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DIPEPTIDE ANALYSIS IN HUMAN URINE

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SUMMARY

Fractions of dipeptides, obtained from human urine by a combination of cation-exchange chromatography, ligand-exchange chromatography and reversed-phase chromatography, were transformed into their N-heptafluorobutyryl methyl ester derivatives and then subjected to capillary gas chromatography. The profiles obtained indicate the presence of many dipeptides in human urine For the first time, α-Asp-Hyp, Pro-Phe and γ-Glu-Phe were detected in the urine of healthy individuals.

INTRODUCTION

In contrast to the excretion of amino acids, peptides are excreted in the urine of healthy individuals only in minute amounts [1]. A strongly increased excretion of dipeptides containing proline or hydroxyproline is observed in individuals suffering from diseases, especially those concerning collagen metabolism [2—4]. In the urine of individuals suffering from phenylketonuria large amounts (35 mg/l) of γ-glutamylphenylalanine have been found [5, 6], but this dipeptide could not be detected in the urine of healthy persons. γ-Glutamyl dipeptides, containing amino acids other than phenylalanine, have also been found in healthy individuals [7, 8]. Further, some β-aspartyl dipeptides [7—9] are known to occur in urine.

The isolation and identification of di- and oligopeptides in urine was reviewed by Lou and Hamilton [1]. Amino acids and small peptides are easily separated from other compounds in urine by cation-exchange chromatography and elution with 1 *M* ammonia solution [1, 3]. Amino acids may be removed by ligand-exchange chromatography [1, 10, 11] using a Chelex ion-exchange column treated with Cu^{2+}. Unfortunately, these columns not only retain amino acids but also dipeptides containing a basic amino acid at the C-terminus, e.g., histidine or lysine [10, 11]. In turn, amino acids

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containing additional acidic groups may pass through the column together with the dipeptide fraction [10]. Thus, further separation steps are required if all dipeptides are to be analysed. If the dipeptide fraction is subjected to a purification on reversed-phase columns [12, 13], two fractions can be obtained: fraction A (aqueous solvent) containing acidic amino acids and dipeptides with hydrophilic groups and fraction B (alcoholic solvent) containing dipeptides with hydrophobic groups [12].

We have achieved a further separation of the dipeptide fractions by glass capillary and fused-silica capillary gas chromatography (GC) using N-heptafluorobutyryl methyl ester (HFBM) derivatives.

EXPERIMENTAL

Dipeptide standards

Ile-Pro, Gly-Pro, α-Asp-Gly and β-Asp-Gly were purchased from Bachem (Bubendorf, Switzerland), Phe-Pro, Leu-Pro, Pro-Phe and Gly-Hyp from Sigma (Munich, F.R.G.) and Pro-Hyp from Serva (Heidelberg, F.R.G.). The dipeptides β-Asp-Ala, β-Asp-Leu, α-Asp-Pro, α-Asp-Hyp, β-Asp-Hyp, γ-Glu-Leu, γ-Glu-Ile, γ-Glu-Phe, γ-Glu-Val, α-Glu-Hyp and Phe-Hyp were prepared as described previously [14, 15]. Standard compounds for retention indices were C_8—C_{26} saturated hydrocarbons.

Enrichment of dipeptides

A 24-h urine volume of 600 ml was acidified to pH 1.8—2.0 by addition of concentrated hydrochloric acid. This solution was poured onto a column (50 × 3 cm I.D.) of Dowex 50W-X8 (H^+) (50—100 mesh) (Serva). After passage of the urine sample, the column was washed with 1.5 l of water to remove salts, acids and neutral compounds. The fraction of bases was then eluted with 1.5 l of 1 *M* ammonia solution. A rotary evaporator was used to remove the solvent from the collected fraction at 40°C. From the dry residue (2.2 g), 1 g was dissolved in 50 ml of distilled water and the pH adjusted to 10.3 ± 0.1 by addition of concentrated ammonia solution. The solution was applied to the top of a column (44 × 1.8 cm I.D.) of a Chelex ion exchanger, IRC 718 (20—50 mesh) (Serva) which was previously loaded with Cu^{2+} ions by treatment with aqueous copper sulphate solution [10]. The chromatographic procedure was performed as described by Boisseau and Jouan [11]. The solutions obtained after passage of the sample through the column and washing with 500 ml of water were combined and evaporated to dryness on a rotary evaporator at 40°C. From the residue (410 mg), 40 mg were dissolved in 10 ml of 1% trifluoroacetic acid (TFA) (EGA, Steinheim, F.R.G.) and pumped onto a column (20 × 1 cm I.D.) filled with LiChroprep RP-18 (40—63 mesh) (Merck, Darmstadt, F.R.G.) [13]. Fraction A was obtained by elution with 100 ml of 1% trifluoroacetic acid and fraction B by elution with 100 ml of *n*-propanol—water—TFA (60:39:1).

