Journal of Chromatography, 164 (1979) 217—221
Biomedical Applications

CHROMBIO. 390

Note

Gas chromatography of urinary N-phenylacetylglutamine

J.P. KAMERLING, M. BROUWER, D. KETTING and S.K. WADMAN

University Children's Hospital "Het Wilhelmina Kinderziekenhuis", Nieuwe Gracht 137, Utrecht (The Netherlands)

(Received April 9th, 1979)

N-Phenylacetylglutamine (PAG) is a normal constituent of human urine [1]. In untreated phenylketonuria (PKU) its excretion is greatly increased, reflecting secondary endogenous metabolism of phenylalanine [2, 3]. Patients with malabsorptive disease may also excrete large amounts of N-phenylacetylglutamine as a result of excessive bacterial metabolism of unabsorbed phenylalanine in the intestinal lumen [4].

When screening for heritable organic acidurias in our laboratory, the acidified urine samples are extracted with ethyl acetate and the organic acids so obtained are analysed as the corresponding trimethylsilyl (Me_3Si) derivatives by gas chromatography (GC) [5]. For PKU patients and patients with malabsorptive disease, a peak with a relatively high retention time is observed in the chromatograms, which turned out to be a non-trimethylsilylated thermal degradation product of PAG.

EXPERIMENTAL

Synthetic N-phenylacetylglutamine was a gift of Dr. R.J. Kleipool (CIVO-TNO, Zeist, The Netherlands). N-Acetylglutamine was obtained from Serva (Heidelberg, G.F.R.).

For the extraction of organic acids, 5 ml of urine were mixed with 5 ml of a saturated NaCl solution and 0.5 mg of 2-phenylbutyric acid (internal standard). The solution was acidified to pH 1—2 with concentrated HCl and, after the addition of 10 mg of vitamin C (antioxidant), extracted twice with 20 ml of ethyl acetate. The combined ethyl acetate phase was dried over anhydrous Na_2SO_4 and then evaporated to dryness under reduced pressure at 40°.

Trimethylsilylation of compounds was performed with 0.5 ml of N,O-bis-(trimethylsilyl)acetamide (BSA) in chloroform (1:3, v/v) at 37° for 30 min.

For the preparation of methyl esters of synthetic N-acyl amino acids, the substances were dissolved in 1 ml of absolute methanol and esterified with diazomethane in ether for 5 min at room temperature.

Gas chromatography (GC) was carried out on a Varian Aerograph 3700 instrument equipped with a dual flame ionization detector and glass columns (8 ft. × 1/8 in.) packed with 5% GESE-52 on Chromosorb W AW DMCS, 100—120 mesh (HP). The column oven temperature was held at 67° for 10 min followed by an increase of 2°/min up to 220° and finally 15 min at 220°. The gas flow-rate for nitrogen was 30 ml/min, the injection port temperature 210° and the detector temperature 310°.

Mass spectra at 70 eV were recorded on a Jeol JGC-20 KP/JMS-D100/W-JMA combination at an ion-source temperature of 150°, an accelerating voltage of 3 kV and an ionizing current of 300 μA. Both the gas chromatograph and direct inlet were used. For routine analysis of Me_3Si derivatives, the GC conditions were the same as described above.

RESULTS AND DISCUSSION

In Fig. 1 a gas chromatogram of the urinary organic acids (Me_3Si derivatives) of a patient with phenylketonuria, having an unknown peak with t_R = 83.72 min, is presented. The product responsible for this peak could be removed from the ethyl acetate extract by anion-exchange chromatography on 100–200 mesh Dowex 2-X8 (OH^-). When the urine was acidified to pH≥4, the compound could not be extracted. These observations suggested the presence of a carboxyl function in the original substance. Alkaline hydrolysis of the urine (after removal of phenylpyruvic acid with dinitrophenylhydrazine), followed by acidification and subsequent ethyl acetate extraction showed the accumulation of phenylacetic acid, whereas the unknown was no longer detected.

