Journal of Chromatography, 294 (1984) 507–512
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,694

Note

Isotachophoretic analysis of a synthetic dipeptide L-alanyl-L-glutamine

Evidence for stability during heat sterilization

PETER STEHLE, PETER PFAENDER and PETER FÜRST
Institute of Biological Chemistry and Nutrition, University of Hohenheim, Stuttgart (F.R.G.)
(First received January 23rd, 1984; revised manuscript received March 2nd, 1984)

There is overwhelming evidence for an intracellular glutamine (Gln) depletion following injury and during episodes of malnutrition[1–6]. Thus, Gln might be a mandatory component of parenteral solutions designed for patients suffering from catabolism. However, attempts to include Gln in parenteral solutions failed because of the instability of this amino acid during storage and heat sterilization. During storage, glutamic acid (Glu) and ammonia are liberated[7], while the amino group of Gln is labile at higher temperatures, yielding toxic pyroglutamic acid (pGlu)[8,9].

Encouraged by recent reports showing that short chain peptides are rapidly transported and utilized[10,11], we have synthesized a Gln-containing peptide, L-alanyl-L-glutamine (Ala-Gln)[12], by applying a modification of the N-carboxy anhydride method[13]. The synthesis and purification was monitored by analytical capillary isotachophoresis[14] and high-performance liquid chromatography (HPLC)[15].

Intravenous injection of the peptide, however, requires that this product is stable during storage and heat sterilization. In the present communication these factors were investigated by applying analytical isotachophoresis.

MATERIALS AND METHODS

Materials

The stability of the synthesized dipeptide Ala-Gln and that of free Gln (Serva, Heidelberg, F.R.G.) were investigated before and after heat sterilization in aqueous solutions (2 m*M*). The solutions were kept at −80°C until analysed. Pyroglutamic acid (pGlu) and L-alanyl-L-glutamic acid (Ala-Glu) were provided by Sigma (Taufkirchen F.R.G.). Glutamic acid (Glu) and other chemicals were obtained from E. Merck (Darmstadt, F.R.G.).

Methods

Heat sterilization was carried out in sealed tubes at 120°C for 30 and 60 min, respectively. The tubes were flushed with nitrogen at −60°C before sealing.

Analytical isotachophoresis was performed by using a LKB 2127 Tachophor[16]. The instrument was equipped with a PTFE capillary (230 × 0.55 mm I.D.) without cooling plate. UV detection was made at 254 nm. A conductivity detector

0021-9673/84/$03.00 © 1984 Elsevier Science Publishers B.V.

TABLE I

OPERATIONAL SYSTEMS FOR ISOTACHOPHORETIC ANALYSES

MES = 2-Morpholinoethanesulphonic acid; Ammediol = 2-amino-2-methyl-1,3-propanediol; HPMC = hydroxypropylmethylcellulose.

System	*Leading electrolyte*	*Terminating electrolyte*
1	0.01 *M* Cl^- Histidine 0.4% HPMC pH 6.0	0.01 *M* MES Tris pH 6.0
2	0.05 *M* MES Ammediol 0.4% HPMC pH 9.1	0.01 *M* β-alanine Ammediol $Ba(OH)_2$ pH 10.2

was employed allowing detection of the "stepped" conductivity signal. The UV transmission and conductivity were monitored by using a two-channel recorder (Kipp & Zonen, Delft, The Netherlands) with a chart speed of 6 cm/min. The separations required about 20 min and the current at detection was 60 μA. The samples were injected at the leading/terminating electrolyte interface.

The electrolyte systems used are outlined in Table I. All chemicals used were of analytical grade. The water was purified with an Elgastat Spectrum water-purification system including reverse osmosis, activated carbon and nuclear grade ionization cartridges (Elga, Lane End, U.K.). Before preparing the electrolyte solutions, the water was boiled for 15 min and cooled without access of air.