Both fractions were lyophilized, yielding residues of 27 mg (fraction A) and 8 mg (fraction B). For the preparation of N-heptafluorobutyryl dipeptide methyl esters, 1 mg of the lyophilized sample was esterified in a Reacti-Vial (Pierce, Rockford, IL, U.S.A.) with 2 ml of methanol—thionyl chloride (9:1)

at −20°C as described previously [16]. After removing of the solvent under a stream of nitrogen at 60°C, the residue was dissolved in 250 μl of dry methylene chloride and again evaporated to dryness under a stream of nitrogen to remove traces of the applied reagents. Acylation was carried out at room temperature for 1.5 h after adding 200 μl of heptafluorobutyric anhydride (Merck) and 500 μl of methylene chloride. The solution was then evaporated under a stream of nitrogen at 60°C, 250 μl methylene chloride were added and the solvent was removed under nitrogen again. Finally, the residue was dissolved in ca. 0.1 ml of methylene chloride and 0.5—1 μl of this solution was used for GC and GC—mass spectrometry (GC—MS).

Gas chromatography—mass spectrometry

For fraction A a Carlo Erba 4160 gas chromatograph was equipped with a fused-silica capillary column (50 m × 0.32 mm I.D.) coated with DB 1701 (I & W Scientific, Rancho Cordova, CA, U.S.A.). The carrier gas was hydrogen at 1 kg/cm^2 and the temperatures were: injector 260°C, detector 282°C and column programme 130°C for 3 min, 130—260°C at 3°C/min and 260°C for 15 min. For fraction B the column was a WCOT glass capillary (30 m × 0.3 mm I.D.) coated with OV-101. The carrier gas was hydrogen at 0.5 kg/cm^2 and the temperatures were: injector 270°C, detector 280°C and column programme 80°C for 3 min, 80—280°C at 3°C/min. Mass spectra were obtained with a Varian-MAT 312 mass spectrometer equipped with a MAT SS 200 data system (PDP 11/34 computer). The ionization energy was 70 eV. The mass spectrometer was connected to a Varian 3700 gas chromatograph equipped with the capillary columns mentioned above.

RESULTS AND DISCUSSION

Different derivatives of dipeptides were investigated with respect to their ability to be separated by capillary GC. As N-trifluoroacetyl derivatives turned out in a previous investigation of amino acids to be sensitive to hydrolysis [17], we investigated higher fluorinated homologues. As was found earlier, N-heptafluorobutyryl derivatives are well suited for amino acid analysis [18, 19]. Propyl or butyl esters were used in these studies as some methylates turned out to be too volatile. Dipeptides are less volatile than amino acids, allowing the preparation of methylates without the risk of sample loss. Therefore, N-heptafluorobutyryl dipeptide methyl esters were prepared. However, not all dipeptides could be analysed in the form of their HFBM derivatives; in particular, dipeptides containing tryptophan or arginine are not volatile enough. These losses were accepted in order to keep the separation uncomplicated. The mass spectrometric degradation reactions of HFBM dipeptides are similar to those of the N-trifluoroacetyl dipeptide methyl esters described by Weygand et al. [20], but the fragments containing the N-terminal amino acid are shifted by 100 mass units to higher mass in comparison with the N-trifluroacetyl derivatives [20, 21].

In the gas chromatograms of fractions A (Fig. 1) and B (Fig. 2) twenty peaks could be attributed to dipeptides. The main dipeptides identified correspond to degradation products of the collagen metabolism, e.g., Pro-Hyp [1]. Proline-