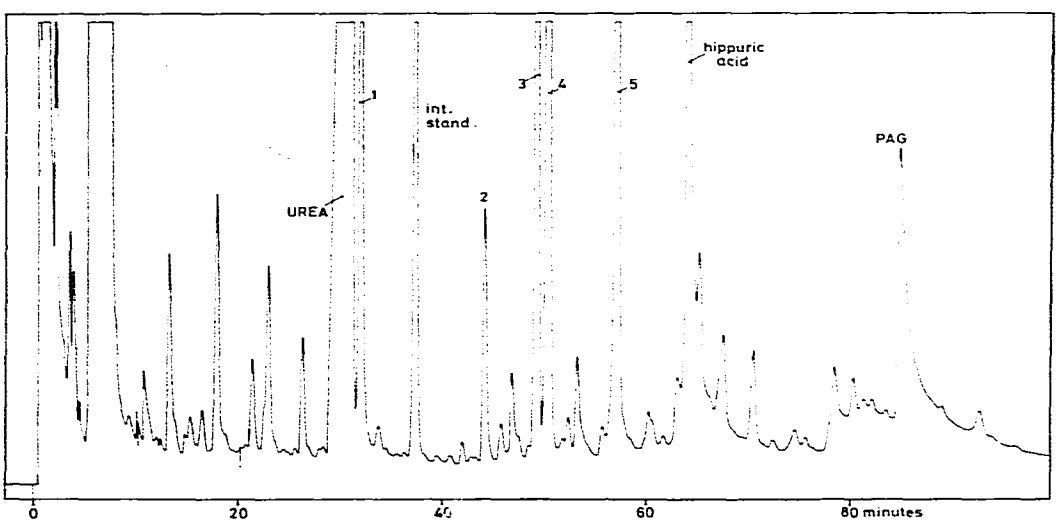

Fig. 1. Gas chromatogram of urinary organic acids (Me_3Si derivatives) of a patient with PKU. 1 = Phenylacetic acid; 2 = mandelic acid; 3 = *o*-hydroxyphenylacetic acid; 4 = phenyllactic acid; 5 = phenylpyruvic acid; internal standard = 2-phenylbutyric acid. The thermal degradation product of PAG is indicated as PAG.

The mass spectrum of the unknown, obtained by combined gas chromatography—mass spectrometry (GC—MS) of the trimethylsilylated urinary extract is given in Fig. 2. The very low abundance of the ions *m/e* 73 and *m/e* 147 indicated that the compound did not contain Me_3Si groups. The abundant ions at *m/e* 91, 92 and 118 were in accordance with the occurrence of a phenylacetyl group. These peaks shifted to higher masses when patients with PKU were loaded with partially deuterated phenylalanine (a mixture of 50% non-deuterated, 40% monodeuterated and 10% dideuterated L-phenylalanine). The various fragment ions present in the mass spectrum suggested the structure of PAG Me_3Si ester, of which Me_3SiOH has been eliminated (M = 246). The mass spectrum was identical to that of synthetic PAG treated with BSA. Because of the silylation procedure used, the formation of a Me_3Si ester has to be expected (see also below for N-acetylglutamine).

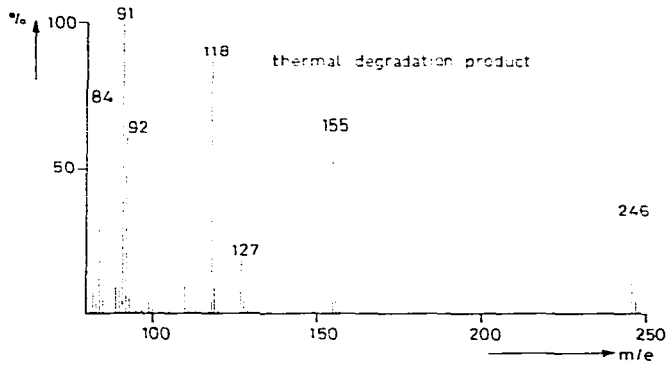

Fig. 2. Mass spectrum at 70 eV of the thermal degradation product of N-phenylacetylglutamine.

To investigate the problem of Me_3SiOH elimination, synthetic PAG was studied in more detail. Using the direct inlet of the mass spectrometer, PAG and its methyl ester showed at low probe temperatures (ca. 80°) the expected mass spectra (acid, Fig. 3; methyl ester, Fig. 4). At higher probe temperatures

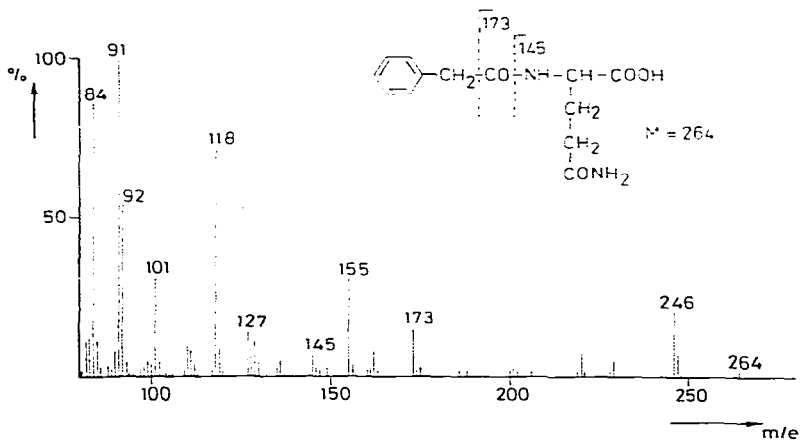

Fig. 3. Mass spectrum at 70 eV of N-phenylacetylglutamine.