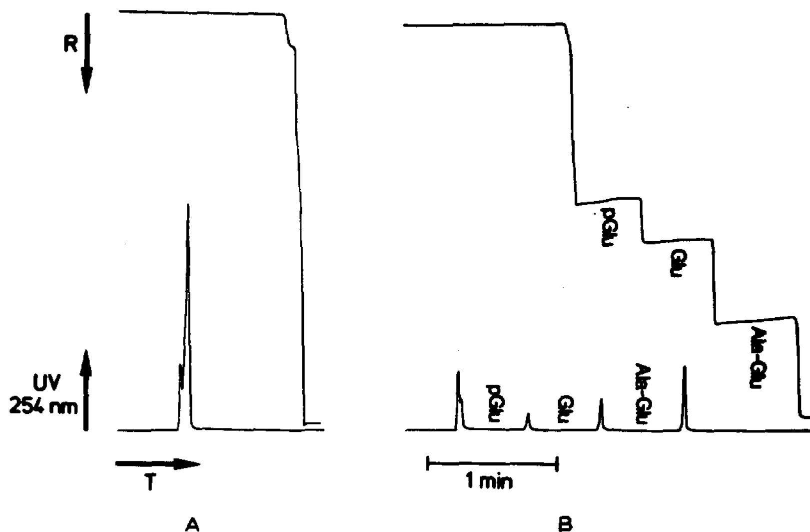

Fig. 1. Isotachophoretic analysis of a reference mixture of pGlu, Glu and Ala-Glu (electrolyte system 1, Table I). A, Electrolyte system; B, reference mixture (5 μl injected corresponding to 1.29 μg pGlu, 1.41 μg Glu and 2.18 μg Ala-Glu).

RESULTS AND DISCUSSION

In the present study special attention was paid to the analysis of the synthesized peptide Ala-Gln before and after heat sterilization. Simultaneous examination of pGlu, Glu and Ala-Glu is feasible by using an anionic electrolyte system at pH 6.0 (system 1, Table I).

The isotachophoretic pattern in electrolyte system 1 is illustrated in Fig. 1A. The presence of several impurities from the chemicals used is apparent. In Fig. 1B the isotachopherogram of an equimolar (2 m*M*) mixture of pGlu, Glu and Ala-Glu is depicted. This revealed three discrete non-UV-absorbing zones with conductivity step heights of 50.6 ± 0.7, 59.4 ± 0.6 and 76.2 ± 0.6% respectively (mean value ± S.D.) in seven consecutive runs. In separate studies, analyses of pGlu and Glu exhibited excellent linearity of the zone lengths at different concentrations, thus allowing quantitation of these amino acids (Fig. 2).

Applying electrolyte system 1, neither free pGlu and Glu nor Ala-Glu could be detected when analyzing equimolar amounts of the peptide Ala-Gln before (Fig. 3A, 1) and after heat sterilization for 30 min (Fig. 3A, 2) and 60 min (Fig. 3A, 3).

However, when applying the electrolyte system 2, analyses of the same preparations revealed a single non-UV-absorbing zone independently of whether the examinations were carried out before or after heat sterilization (Fig. 3B). Electrolyte system 2 (Table I) is especially suited to the detection of peptides as well as free Gln, as reported earlier[14]. Previous studies with field desorption mass spectrometry and nuclear magnetic resonance spectroscopy provided evidence that the single zone detected corresponds to the peptide Ala-Gln[12]. Compared with the untreated sample (Fig. 3B, 1), the zone length of Ala-Gln was unaltered after heat sterilization for 30 min (Fig. 3B, 2) and was slightly increased (about 10%) after 60 min (Fig. 3B, 3).

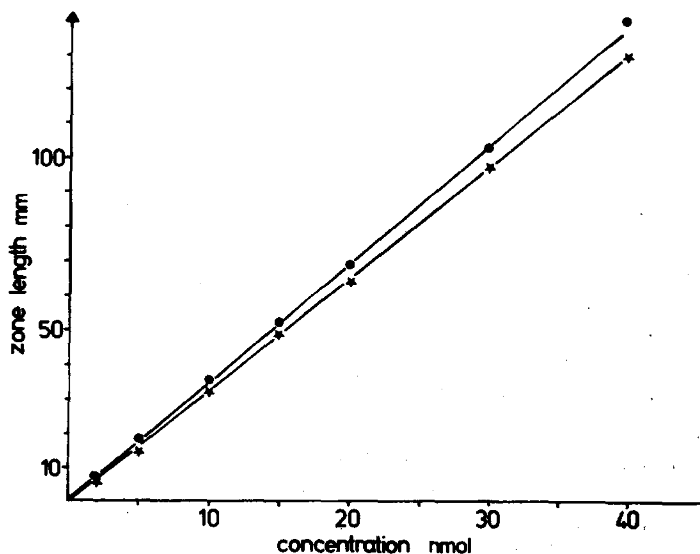

Fig. 2. Standard curves for pGlu (★—★) and Glu (●—●) in electrolyte system 1 (Table I).

