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CHROMATOGRAPHIC DETERMINATION AND MASS SPECTROMETRIC IDENTIFICATION OF γ -GLUTAMYLPHENYLALANINE, A URINARY CONSTITUENT IN PHENYLKETONURIA

J.P. KAMERLING, G.J. AARSEN, M. DURAN, P.K. DE BREE, F.J. VAN SPRANG
and S.K. WADMAN *

*University Children's Hospital "Het Wilhelmina Kinderziekenhuis", Nieuwe Gracht 137,
Utrecht (The Netherlands) and Department of Bio Organic Chemistry, Croesestraat 79,
Utrecht (The Netherlands)*

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Summary

The occurrence of γ -glutamylphenylalanine in the urine of patients with phenylketonuria could be demonstrated using chromatographic techniques and mass spectrometry. Concentrations ranged up to 35 mg/l. Only a weak correlation between the urinary excretion of this compound and phenylalanine was seen. The ages of the patients investigated ranged from 2 weeks to 18 years. The origin of the dipeptide is discussed.

Introduction

When screening urinary amino acids by micro-scale two-dimensional thin-layer chromatography [1], we noticed that in the urine of many patients with phenylketonuria (PKU) an unknown ninhydrin-positive compound was present, which was not seen on chromatograms of other patients. The position of the spot was between alanine and tyrosine; its magnitude and intensity varied considerably.

In this report we describe the isolation and mass spectrometric identification of the abnormal compound which appeared to be γ -glutamylphenylalanine (γ -GluPhe). The occurrence of this peptide in urine of patients with PKU prompted us to investigate its connection with abnormal phenylalanine overflow.

* Corresponding should be directed to S.K. Wadman, University Children's Hospital "Het Wilhelmina Kinderziekenhuis", Nieuwe Gracht 137, Utrecht, The Netherlands.

Materials and methods

1. General

γ -Glutamylphenylalanine was obtained from Aldrich Chemical Company, Inc., Milwaukee, WI, U.S.A.

Micro-scale two-dimensional thin-layer chromatography for the routine analysis of ninhydrin-positive compounds was performed as described previously [1]. Gas-liquid chromatography (GLC) and gas-liquid chromatography-mass spectrometry (GLC-MS) of derivatized amino acids and peptides were carried out essentially as reported earlier [2], except that the column oven temperature was programmed from 135°C at 2°C/min.

2. Column chromatographic determination of urinary phenylalanine and of γ -GluPhe

Determination of both compounds was performed with a Technicon TSM1[®] automatic amino acid analyzer. A sample of 100 μ l urine was applied to the cartridge.

For urinary phenylalanine a short program was used. Column: 41 cm \times 0.5 cm filled with Chromobeads C3. Temperature 47°C. Elution with lithium citrate buffer: pH 4.40; citrate 0.05 mol/l; Li 0.3 mol/l; flow 0.45 ml/min. The buffer pH was adjusted to 4.40 by titration with 6 mol/l HCl. One litre of buffer contained 1.0 ml thiodiglycol and 10 ml Brij-35[®], which is a wetting agent. Phenylalanine elutes at 57 min, tyrosine at 49 min. For γ -GluPhe a special program was used also, because this compound coincided with homocitrulline in the standard method for physiological fluids. The column was the same as described above. γ -GluPhe was eluted with a lithium citrate buffer, pH 3.10 (citrate 0.05 mol/l; Li 0.3 mol/l; flow 0.45 ml/min). One litre of buffer contained 1.0 ml thiodiglycol and 10 ml Brij-35[®]. γ -GluPhe was eluted as a broad peak after 90 min between valine and homocitrulline.

The amino acids were detected with a standard ninhydrin procedure. Absorbances of the coloured products were continuously recorded at 570 and 440 nm. The 570/440 nm colour ratio (7.0) for γ -GluPhe did not differ from that of the common amino acids. The molar extinction coefficient at 570 nm compared with that of norleucine was 0.93.

The coefficient of variation of the γ -GluPhe procedure as determined in a series of five analyses was found to be 3.3%. For the phenylalanine determination the same reproducibility as for the common amino acids (c.v. 2.9%) was observed.

