protective substance in TLC and GC assays of PPA [13]. Our experience with this reducing substance in the GC assay of aromatic α-keto acids is shown in Table II. At biomedically relevant concentrations these acids are stable for at least four days when urine is kept (and mailed) frozen in the presence of $Na_2S_2O_4$. Clearly, PHPPA is more labile than PPA.

The molar response factors on OV-1 with α-ketovaleric acid as internal

TABLE I

MU VALUES OF O-TMS-QUINOXALIN-2-OLS SUBSTITUTED IN POSITION 3 AND 6 OR 7

Temperature program was run at 2°/min starting at 50°.

Substituent in position 3	Substituent in position 6 or 7	Parent α-keto acid	MU values OV-1	OV-17	Dexsil 300	ΔMU (OV-17)—(OV-1)
Benzyl	H	PPA	20.63*	23.31	21.32	2.68
Benzyl	Cl	PPA	22.49**	25.01	23.31, 23.41	2.52
p-Hydroxybenzyl	H	PHPPA	24.06	26.52	24.67	2.46
p-Hydroxybenzyl	Cl	PHPPA	26.0**	28.26	n.d.	2.3

*Taken from ref. 2.
**Peak broadened.
n.d. = Not determined.

TABLE II

STABILITY OF AROMATIC KETO ACIDS IN URINE

4 mg sodium dithionite were added per ml. Numbers denote % of initial concentration.

Days storage	Room temperature		Refrigerator		Freezer (—20°)	
	PPA	PHPPA	PPA	PHPPA	PPA	PHPPA
0	100	100	100	100	100	100
1	102	86	107	113	119	134
2	92	79	124	114	119	100
3	68	7	111	121	112	121
4	71	7	103	86	111	105
Conc.	1.7 m*M*		1.5 m*M*		1.5 m*M*	

standard [3] were 0.769 ± 0.057 (n = 11) for PPA and 0.769 ± 0.062 (n = 5) for PHPPA. The data obtained for estimation of these response factors yielded mean coefficients of correlation [3] of r = 0.997 (0.989—0.999) for PPA and r = 0.997 (0.996—0.999) for PHPPA.

In Table III it is demonstrated that the final O-TMS-quinoxalinols are stable at room temperature for at least four weeks. It is expected that the stability would be found to be much greater if the vials are opened only once.

Finally, in Table IV parameters of quality control [14] for our assay are given. The specificity of the GC assay is determined by the size of contaminating peaks in the chromatographic position of PPA and PHPPA. In normal controls on OV-1 it never corresponds to more than 30 μM. Specificity for PPA is still better by a factor of 3 on OV-17 (not shown). As also shown in Table IV, there is no major influence of the initial concentration of the keto acid on the accuracy and precision of the assay.

Application of the procedure to the study of phenylketonuria (PKU)

We have applied our method to the analysis of urinary aromatic acids in

TABLE III

STABILITY OF O-TMS-QUINOXALINOLS FROM URINARY AROMATIC KETO ACIDS

Concentration of keto acids in urine was 1.5 m*M*. The sample was stored at room temperature. Until day 66 the sample vial was opened 11 times. Numbers denote % of initial peak height ratios of compound vs. internal standard.

Days	PPA	PHPPA
1	100	100
3	102	111
4	99	103
14	98	102
20	95	96
26	97	94
48	84	102
66	90	91

TABLE IV

RELIABILITY OF THE O-TMS-QUINOXALINOL METHOD FOR QUANTITATIVE DETERMINATION OF URINARY AROMATIC KETO ACIDS

Determinations were made on OV-1 under the chromatographic conditions described in ref. 3 and the legend to Fig. 2.

	PPA	PHPPA
Specificity	Very high at concentrations $> 30\ \mu M$	Very high at concentrations $> 30\ \mu M$
Accuracy	99.7% at 280 μM ($n = 7$) 98.8% at 710 μM ($n = 6$)	98.4% at 290 μM ($n = 7$) 92.6% at 760 μM ($n = 6$)
Precision (S.D./mean)	4.1% at 280 μM ($n = 7$) 2.8% at 710 μM ($n = 6$)	7.8% at 290 μM ($n = 7$) 8.8% at 760 μM ($n = 6$)

mentally retarded patients with PKU who had never been treated, and who were on a normal, unrestricted diet. The first urine in the morning was sampled for analysis. Fig. 2 shows an example. In 15 patients aged 13—40 years we found PPA in a concentration of 409.9 ± 149.8 μmoles/mmole creatinine (range 104—720). PHPPA was detected in all PKU urines. The mean concentration was 16.6 ± 6.5 μmoles/mmole creatinine (range 8—30). The detailed presentation of these and other relevant data will be the subject of a forthcoming paper.

