Journal of Chromatography, 152 (1978) 546–550

CHROM. 10,685

Note

Simultaneous estimation of dicarboxylic amino acids and their amides by gas chromatography

PETR HUŠEK and VLADIMÍR FELT
Research Institute of Endocrinology, Národní třída 8, 11694 Prague 1 (Czechoslovakia)
(Received October 11th, 1977)

In gas chromatography of amino acids there is a lack of convenient procedure enabling asparagine and glutamine to be estimated separately from their corresponding acids. Today the most commonly used, and practically the only successful approaches, treat amino acids with acidified alcohols at high temperatures, leading to conversion of the amide to the acid. Hediger *et al.*[1] studied the rate of this conversion in acidified butanol, and by combination of esterification time and temperature they found optimal reaction conditions at which both forms exist in approximate equilibrium. Even when the technique is applied to the determination of asparagine and glutamine in enzymic hydrolysates it seems impractical.

Condensation of amino acids with 1,3-dichlorotetrafluoroacetone (DCTFA) proceeds in mild, weakly-basic media[2] which do not attack the amido groups of the two mentioned protein amino acids. This fact provides possibilities for distinguishing between the dicarboxylic amino acids and their amides when a convenient treatment for the side-chain polar grouping can be found. A suitable procedure for treatment of the second carboxyl group in molecules of aspartic and glutamic acids converted to oxazolidinones was given in a preceding study[3]. In relation to that report we present a method which enables asparagine and glutamine to be determined simultaneously with their corresponding acids, after a simple and rapid derivatization step.

EXPERIMENTAL

Material and equipment

L-Asparagine monohydrate (ASN), L-glutamine (GLN), DL-aspartic (ASP) and L-glutamic acid (GLU) and L-phenylalanine (PHE) all grade A quality, were obtained from Calbiochem (Lucerne, Switzerland). Reagents, chemicals, glassware and apparatus were the same as reported earlier[3].

Analysis was performed on a 1.5 m × 2 mm I.D. glass column filled with 3% SP-2250 on Supelcoport (80–100 mesh) in temperature range 130–210° (rate 8°/min). Injection port and detector temperatures were 200 and 250°. Nitrogen, hydrogen and air flow-rates were 20, 20 and 240 ml/min respectively.

Procedure

Condensation. 10–100 nmoles of each amino acid in the mixture (phenylalanine

was used as internal standard) were condensed with 100 μl of mixed solvent, consisting of benzene, acetonitrile and pyridine in volume ratio 60:32:8 and 20 μl DCTFA at 40° for 5 min.

First acylation. The sample was subsequently treated with 20 μl of 20% (v/v) of methanol in benzene followed by 12 μl of heptafluorobutyric anhydride (HFBA) and after at least 10 sec an additional 24 μl HFBA were added.

Extraction. 500 μl of light petroleum–dichloromethane mixture (4:1, v/v) were added to the sample and the contents were shaken for 10–15 sec with 400 μl each of: 1 *M* sodium carbonate, 1 *M* hydrochloric acid (twice) and 1 *M* sodium bicarbonate. The water phase was always removed using a Pasteur pipet and, after drying of the organic phase with anhydrous sodium sulfate, the extraction medium was transferred into another tube, leaving the sulfate in the original tube, and evaporated just to dryness (avoid excessive blowing of gas into the dry residue).

Second acylation. To the evaporated residue 100 μl of hexane and 5 μl of HFBA were added, and after heating the sample at 70° for 10 min, an aliquot of 2–3 μl was injected into the chromatographic column.

RESULTS AND DISCUSSION

According to the preceding study[3] esterification of the second carboxyl group in molecules of aspartic and glutamic acids requires an exactly defined ratio of methanol, pyridine and anhydride in order to achieve maximal derivatization yields. When HFBA is employed this ratio should be 1:2:3 (v/v/v). Methanol converts DCTFA in the medium to DCTF-methoxypropan-2-ol and the secondary alkoxyalcohol formed is then the inherent esterification agent. Acylation with anhydride only (Fig. 1), without prior addition of methanol, leads to unsatisfactory results especially with aspartic acid. Compared with the preceding study[3], aspartic acid gives higher derivatization yields due to the second acylation step but the double peak formation is impractical and under the employed conditions the second peak of GLU co-elutes with the first peak of GLN (t_R 5.36 min on Fig. 1B).