3. Isolation of γ -glutamylphenylalanine from urine

Urine (200 ml) from a PKU patient was filtered and then applied to a column of Dowex 50W \times 8, 50–100 mesh, H⁺-form (20 cm \times 2.5 cm). The resin was washed with 1 l of H₂O and the peptide-containing fraction eluted with 600 ml of 1 mol/l pyridine. After evaporation of the solvent the residue was dissolved in 2 ml of H₂O and fractionated on a column of Aminex-MS, fraction B, H⁺-form (110 cm \times 2.5 cm). The elution was performed with 0.1 mol/l pyridine/formic acid, pH 3.1, at a flow rate of 7.3 ml/min and a column temperature of 48.2°C. The compound eluted between 2370 and 2740 ml.

Pyridine and formic acid were removed by concentration in vacuo followed by treatment with Dowex 50W \times 8. The peptide was eluted from the cation exchange resin with 2 mol/l ammonia and obtained as dry residue after evaporation of the ammonia. The material so obtained was subjected once more to chromatography on Aminex-MS using 0.1 mol/l pyridine/acetic acid, pH 3.5. The peptide was present in the fractions between 960 and 1110 ml of the eluate and proved to be pure by thin-layer chromatography. The total yield was about 2 mg.

4. Derivatization procedures

Esterification. (a) The peptide (1 mg) was treated with methanolic 1 mol/l HCl (1 ml) for 3 h at 20°C or at 100°C [2]. (b) The peptide (1 mg) was dissolved in methanol (1 ml) and treated with a small excess of diazomethane in ether for 5 min at 20°C. The solution had a faint yellow colour.

Acylation. Methyl ester derivatives were acylated with trifluoroacetic anhydride (1 ml) for 1 h or 18 h at 20°C [2,3].

Methodological investigations were performed with the commercially obtained peptide.

Results

Identification of γ -GluPhe

In Fig. 1 a two-dimensional thin-layer chromatogram (5 cm \times 5 cm) of the urinary amino acids including the unknown compound (see arrow) is given. Hydrolysis of the isolated peptide in 6 mol/l HCl for 24 h at 110°C yielded equimolar amounts of glutamic acid (Glu) and Phe. The structure of the peptide was investigated further by GLC-MS using the *N*-trifluoroacetyl (*N*-TFA) methyl ester derivative [4]. Fig. 2 shows the gas chromatogram of the derivatized peptide using diazomethane and trifluoroacetic anhydride (18 h). Peak 5 corresponds with the presence of the *N*-TFA methyl ester derivative of γ -GluPhe (*N*-TFA- γ Glu(OCH₃)-Phe-OCH₃), as can be deduced from the mass

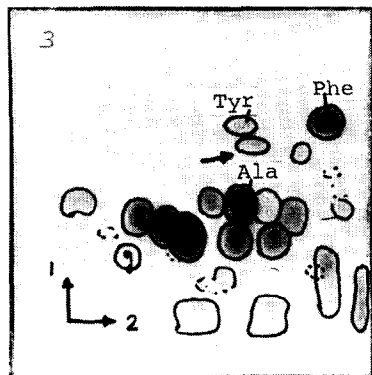


Fig. 1. Two-dimensional thin-layer chromatography of urinary amino acids in patient A.S. with phenylketonuria. γ -GluPhe is indicated with an arrow. Solvent (1) 1-butanol/pyridine/water (1 : 1 : 1, v/v) (30 min) and solvent (2) 88% phenol/25% ammonia/water (10 : 0.05 : 1, v/v).