Preliminary data on four controls and three obligate heterozygotes indicate the value of urinary PPA measurement in the detection of carriers by an oral load of L-phenylalanine (100 mg/kg body weight). In the first six hours after loading, controls excreted a total of 6.52, 8.13, 4.09 and 3.70 μmoles PPA. The three parents of PKU patients excreted 16.21, 47.19 and 19.18 μmoles PPA. In this small amount of material we have already found an overlap in

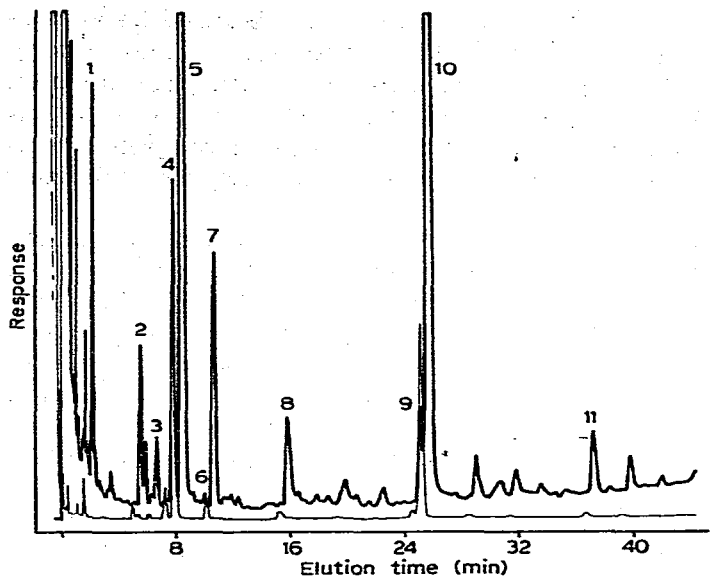

Fig. 2. Gas chromatogram of urinary acids from a 26-year-old mentally retarded male patient with untreated PKU (B. Th., Neuerkeröder Anstalten). Urine with $Na_2S_2O_4$ was received by post at ambient temperature and analysed 24 h after sampling. GC was carried out on 3% OV-1 in a glass column (180 cm long) with N_2 at 60 ml per min. Temperature program was from 70° to 160° at 2°/min. The lower trace is recorded at 10 mV recorder span, the upper one at 1 mV. The following acids are indicated in the figure (micromolar concentrations in brackets): 1 = phenylacetic acid; 2 = mandelic acid (319); 3 = pyruvic acid (91, determined on Dexsil 300 from urine treated with toluene—acetic acid [3]); 4 = *o*-hydroxyphenylacetic acid (855); 5 = phenyllactic acid (5.434); 6 = *p*-hydroxyphenylacetic acid; 7 = ketovaleric acid (internal standard); 8 = hippuric acid; 9 = ketoglutaric acid (177, determined on Dexsil 300 from urine treated with toluene—acetic acid); 10 = phenylpyruvic acid (4.912); 11 = *p*-hydroxyphenylpyruvic acid (166). Creatinine concentration was 10.5 m*M*. 500 µl urine were taken for derivatization. An equivalent of 5 µl urine was injected for analysis. *p*-Hydroxyphenyllactic acid remains in the aqueous phase on extraction with chloroform (unpublished results).

the excretion *o*-hydroxyphenylacetic acid (OHPAA). Further work on this topic is in progress [15].

We apply our method also to monitoring children with PKU who are dietetically treated at the Pediatric Department, University of Göttingen. Due to the sensitivity of the PPA assay it is possible to distinguish between acute and chronic derangements of dietary control [16].

DISCUSSION

Hemmerle [17] clearly demonstrated the great lability of PPA, which on contact with air is decomposed to benzaldehyde and oxalic acid. Also, spontaneous decarboxylation to phenylacetic acid is observed on extraction of free PPA [18].

The most successful procedure to prevent loss of PPA on derivatization for GC has been the addition of excess quantities of ascorbic acid to the urine, as

described by Wadman et al. [19]. This method could not be used here, as *o*-phenylenediamine reacts with ascorbic acid [20]. Because of smell and toxicity we did not try the sodium hydrosulphide procedure of Nielsen [21] but used sodium dithionite as a protective substance [13] instead. This procedure is fully evaluated in the present paper. While our work was in progress 2-mercaptoethanol was also tried successfully by Hayashi et al. [8] for stabilizing PPA.

Hinsberg [22], in 1887, suggested the use of *o*-phenylenediamine for the determination of α-keto acids. Wieland first reported such a procedure in 1949 [23]. We have shown in the present and three previous [2—4] papers that the GC determination of α-keto acids, both aliphatic and aromatic, can be performed very reliably after formation of the stable O-TMS-quinoxalinols with *o*-phenylenediamine and a silylating agent. In a carefully documented study Chalmers and Watts [24] found in 34 PKU patients almost the same urinary levels of PPA and PHPPA as we did. The concentrations (given as μmoles/mmole creatinine) they found were, for PPA 517.0 ± 182.7 (range 282—916), and for PHPPA 22.1 ± 26.7 (range 4—35). The correlation between the levels of both acids is +0.19 ($p>5\%$) in the study of Chalmers and Watts [24], and +0.88 ($p<0.1\%$) for our data. Evidently, due to stabilization of the urinary acids and to the very high stability of the final GC derivatives, our assay procedure for aromatic α-keto acids and especially for PHPPA is better controlled.

In the field of PKU research due credit can now be given to the importance of PPA as the major abnormal metabolite of phenylalanine. In the previous literature this view had been somewhat distorted because OHPPA was much more easily quantitated than PPA. Thus, without obvious biochemical reason, OHPPA was taken as the most important metabolite to diagnose heterozygotes [25—27; see also ref. 28] and to monitor dietary treatment [29]. Our own data [15, 16] indicate that a fresh look at these problems should be taken. Progress in human biochemical genetics is not only a consequence of the detection of new metabolites but also of careful quantitative analysis of known compounds [30].

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