The second acylation with anhydride in hexane was necessary to restore the amide derivatives. These are formed during the first acylation process and, unlike the derivatives of the acids, which can be chromatographed directly after extraction, the derivatized amides are destroyed in the course of the extraction procedure. Derivatization of asparagine only was found to be influenced by, both total amount of anhydride added into the first reaction medium and time of the second acylation (Fig. 2). As the second anhydride portion does not alter the yields for the dicarboxylic amino acids, provided that the interval between the first and second HFBA addition is at least 10 sec, the additional anhydride amount can be altered arbitrarily. The yield of asparagine was influenced by the reaction time of the second acylation at lower molar ratios of HFBA to methanol and the maximal value could not be achieved even after prolonged heating. The highest relative molar response (*RMR*) value was obtained when the molar ratio of HFBA to methanol was equal to 1.5:12 + 24 μl HFBA added (Fig. 2d). The effect of acylation time under such conditions was minimal and heating of the sample for 10 min at 70° was found to be sufficient. The amount of HFBA in hexane in the range 2–10 vol% did not influence the results substantially and addition of 4–5 μl HFBA to hexane led to optimal reaction yields.

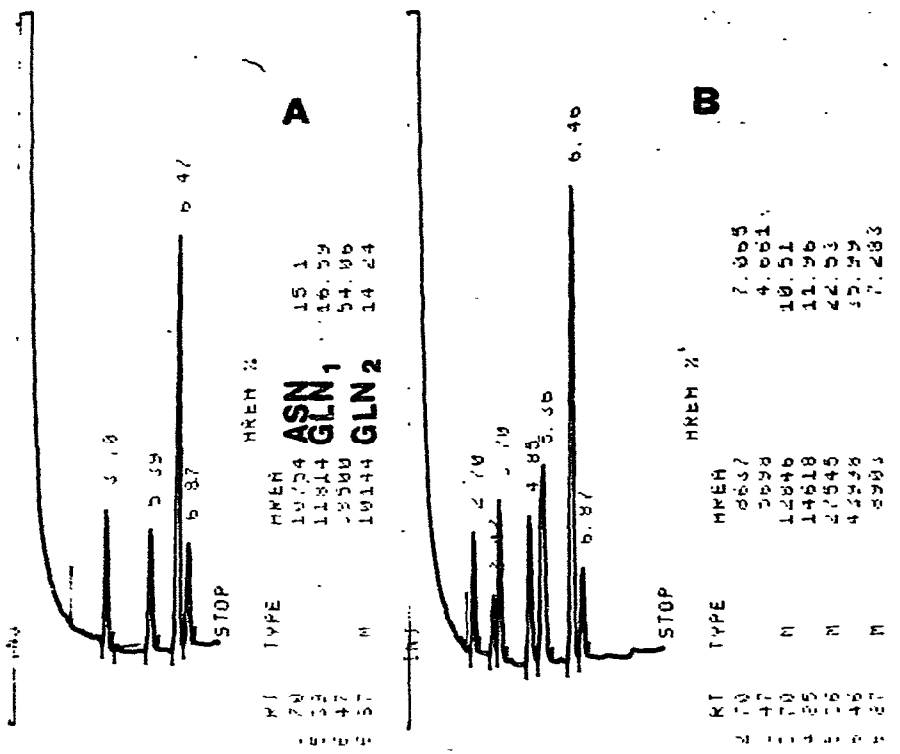

Fig. 1. Gas chromatograms of: (A) equimolar mixture of phenylalanine with asparagine and glutamine alone, (B) in mixture with aspartic and glutamic acids treated with 10 μl HFBA after the condensation step (alcohol addition was omitted). After extraction and second acylation two peaks appeared for each of the following amino acids: ASP (retention time 2.70 and 3.47 min), GLU (4.85 and 5.36 min) and GLN (5.39 and 6.67 min). ASN gave single peak only (3.70 min).

Moreover, the yields were slightly better and more consistent when methanol (4 μl) was not added alone but with benzene in a mixture of 1:4. It was interesting to note that glutamine did not exert similar dependence in any way and that the highest *RMR* value (0.48) could be obtained with only 12 μl HFBA added, after methanol, to the condensation medium. Therefore, for estimation of ASP, GLU and GLN only, the second portion of HFBA (24 μl) can be omitted.