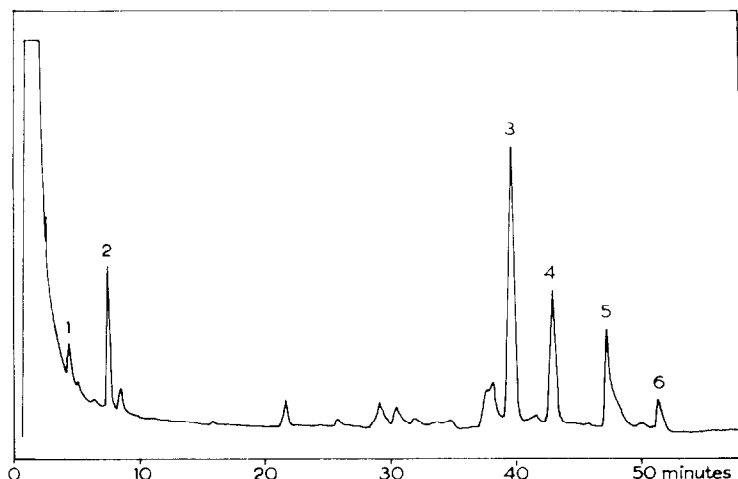


Fig. 2. Gas chromatogram of the isolated urinary dipeptide treated with diazomethane (5 min, 20°C) and trifluoroacetic anhydride (18 h, 20°C): 1, *N*-TFA-Pyroglu-OCH₃; 2, *N*-TFA-Phe-OCH₃; 3, *N*-TFA-γGlu(OCH₃)-(TFA)Phe-OCH₃; 4, *N*-CH₃-*N*-TFA-γGlu(OCH₃)-(TFA)Phe-OCH₃; 5, *N*-TFA-γGlu(OCH₃)-Phe-OCH₃; 6, *N*-CH₃-*N*-TFA-γGlu(OCH₃)-Phe-OCH₃. Column material: 3.8% SE-30 on Chromosorb W/AW-DMCS-HP, 80–100 mesh (2.00 m × 4 mm). Oven temperature: 135°C → 250°C at 2°C/min. Nitrogen flow rate: 40 ml/min.

spectrum (Fig. 3). The results of the application of diazomethane instead of methanolic HCl for the esterification prove the occurrence of glutamic acid in the native dipeptide and exclude glutamine. The peaks at *m/z* 162 (163 – 1 H) (degradation sequence: *m/z* 162 → *m/z* 131 → *m/z* 103) and *m/z* 180 (178 + 2 H) (degradation sequence: *m/z* 180 → *m/z* 120) indicate the C-terminal position of Phe [4]. The N-terminal position of Glu is evident from the peaks at *m/z* 240 and *m/z* 212 (degradation sequence: *m/z* 212 → *m/z* 180 → *m/z* 152) [4]. It has also been reported [4] that α-Glu- and γ-Glu-peptides can be differentiated on the basis of their mass spectra. α-Glu-peptides give rise to mass spectra which contain in addition to the peaks at *m/z* 212 and *m/z* 180 also

