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QUANTITATIVE ANALYSIS OF β-PHENYLPYRUVIC ACID BY SINGLE ION MONITORING

EVALUATION OF ISOMERIC INTERNAL STANDARDS*

U. LANGENBECK, A. MENCH-HOINOWSKI and I. RØD-URBAN**

Institute of Human Genetics, University of Göttingen, D-3400 Göttingen (G.F.R.)

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SUMMARY

Quantitative single ion monitoring of β-phenylpyruvic acid at high sensitivity is possible after derivatization first with *o*-phenylenediamine and then with a silylating reagent. The resulting O-trimethyl-silyl-quinoxalinol (O-TMS-Q) has previously been shown to be highly stable during storage and on chromatography. As an internal standard the isomeric *o*-methylphenylglyoxylic (*o*-toluylformic) acid is introduced. The mass spectra of both O-TMS-Q's are characterized by abundant $[M]^+$ at *m/e* 308. The concept of "class specific metabolic profiling" is discussed in relation to quantitative gas chromatography—mass spectrometry detection of aliphatic and aromatic α-ketoacids.

INTRODUCTION

Gas chromatographic (GC) and mass spectrometric (MS) methods in combination with electronic data acquisition and handling (Comp) allow very efficient detection of primary and secondary metabolic anomalies in patients with inherited disease. Such "profiling" of human body fluids [1] has greatly contributed to our understanding of a group of diseases known as the organic acidurias [2, 3].

By definition, profiling techniques are qualitative or semiquantitative in nature, i.e., deviations of metabolite levels in the positive or negative direction are indicators of possible metabolic disease [4]. If general profile data are also analyzed quantitatively [5], erroneous conclusions may be reached.

However, GC—MS—Comp profiling techniques can yield reliable quantitative

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data if only a single class of metabolites is detected, e.g. steroids [6, 7] or ketoacids [8, 9]. In such cases, chromatographic properties and quantitative behavior are more uniform and better understood, provided also that the internal standards belong to the same chemical class. Such "class specific metabolic profiling" as we would like to call it, can be made possible either through specific purification [6, 7] or through specific detection [8, 9].

Aliphatic α-ketoacids can be specifically detected as O-TMS-Q derivatives [8, 9, 10] either with nitrogen selective detectors [11] or even more specifically with multiple ion detection [9, 12]. In this case *o*-phenylenediamine serves as a label: it imposes a characteristic fragmentation pattern on the small molecular weight keto acids by converting them to hetero-aromates [9]. With the resulting high mass fragments (*m/e* 217, 232, 245) relatively little interference from other compounds is observed.

Recently, we have extended the O-TMS-Q procedure to the quantitative GC analysis of aromatic α-ketoacids in urine [13]. In the following lines we introduce a suitable internal standard which allows β-phenylpyruvic acid (PPA) to be included in a ketoacid specific profiling of body fluids using GC—MS—Comp techniques.

MATERIALS AND METHODS

The gas chromatograph and other equipment, the source of chemicals, the derivatization of aliphatic and aromatic ketoacids and the quantitative evaluation, have been fully described in previous communications [8, 13]. An O-TMS-Q procedure is also available for ketoacids in blood [14]. In short, the procedure consists of reacting unextracted ketoacids in 2 *N* HCl for 30 min at 70° with *o*-phenylenediamine, extraction of the quinoxalinols with chloroform (the aqueous phase being saturated with $(NH_4)_2SO_4$), evaporation of the organic phase, and final derivatization for 30 min at 70° with 50 μl of pyridine and 50 μl of bis-(TMS)-trifluoro acetamide. Care is taken to condense the chloroform fully by feeding the rotary evaporator with an ethylene glycol—water (1:1, v/v) mixture at −10° [14].

The deutero-TMS-Q's were prepared as described in ref. 10.

Low resolution 70-eV mass spectra were obtained on a Varian Model CH7 magnetic mass spectrometer. Samples were introduced via a Varian Model 1700 gas chromatograph with a 150-cm 3% SE-30 column and a Biemann—Watson separator. Parameters of operation have been given previously [13].

Single ion monitoring with α-ketocaprylic acid as internal standard was performed on a Finnigan Model 3000 quadrupole mass spectrometer coupled to a Varian Model 1400 gas chromatograph. The samples were introduced via a 180 cm × 1/8 in. O.D. spiral glass column filled with Dexsil 300 on Supelcoport. The temperature program was from 180 to 220° at 2°/min. The injection port temperature was 200°, the glass-jet separator and metal transfer line were kept at 250°. Electron energy was 70 eV and ion energy 5 V.