Results of simultaneous estimation of the dicarboxylic amino acids and their amides are given in Fig. 3. *RMR* values are given in the legend under the figure. The value for aspartic acid is about 5% higher when HFBA is co-injected, but the reason for this is unclear; the only explanation is that HFBA diminishes column adsorption or possible derivative degradation.

Acylation using TFAA produces worse results with asparagine than does HFBA (Fig. 4A). When the sample was acylated first with TFAA and then with HFBA (Fig. 4B) the results were better, although still worse than when HFBA was used alone. This indicates that chemical changes occurring in the polar condensation medium (first acylation) are of prime importance. Moreover, as after the dicarboxylic amino acid esterification, the retention times of the derivatized amides do not alter

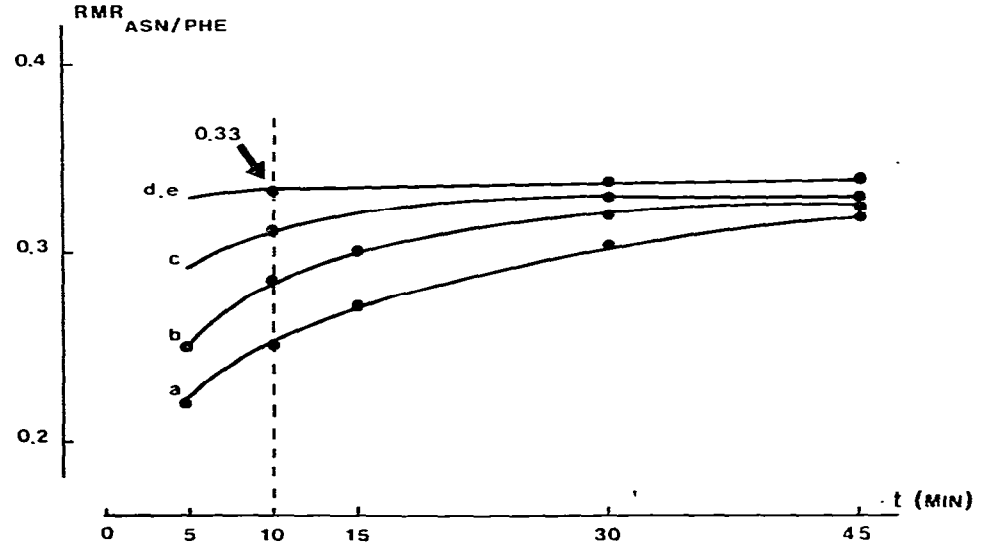

Fig. 2. Effect of amount of HFBA added to the first reaction medium and the time of the second acylation (with 5 vol% HFBA in hexane) on *RMR* value of asparagine. After treatment of the condensation medium with methanol (20 μl of 1:4 mixture with benzene) the following amount of HFBA was added (interval between the two portions was 10 sec at least): (a) 12 μl, (b) 12 + 12 μl, (c) 12 + 18 μl, (d) 12 + 24 μl. In (e) 10 μl of HFBA were added to the medium without alcohol addition.