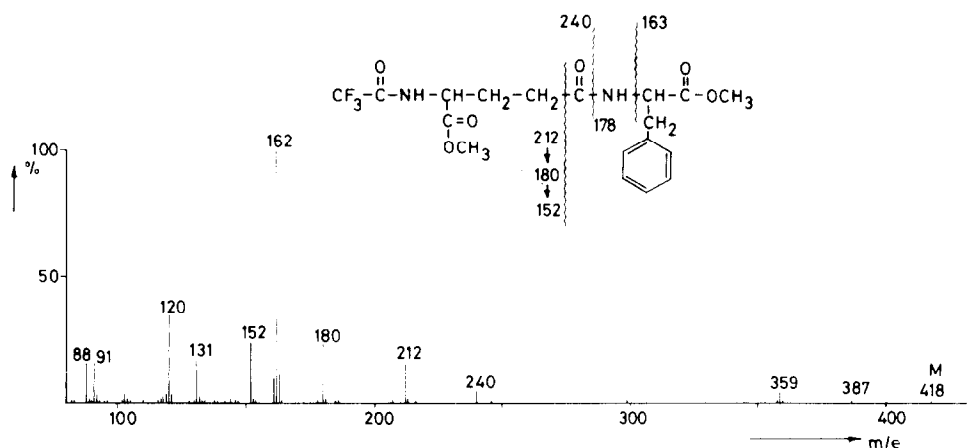
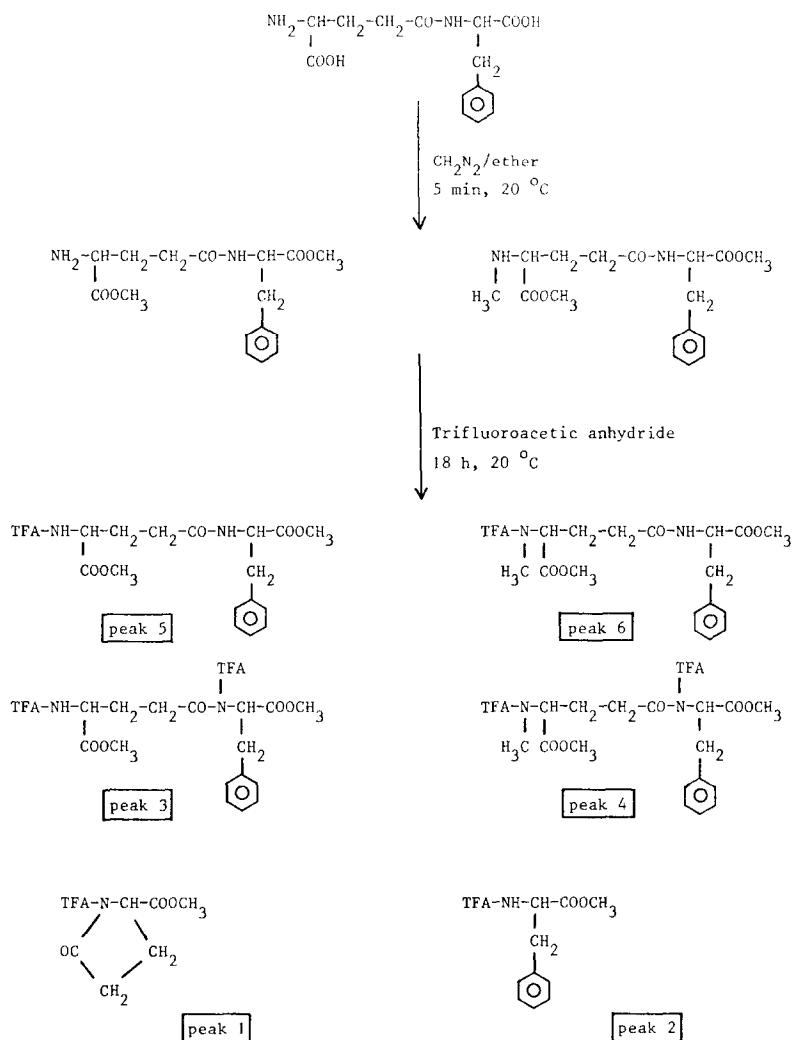


Fig. 3. 70 eV Electron impact mass spectrum of *N*-TFA-γGlu(OCH₃)-Phe-OCH₃.



Scheme 1.

non-isotopic peaks at m/z 213 and m/z 181. The latter fragment ions originate from a H-rearrangement; γ -Glu-peptides do not show this rearrangement. Using these rules, one has to conclude that the isolated peptide contains a γ -Glu-unit. The identity of the urinary γ -GluPhe was verified by comparison with synthetic γ -GluPhe. The chromatographic as well as the mass spectrometric data of both compounds are in full agreement with each other. As becomes evident from Fig. 2, the derivatization procedure used gives rise to several by-products (see Scheme 1). Esterification with diazomethane causes partial N-methylation of the terminal amino function (peak 6: $N\text{-CH}_3\text{-N-TFA-}\gamma\text{Glu(OCH}_3\text{)-Phe-OCH}_3$; the mass spectrum of this derivative was helpful for the interpretation of the mass spectrum of peak 5). However, use of methanolic HCl at 100°C leads to a substantial cleavage of the peptide bond and formation of $\text{Glu(OCH}_3\text{)-OCH}_3$,