Preparation of isomeric methylphenyl glyoxylic (toluylformic) acids

In very low yields the acids could be obtained from the corresponding methylacetophenones (EGA Chemie, Steinheim, G.F.R.) by reacting them for

6 h in icecold 1% KOH with $K_3[Fe(CN)_6]$ [15].

For preparation of larger amounts with optimal purity we started from the isomeric methylbenzoic acids. These are converted: (i) to the chloride [16]; (ii) to the cyanide [17]; and (iii) by acid hydrolysis to the free ketoacid.

All chemicals were from Riedel de Haën (Seelze, G.F.R.).

(i) Dimethylformamide (in traces) was dropped into a stirred suspension of methylbenzoic acid in thionylchloride (molar ratio 1.0:1.5) and the suspension was refluxed for 1 h. After stirring at room temperature overnight, the excess thionylchloride was removed under vacuum, using a waterpump and a 15-cm Vigreux column to facilitate separation. By distillation of the residue the acid chloride was obtained. Yield 95%. $B.p._{.10}$: 2-methylbenzoylchloride, 100—103°; 3-methylbenzoylchloride, 99—101°; 4-methylbenzoylchloride, 95°.

(ii) Anhydrous cuprous cyanide and methylbenzoylchloride in a molar ratio of 1.2:1 are thoroughly mixed and heated. The temperature is raised to 220—230° and maintained between these limits for 1.5 h. At the end of this time the crude toluylcyanide is purified by fractional distillation through a column. The distillate of the *meta* and *para* derivatives solidifies to colorless crystals. The *ortho* derivative is obtained as an oil. Yield 60—65%. $B.p._{.10}$: 2-toluylcyanide, 109—111°; m.p.: 3-toluylcyanide, 24°; 4-toluylcyanide, 46°.

(iii) Toluylformic acid is prepared by the hydrolysis of toluylcyanide with concentrated hydrochloric acid. The mixture is shaken occasionally until the cyanide is dissolved completely and is then allowed to stand at room temperature for 5 days. The clear solution is poured into water and extracted with ether. The ether is removed by distillation. The residual oil is placed in a vacuum desiccator containing phosphorus pentoxide and solid sodium hydroxide and allowed to remain there until dry. The crude acid is dissolved in hot carbon tetrachloride and cooled until crystallization is complete.

The free *ortho* acid is a high-boiling oil. Equimolar amounts of the *ortho* acid and concentrated ethanolic sodium hydroxide were heated. The resulting sodium salt was recrystallized from 70% ethanol. Yield 73—77%. M.p.: sodium salt of 2-toluylformic acid, 297. 3—297.9°; 3-toluylformic acid, 71°; 4-toluylformic acid, 95—97°. All melting points are uncorrected.

RESULTS

Mass spectra

In Fig. 1 the mass spectra of the β-phenylpyruvic acid (PPA) derivative, O-TMS-benzylquinoxalinol (BQ) (data from ref. 13), and of O-TMS-*p*-methylphenylquinoxalinol (*p*MPQ) are shown. A high similarity with regard to high abundance of the molecular ion was expected and was indeed found. The MS of *m*MPQ is almost identical to the one of *p*MPQ. The peculiarities of *o*MPQ are described below.

The spectrum of BQ has been interpreted in ref. 13. Major ions of *p*MPQ and *m*MPQ are (*m/e* of the deuterated derivative in brackets): *m/e* 235 (235), 219 (219), and 217 (226). The ion *m/e* 217 is formed by the loss of a methylphenyl radical from the molecular ion (metastable ion at *m/e* 152.8). The ion *m/e* 219 is formed by loss of $(CH_3)_2SiO$ from the [M—15] ion (metastable ion