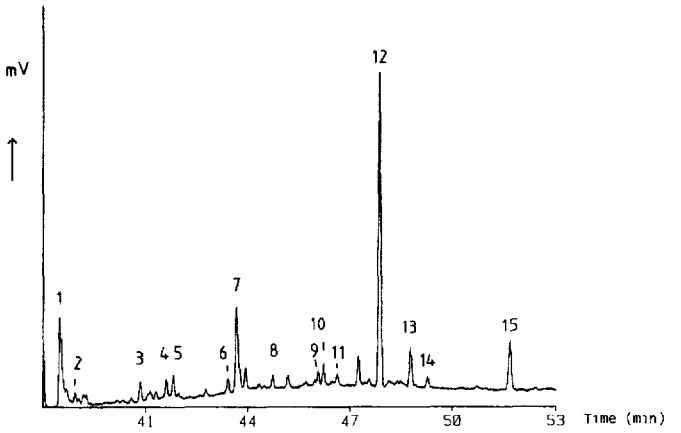

Fig. 1. Fused-silica capillary gas chromatogram of fraction A. The dipeptides were separated as their N-heptafluorobutyryl methyl ester derivatives. Peaks: 1 = hippuric acid; 2 = α-Asp-Gly; 3 = Gly-Pro; 4 = aminosuccinimide of Asp-Gly; 5 = β-Asp-Ala; 6 = Gly-Hyp; 7 = β-Asp-Gly; 8 = α-Asp-Pro; 9 = α-Asp-Hyp; 10 = N_ε-acetyllysine; 11 = β-Asp-Leu; 12 = Pro-Hyp; 13 = α-Glu-Hyp; 14 = γ-Glu-Val; 15 = diketopiperazine of Pro-Hyp.

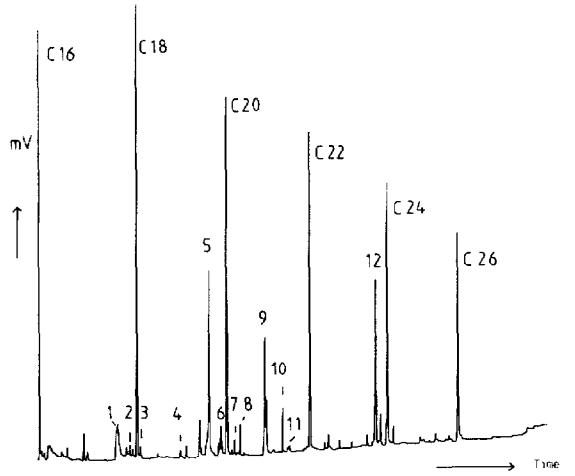

Fig. 2. Glass capillary gas chromatogram of fraction B. The dipeptides were separated as their N-heptafluorobutyryl methyl ester derivatives. Peaks: 1 = caffeine; 2 = Leu-Pro; 3 = Ile-Pro; 4 = β-Asp-Leu; 5 = diketopiperazine of Pro-Hyp; 6 = Pro-Hyp; 7 = γ-Glu-Leu; 8 = γ-Glu-Ile; 9 = Phe-Pro; 10 = Phe-Hyp; 11 = Pro-Phe; 12 = γ-Glu-Phe.

containing compounds were partly converted during derivatization into the corresponding diketopiperazines [2, 16]. While Pro-Pro is converted quantitatively to the diketopiperazine derivative, these derivatives were formed only in minute amounts in the case of Pro-Leu, Ala-Pro and Ile-Pro. Another side-reaction was observed on derivatization of β-Asp-Gly. This dipeptide suffers an intramolecular cyclization to produce the amino succinimide derivative [22, 23] (compound 4, fraction A). These side-reactions could not be avoided. Proline- and hydroxyproline-containing dipeptides, with an N-terminal acidic amino acid, are always connected to the proline or hydroxyproline residue via the α-carboxylic groups. In addition to these dipeptides with an α-glutamyl

or α-aspartyl bond, dipeptides with γ-glutamyl or β-aspartyl amino acid residues are present in the urine of healthy persons. These β-aspartyl and γ-glutamyl dipeptides are probably not produced in the course of collagen metabolism, as such sequences have not been described for these peptides. Therefore, we have to assume that their occurrence is the result of the enzymatic degradation of larger peptides, containing an unusual peptide bond (γ-glutamyl or β-aspartyl peptide bond) that is not attacked by the usual peptidases.

The identification of these dipeptides requires isomeric α- and β-aspartyl dipeptides and α- and γ-glutamyl dipeptides to be distinguished unambiguously. In contrast to γ-glutamyl dipeptides, the mass spectra of the α-isomers show an ion of mass 74, originating from fragmentation of the glutamyl side-chain [20]. As already pointed out by Weygand et al. [20], α-glutamyl dipeptides usually show in their mass spectra ions of mass 313, produced as shown in Fig. 3.

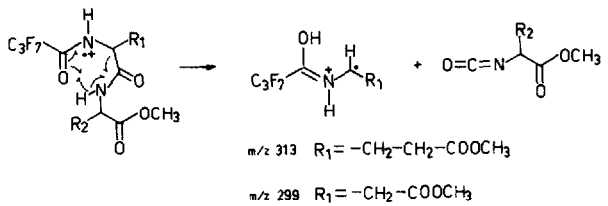

Fig. 3. Reaction scheme showing the production of ions of mass 313 by α-glutamyl dipeptides and ions of mass 299 by α-aspartyl dipeptides.