Phe-OCH₃ and a small amount of pyroglutamic acid methyl ester (Pyroglu-OCH₃). It is known that γ -Glu derivatives are more labile towards acid solvolysis than α -Glu derivatives [5]. The methanolysis can be suppressed by carrying out the reaction at room temperature, but then the esterification is poor. Acylation with trifluoroacetic anhydride of γ -Glu(OCH₃)-Phe-OCH₃ for 18 h [3] gives besides the expected *N*-TFA peptide derivative some additional components, namely *N*-TFA-Pyroglu-OCH₃ (peak 1), *N*-TFA-Phe-OCH₃ (peak 2) and *N*-TFA- γ Glu(OCH₃)-(TFA)Phe-OCH₃ (peak 3). The molecular weight of the third substance was determined by chemical ionization mass spectrometry using methane as reactant gas. The formation of these derivatives is due to over-trifluoroacetylation of the peptide bond. The presence of *N*-TFA-Pyroglu-OCH₃ and *N*-TFA-Phe-OCH₃ can be explained as the result of a transamidation reaction at the NH group of the peptide bond. Moreover, it proves the presence of a γ -Glu-unit in the native dipeptide, because *N*-TFA-Pyroglu-OCH₃ cannot be formed from α -Glu peptides. The peptide derivative is formed by the introduction of one additional TFA group at the peptide bond (see also peak 4 starting with the *N*-CH₃ derivative: *N*-CH₃-*N*-TFA- γ Glu(OCH₃)-(TFA)Phe-OCH₃). These side-reactions can be greatly reduced using a reaction time of 1 h.

Excretory values

As γ -GluPhe seems to occur especially in the urine of PKU-patients it is tempting to try to correlate its excretory level with phenylalanine concentrations in urine and plasma. By column chromatography we analyzed 42 urine samples obtained from eight PKU-patients, whose ages ranged from several weeks to 18 years; 24-h urine samples were collected either at random or during loading studies: chronic loading with L-phenylalanine (50 or 100 mg/kg body weight) was performed in four PKU-patients (F.K. (m) 5 7/12 years,

TABLE I

URINARY γ -GluPhe, URINARY Phe AND FASTING SERUM Phe IN PATIENTS WITH PHENYLKETONURIA

Patient	Creatinine (g/l)	γ -GluPhe (μ mol/l)	γ -GluPhe (μ mol/24 h)	Phe (mmol/24 h)	Fasting serum Phe (mmol/l)
1. A.S.	1.00	95	31	1.30	1.74
2. C.v.d.B.	1.05	25	35	2.58	2.78
3. J.v.D.	1.58	23	4	0.15	0.59
4. J.v.D.	1.91	65	12	0.34	1.05
5. J.v.D.	1.95	104	23	0.69	1.44
6. J.v.D.	1.56	111	28	0.90	1.97
7. J.v.D.	1.65	118	28	0.90	2.21
8. J.v.D.	1.38	116	26	0.75	1.89
9. M.P.	1.31	18	2	0.05	0.25
10. M.P.	0.53	50	21	1.53	1.79
11. M.P.	0.87	118	32	2.23	2.60
12. M.P.	0.40	30	16	1.81	2.70
13. F.K.	0.53	18	9	0.73	1.76
14. F.K.	0.45	24	17	1.05	1.94
15. F.K.	0.37	28	14	0.97	1.88
16. F.K.	0.60	31	11	0.67	1.96
17. F.K.	0.58	42	15	0.58	1.69

J.v.D. (m) 7 11/12 years, M.P. (m) 7 7/12 years, C.v.d.B. (m) 18 1/12 years) on treatment. The effect of this loading was monitored by daily recording of a computerized electroencephalogram [6]. The objective of this test was to look for suitable parameters which can be followed when taking patients off treatment. In 17 out of 42 quantitative analyses the column chromatogram showed a measurable peak of γ -GluPhe, its concentration ranging up to 0.12 mmol/l. In the remaining 25 samples the concentrations were lower than 0.02 mmol/l, but not zero. In Table I the values for urinary γ -GluPhe and Phe, as well as for Phe in fasting serum are given.

A weak correlation between urinary γ -GluPhe and Phe ($r = 0.75$) was observed. During loading of J.v.D. there was an increase of γ -GluPhe parallel with phenylalanine overflow and this was also seen more or less in the other patients. In patient C.v.d.B. (18 years old) during loading, eight out of nine values were lower than 24 μ mol/l despite high serum Phe values.

No clear correlation of γ -GluPhe with age was observed. Two infants of two weeks old and an untreated patient of three years of age had very low excretions despite serum Phe values of 1.47, 1.38 mmol/l and 1.82 mmol/l respectively. A.S. (Table I), two years old, was in the high range.