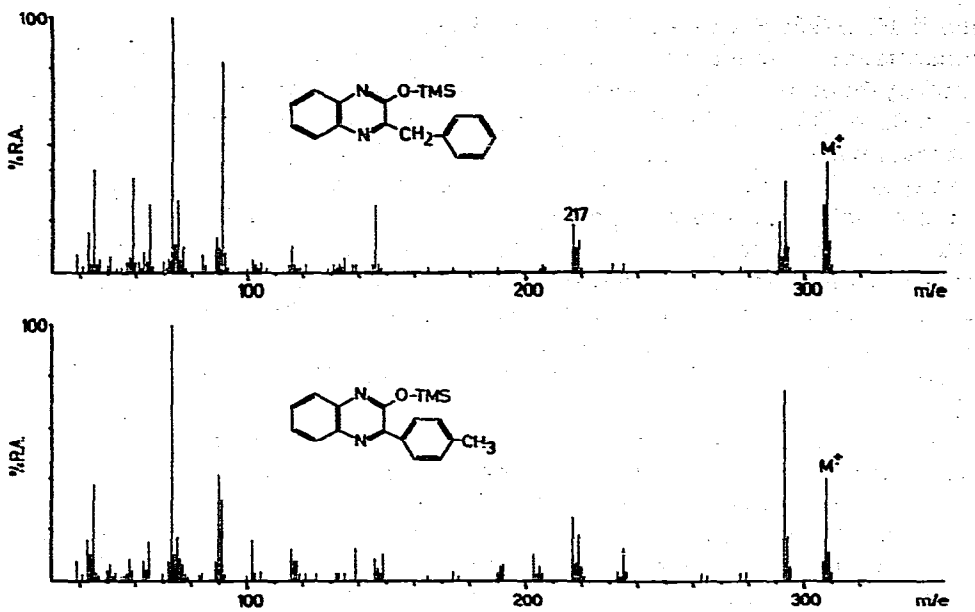

Fig. 1. 70-eV Mass spectra of O-TMS-Q's from phenylpyruvic acid (upper panel; data from ref. 13) and *p*-methylphenylglyoxylic (*p*-toluylformic) acid (lower panel). See text for interpretation.

at *m/e* 163.7). Ion *m/e* 235 is probably the result of loss of a TMS radical from the molecular ion. As evidenced by very low abundance of *m/e* 226 in the deuterated derivative, there is almost no cleavage of a methylphenyl radical from the molecular ion of *o*MPQ.

The ion *m/e* 135 (141) of BQ has been interpreted as a rearrangement product with the composition $[C_6H_5\text{-}Si(CH_3)_2]$ (ref. 13). All three isomers have a conspicious ion *m/e* 149 (155) which may be due to a similar rearrangement.

Evidence from isotope peaks shows that *p*MPQ and *m*MPQ have two doubly charged ions: *m/e* 139 (140.5) and 146.5 (149.5). The latter probably represents $[M-15]^{++}$. The first should be $[M-30]^{++}$. Its relative abundance is 3.8%, and that of $[M-30]^{+}$ is 1.1%. Ion *m/e* 139 (140.5) is practically absent from the MS of *o*MPQ.

The tropylium ion, *m/e* 91, is relatively low in abundance in all three isomers. The ions *m/e* 90 (90) and 102 (102) are similarly found in O-TMS-Q's with an aliphatic side chain [9].

Gas chromatographic properties

In Table I methylene units (MU) [18] on two silicone phases are given for the O-TMS-Q derivatives of PPA, the three isomeric methylphenylglyoxylic acids and phenylglyoxylic acid (PGA), respectively. O-TMS-phenylquinoxalinol (from PGA) and the PPA derivative have nearly identical elution times on OV-1 as well as on OV-17. There is a striking chromatographic *ortho*-effect in the elution time of the MPQ's: *o*MPQ comes out even earlier than the non-

methylated compound. The trend in ΔMU values (MU_{OV-17}—MU_{OV-1}) as shown in the table is not consistently found with different OV-1 samples. On OV-1 all derivatives yield perfectly symmetrical peaks. Multiple peaks do not occur.