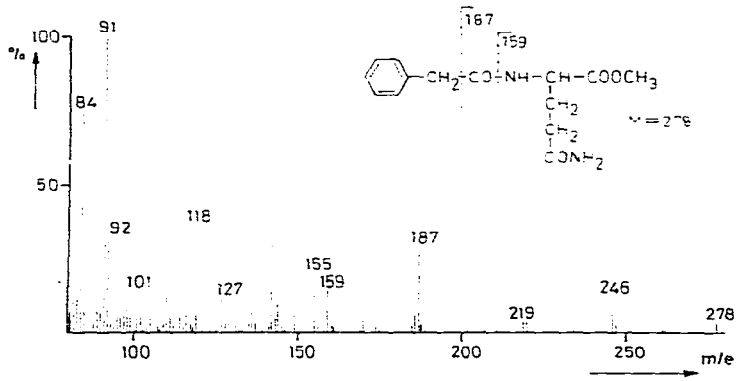

Fig. 4. Mass spectrum at 70 eV of N-phenylacetylglutamine methyl ester.

both mass spectra became identical to that shown in Fig. 2. For practical reasons the Me_3Si ester was not studied using the direct introduction technique. When analysed via the GC inlet system, PAG as well as the corresponding Me_3Si and methyl ester gave rise to the same mass spectrum (Fig. 2). Thermal degradation of organic compounds is known to occur in the ion source of the mass spectrometer. However, from the GC (—MS) data it can be concluded that in the latter cases thermal degradation of PAG (loss of H_2O), its Me_3Si ester (loss of Me_3SiOH) and its methyl ester (loss of CH_3OH) has to take place in the injection port of the gas chromatograph. Especially, PAG itself is not volatile enough to be analysed by GC (m.p. 105°—107°).

In addition, also the behaviour of N-acetylglutamine was studied. Analysis of N-acetylglutamine (M = 188) and its methyl ester (M = 202), using the direct introduction method, showed the expected corresponding mass spectra. The occurrence of a thermal degradation product could not be demonstrated clearly. N-Acetylglutamine, analysed via the GC inlet gave rise to a mass spectrum corresponding to the same type of degradation product (M = 170) as observed for PAG. For N-acetylglutamine methyl ester a broad and a sharp peak were seen on the gas chromatogram. The broad peak with lower retention time led to the mass spectrum of the substance M = 170, whereas the sharp peak gave the expected mass spectrum of the methyl ester (M = 202). As was evident from its mass spectrum obtained via the GC inlet, N-acetylglutamine treated with BSA gave the bis-Me_3Si derivative (M = 332); also the free amide function bears a Me_3Si group. The degradation product could not be detected. In this context it is unknown why for PAG a bis-Me_3Si derivative could not be detected.

In conclusion, GC analysis of the studied glutamine derivatives not protected at the amide function gives rise to the observed degradation; a free amide function seems to be essential. Two possibilities of elimination can be considered, namely that leading to a ketene structure or to an imide structure, as given in Fig. 5 for the degradation product of PAG (M = 246). To discriminate between these structures further investigations are necessary, as are preparative gas chromatography followed by more sophisticated mass spectrometry and nuclear magnetic resonance spectroscopy.

Finally, it has to be noted that our screening method [5] is not

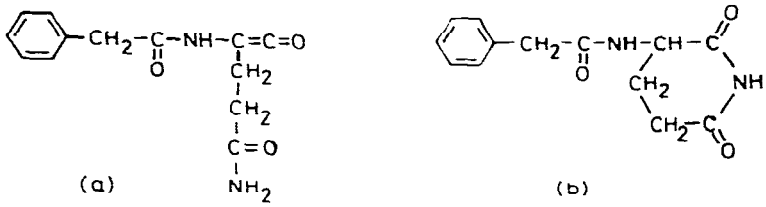

Fig. 5. Ketene (a) and imide (b) structures deduced from PAG.

recommended for the quantitative determination of urinary PAG. Although the extraction yield is 70%, the response with the flame ionization detector is very low. Only a significant asymmetric peak is obtained when the excretion is greatly increased: normal excretion levels are below the level of detection. Therefore, the determination of PAG can be carried out in a more reliable way, using alkaline hydrolysis [3, 4] followed by determination of liberated phenylacetic acid. Publication of our observations were thought to be useful for those who deal with screening for organic acidurias, as elevated PAG concentrations are encountered rather frequently.

ACKNOWLEDGEMENT

This investigation was supported by "Het Praeventiefonds".

REFERENCES

1 W.H. Stein, A.C. Paladini, C.H.W. Hirs and S. Moore, J. Amer. Chem. Soc., 76 (1954) 2848—2849.
2 L.I. Woolf, Biochem. J., 49 (1951) ix—x.
3 S.K. Wadman, C. van der Heiden, D. Ketting and F.J. van Sprang, Clin. Chim. Acta, 34 (1971) 277—287.
4 C. van der Heiden, E.A.K. Wauters, D. Ketting, M. Duran and S.K. Wadman, Clin. Chim. Acta, 34 (1971) 289—296.
5 J.P. Kamerling, M. Duran, L. Bruinvis, D. Ketting, S.K. Wadman, C.J. de Groot and F.A. Hommes, Clin. Chim. Acta, 77 (1977) 397—405.