γ -GluPhe was absent in urine samples from patients with generalized aminoaciduria, as well as in patients with a defective γ -glutamyl cycle due to glutathione synthetase deficiency.

No attempts were made to estimate the γ -GluPhe concentrations in plasma.

Discussion

Human urine may contain a number of dipeptides. Aspartic and glutamic acid have been shown to be among the most abundant N-terminal amino acids in these peptides [7,11]. The glutamic acid involved in peptide formation may react with its α -carboxyl group or with its γ -carboxyl group. γ -Glutamylvaline, γ -glutamylleucine and γ -glutamylisoleucine [7], N^α -(γ -glutamyl)ornithine [8], N^ϵ -(γ -glutamyl)lysine [9], γ -glutamylcysteine [10], γ -glutamylthreonine and γ -glutamylglycine [11] and α,γ -glutamylcystinylbisglycine [12] have as yet been identified in human urine (or tissue). The biological function of most of these γ -glutamyl peptides is still unclear; only the N^ϵ -(γ -glutamyl)lysine moiety has been pinpointed as crosslink in various proteins, especially fibrin [13,14].

γ -Glutamyl amino acid residues may originate from endogenous protein catabolism. It is possible that they are substrates for γ -glutamyl cyclotransferase [15] and metabolized for the major part. A small portion may escape degradation and be excreted in the urine. This mode of formation, however, does not explain the exclusive occurrence of γ -GluPhe in the urine of PKU patients.

Also an exogenous origin seems possible. γ -GluPhe is present in the non-protein fraction of soybeans (0.4 mg/g beans fresh wt) [16], however, PKU-patients are not allowed to consume this foodstuff. It has been mentioned that γ -glutamyl peptides — and β -aspartyl peptides — may be derived from dietary protein [17]. Others [11] also found excretion of these peptides in subjects receiving complete intravenous alimentation, thereby demonstrat-

ing an endogenous formation of these peptides. Endogenous formation of γ -GluPhe in PKU-patients is likely, because most of these patients are fed a protein hydrolysate that does not contain peptides. The endogenous formation of β -aspartyl dipeptides has been reported to be catalyzed by an enzyme which resembles asparaginase [18]. It can be hypothesized that a similar enzyme catalyses the synthesis of γ -glutamyl dipeptides. The formation of γ -GluPhe as a detoxification mechanism in PKU is of no significance, because of the small amounts produced.

An attractive hypothesis is the formation of γ -GluPhe by transpeptidation in the kidney, a step in the tubular reabsorption process of amino acids, known as the γ -glutamyl cycle [19,20]. Glutathione breakdown is catalyzed by membrane-bound γ -glutamyl transpeptidase, which transfers the γ -glutamyl moiety of glutathione to extracellular acceptor amino acids to form γ -glutamyl amino acids. These are released into the intracellular fluid and may be hydrolysed, or undergo additional transpeptidation reactions or serve as substrates of γ -glutamyl cyclotransferase, which converts them to 5-oxoproline and the corresponding amino acids. In untreated PKU the capacity of Phe-reabsorption is exceeded and presumably the γ -glutamyl transpeptidation of Phe is maximal. Consequently the intracellular concentration of γ -GluPhe may be increased and a small fraction of this compound may leak out of the tubular cells back into the luminal fluid. Furthermore it is known that γ -GluPhe has a low affinity for γ -glutamyl cyclotransferase [21], which will also contribute to an increase of the intracellular concentration of this dipeptide.

From the data given in Table I it can be concluded that the excretion of γ -GluPhe is highest when the overflow of Phe is maximal. This is especially true in J.v.D. (numbers 3–8) during loading. The same tendency, though less clearly, was seen in M.P. and F.K. during loading. The appearance of γ -GluPhe during conditions of overloading of the tubular Phe-transport system in PKU might thus be a reflection of the evidence of an operative γ -glutamyl cycle in man.

Note

After the final preparation of this manuscript we became aware of the article by H. Peck and R.J. Pollitt (*Clin. Chim. Acta* 94 (1979) 237–240) which also describes the occurrence of γ -GluPhe in PKU-patients.

Acknowledgements

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