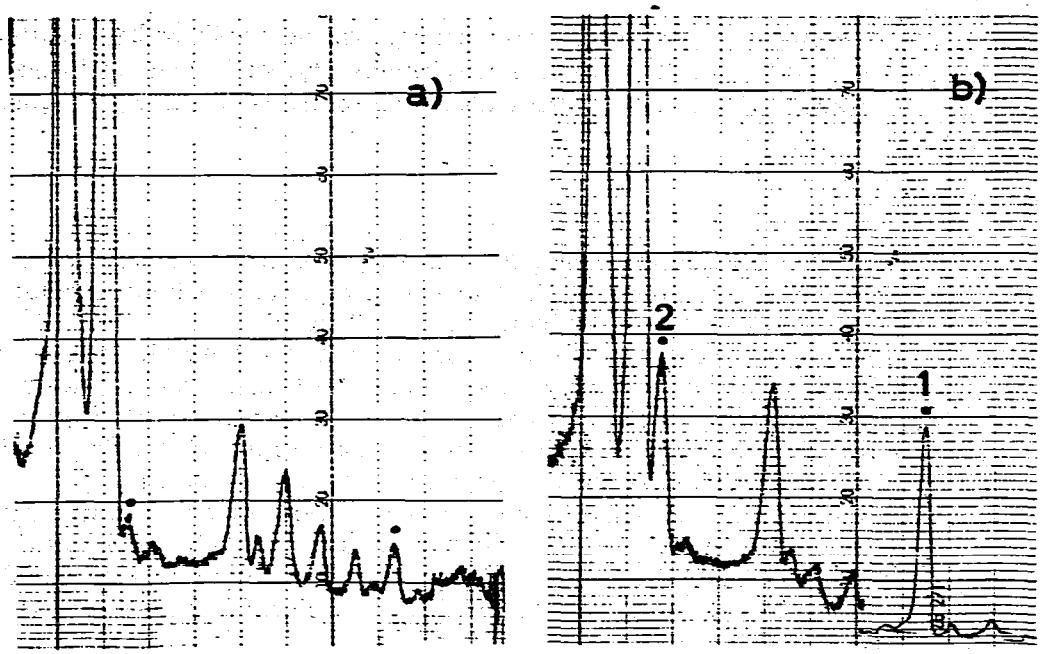

Fig. 2. Single ion monitoring of phenylpyruvic acid (2) in cerebrospinal fluid with α-ketocaprylic acid (1) as internal standard. O-TMS-Q procedure. The quadrupole mass spectrometer was focused on *m/e* 217. (a) 1 ml CSF was processed without added ketoacids. (b) To 1 ml CSF 5.3 nmoles of both ketoacids were added. Final sample volume 40 μl. 1 μl was used for GC—MS.

TABLE I

METHYLENE UNITS OF O-TMS-Q'S FROM AROMATIC α-KETOACIDS

Separation was performed with a Hewlett-Packard Model 7611 A gas chromatograph with 180 × 3 mm I.D. U-shaped glass columns. OV-1 and OV-17 both on 100—120 mesh Supelcoport. Temperature program from 60° to 180° at 2°/min. Carrier gas was nitrogen at 60 ml/min.

O-TMS-Q	OV-1	OV-17	ΔMU
o-Methylphenyl-	20.25	22.94	2.69
Benzyl-	20.63	23.31	2.68
Phenyl-	20.70	23.40	2.70
m-Methylphenyl-	21.66	24.38	2.72
p-Methylphenyl-	21.85	24.61	2.76

Single ion monitoring

Our earlier application of this technique with α-ketocaprylic acid as internal standard is shown in Fig. 2. Although partial overlap is observed between BQ and a peak of unknown identity, very high sensitivity is already obtained. With this method we showed in an untreated female patient with phenylketonuria that only traces of PPA (about 1 μmole/l) were present in her lumbar cerebrospinal fluid [19].

It is evident from Fig. 1 that greater sensitivity will be obtained by monitoring fragment *m/e* 308 or 293 with a methylphenylglyoxylic acid as internal standard. Monitoring these high masses also will improve accuracy of quantitative data.

DISCUSSION

To our knowledge, Narasimhachari [20] was the first to use isomeric internal standards for single ion monitoring. In the present paper we have evaluated this principle for the quantitative determination of PPA.

Because of its close proximity in the chromatogram *o*-methylphenylglyoxylic acid appears to be suited best as an internal standard for single ion monitoring of PPA. The stability of the derivatives is very high: down to 5 pmoles per injection we found no differential chromatographic loss of the PPA derivative when nitrogen-selective detection was used (unpublished data).

Our earlier data [8, 9] and the results of this report reveal that quantitative, α-ketoacid-specific profiling can be performed with GC—MS multiple ion detection of O-TMS-Q's on only four channels, namely *m/e* 217, 232, 245 and 308. Internal standards should be α-keto-*n*-valeric acid and *o*-methylphenylglyoxylic acid.

Such profiiing studies eventually will prove helpful in interpretation of secondary metabolic derangements in phenylketonuria [21] as well as in the assessment of animal models for that disease [22, 23]. Our present studies are concerned with these topics.

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NOTE ADDED IN PROOF

Since this paper went to press we have established a GC—MS method for quantitative determination of PPA in the blood of patients with phenylketonuria. Dual ion monitoring at *m/e* 293 and 308 is performed with *ortho*-methylphenylglyoxylic acid as internal standard [24].

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