Similarly, α-aspartyl dipeptides show an analogous fragment of mass 299 [20]. This hydrogen shift reaction is not possible in the mass spectrometric degradation of β-aspartyl and γ-glutamyl dipeptides. Unfortunately, if proline or hydroxyproline is located at the C-terminus of an α-aspartyl or α-glutamyl dipeptide, the corresponding fragments are also lacking, as in those dipeptides the hydrogen necessary for the rearrangement is missing. However, we have found that α- and β-aspartyl dipeptides can be differentiated unambiguously, even if proline or hydroxyproline is present, due to an ion of mass 113 ($C_5H_5O_3$), which can only be produced by β-aspartyl dipeptides. This criterion proved helpful in identifying the previously unknown dipeptide α-Asp-Hyp (Fig. 4). Ions of mass 282, 126 and 68 are characteristic of a C-terminal hydroxyproline, while ions of mass 298, 266 and 256 indicate a N-terminal aspartyl residue. The lack of the ion of m/z 113 points to an α-linkage, so the structure of the compound should be α-Asp-Hyp. In addition, comparison of the retention time and mass spectrum with those of synthetic α-Asp-Hyp and β-Asp-Hyp confirmed the proposed structure (Figs. 4 and 5).

In fraction B, containing mainly hydrophobic dipeptides, two dipeptides were detected, which were not previously known to occur in the urine of healthy individuals.

The mass spectrum of compound 11 (Fig. 6) shows an ion of m/z 266, typical of an N-terminal proline, while the ion of m/z 162 is characteristic of a C-terminal phenylalanine, especially in combination with another ion of m/z 120. The compound was therefore suggested to be Pro-Phe. Thus suggestion was confirmed by the investigation of a synthetic sample, for which the

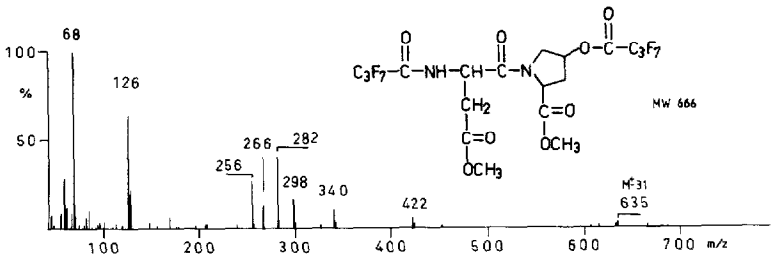

Fig. 4. Mass spectrum of HFBM α-Asp-Hyp.

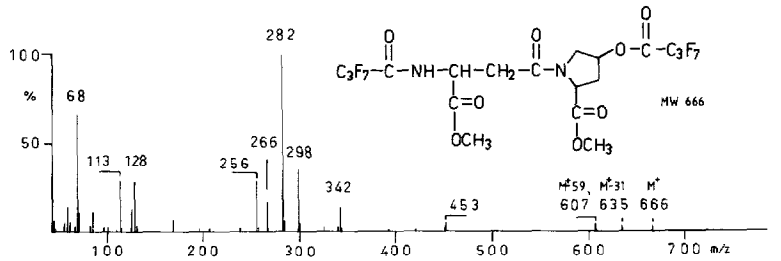

Fig. 5. Mass spectrum of HFBM β-Asp-Hyp.

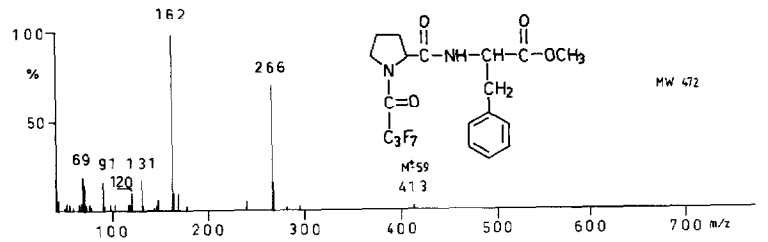

Fig. 6. Mass spectrum of HFBM Pro-Phe.