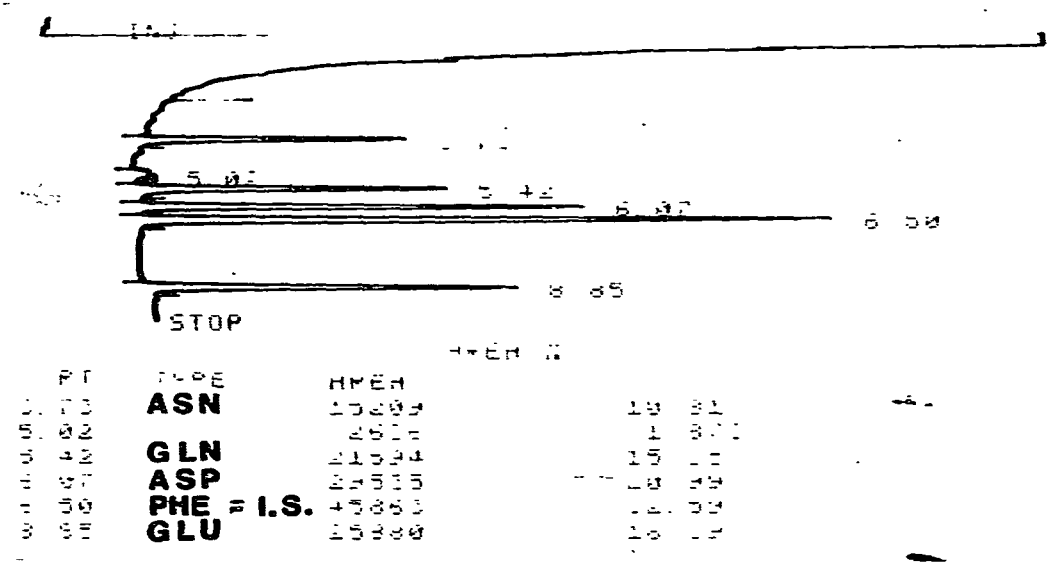

Fig. 3. Gas chromatogram of equimolar mixture of aspartic and glutamic acids and their amides together with phenylalanine derivatized according to described procedure. The following average *RMR* values were obtained: ASN 0.33, GLN 0.48, ASP 0.65, GLU 0.60.

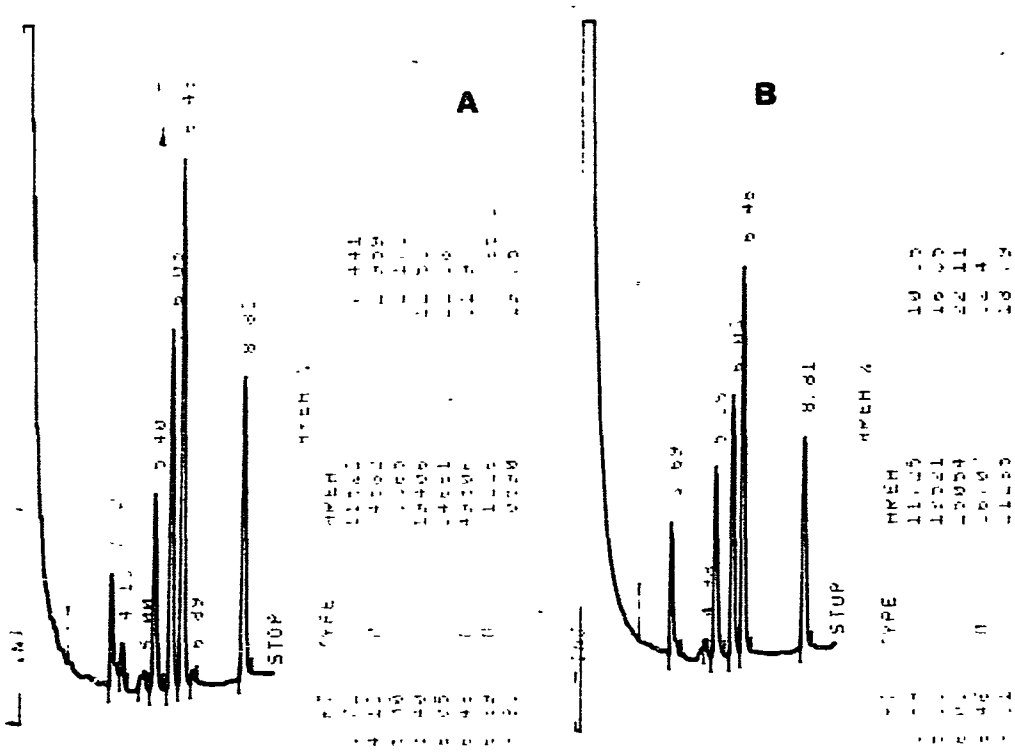

Fig. 4. The same mixture of amino acids as in Fig. 3 was treated according to described procedure with the following exceptions: (A) first and second acylation performed with TFAA: 7 + 14 μl; 5% (v/v) TFAA in hexane. (B) First acylation performed with 7 + 14 μl TFAA, second with 5% (v/v) HFBA in hexane.

with a change of anhydride. This probably means that the anhydride moiety is not included in molecules of the derivatized compounds. Elucidation of their structure by mass spectrometry will be treated in a later report.

REFERENCES

1 H. Hediger, R. L. Stevens, H. Brandenberger and K. Schmid, *Biochem. J.*, 133 (1973) 551.
2 P. Hušek, *J. Chromatogr.*, 91 (1974) 475.
3 P. Hušek and V. Felt, *J. Chromatogr.*, 152 (1978) 363.