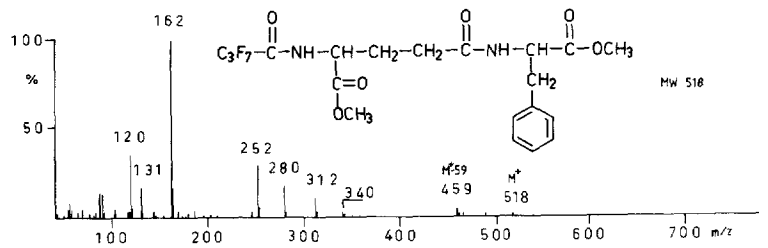

Fig. 7. Mass spectrum of HFBM γ-Glu-Phe.

mass spectrum and retention index proved to be identical with those of the natural product. In the mass spectrum of compound 12 (Fig. 7), a C-terminal phenylalanine is indicated by ions of mass 162 and 120, as mentioned above. The N-terminal glutamyl residue is recognized by the typical key ions of mass 312, 280 and 252. Important for the distinction between α-Glu-Phe and γ-Glu-Phe is the absence of the fragments of *m/z* 74 and 313, which are only present in the α-isomers [5, 20]. These results point to a γ-glutamylphenylalanine. Comparison of the retention index and mass spectrum of a synthetic sample established the correctness of the structural assignment. γ-Glu-Phe was

previously found in the urine of patients suffering from phenylketonuria, but was not detected in the urine of healthy persons [6].

In conclusion, capillary GC proved to be a powerful tool for the final separation of dipeptide fractions isolated from urine. Combination with mass spectrometry allows the identification of dipeptides, not previously known to be present in human urine. The detection of γ-Glu-Phe in the urine of healthy people confirms our experience that there are actually no qualitative differences between the metabolism of healthy and sick individuals. In many instances a metabolite, which is regarded as unique for a chronic disease, is usually also excreted in normal individuals in minute amounts.

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REFERENCES

1 M.F. Lou and P.B. Hamilton, Methods Biochem. Anal., 25 (1979) 203.
2 R.A.W. Johnstone, T.J. Povall, J.D. Baty, J.L. Pousset, C. Charpentier and A. Lemonnier, Clin. Chim. Acta, 52 (1974) 137.
3 K.F. Faull, G.M. Schier, P. Schlesinger and B. Halpern, Clin. Chim. Acta, 70 (1976) 313.
4 C. Charpentier, R.A.W. Johnstone, A. Lemonnier, I. Myara, M.E. Rose and D. Tuli, Clin. Chim. Acta, 138 (1984) 299.
5 J.P. Kamerling, G.J. Aarsen, M. Duran, P.K. De Bree, F.J. van Sprang and S.K. Wadman, Clin. Chim. Acta, 102 (1980) 137.
6 H. Peck and R.J. Pollitt, Clin. Chim. Acta, 94 (1979) 237.
7 T. Tanaka and T. Nakajima, J. Biochem., 84 (1978) 617.
8 D.L. Buchanan, E.E. Haley and R.T. Markiw, Biochemistry, 1 (1962) 612.
9 F.E. Dorer, E.E. Haley and D L. Buchanan, Biochemistry, 10 (1966) 3226.
10 J.F. Bellinger and N.R.M. Buist, J. Chromatogr., 87 (1973) 513.
11 J. Boisseau and P. Jouan, J. Chromatogr., 54 (1971) 231.
12 P. Böhlen, F. Castillo, N. Ling and R. Guillemin, Int. J. Pept. Protein Res., 16 (1980) 306.
13 H. Mabuchi and H. Nakahashi, J. Chromatogr., 233 (1982) 107.
14 G.W. Anderson, J.E. Zimmerman and F.M. Callahan, J. Am. Chem. Soc., 85 (1963) 3039.
15 G.W. Anderson, J.E. Zimmerman and F.M. Callahan, J. Am. Chem. Soc., 86 (1964) 1839.
16 W. Steiner and A. Niederwieser, Clin. Chim. Acta, 92 (1979) 431.
17 A. Darbre and K. Blau, Biochim. Biophys. Acta, 100 (1965) 298.
18 C.W. Moss, M.A. Lambert and F.J. Diaz, J. Chromatogr., 60 (1971) 134.
19 K. Schneider, M. Neupert, G. Spiteller, H.V. Henning, D. Matthaei and F. Scheler, J. Chromatogr., 345 (1985) 19.
20 F. Weygand, A. Prox, H.H. Fessel and K.K. Sun, Z. Naturforsch., Teil B, 20 (1965) 1169.
21 B.A. Andersson, Acta Chem. Scand., 21 (1967) 2906.
22 M.A. Ondetti, A. Deer, J.T. Sheehan, I. Pluscec and O. Kocy, Biochemistry, 7 (1968) 4069.
23 M. Bodansky, Principles of Peptide Synthesis, Springer Verlag, Berlin, Heidelberg, New York, Tokyo, 1984.