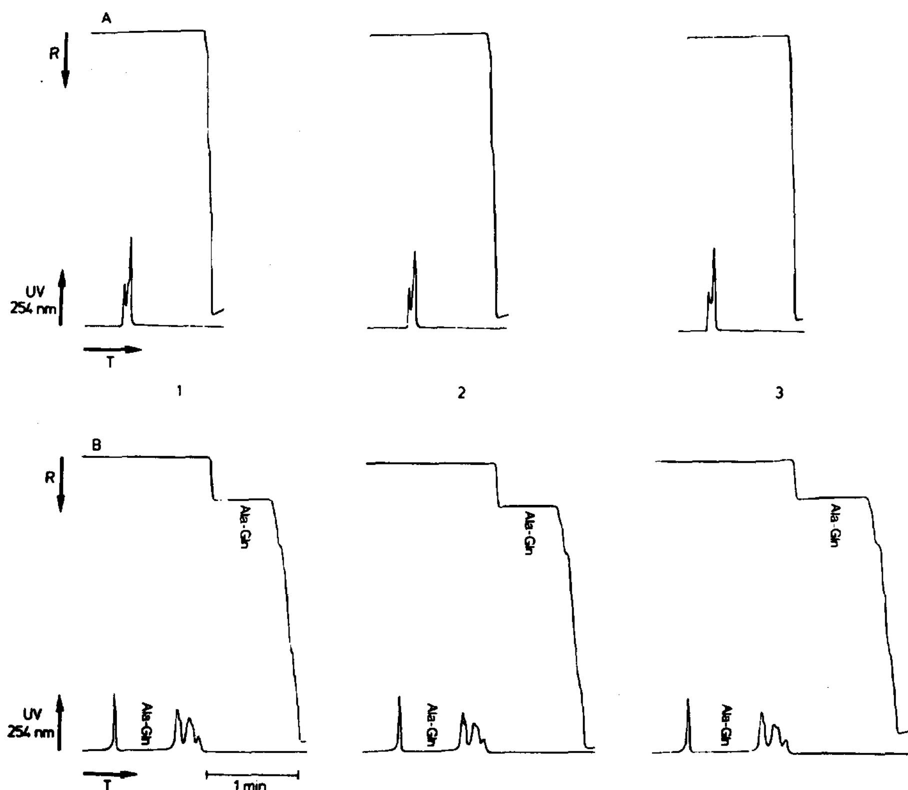

Fig. 3. Isotachophoretic analyses of Ala-Gln before and after the sterilization procedure using electrolyte systems 1 (A) and 2 (B) (see Table I). 1, Before the sterilization procedure; 2, after heat sterilization for 30 min; 3, after heat sterilization for 60 min. For each separation, 5 μl were injected corresponding to 2.17 μg Ala-Gln in the untreated sample.

This increase might be due to a loss of condensed water in the glass tubes. Contaminations from the electrolyte system are clearly apparent in Fig. 3B. Minor UV-absorbing impurities derived from β-alanine and 2-amino-2-methyl-1,3-propanediol have to be taken into account[14].

Several reports have been made of the instability of free Gln[7–9], but to our knowledge carefully conducted investigations are still lacking. Consequently, it is also of interest to investigate the effect of heat sterilization of free Gln in aqueous solution with the aid of analytical isotachophoresis. The results of analyses with the electrolyte system 1 are outlined in Fig. 4A which shows that no pGlu or Glu was present in the untreated sample (Fig. 4A, 1). However, a single non-UV-absorbing zone corresponding to pGlu could be detected after heat sterilization for 30 and 60 min respectively (Fig. 4A, 2 and 4A, 3). Using the calibration curve for pGlu (Fig. 2), it was possible to evaluate the absolute amount of pGlu formed; thus, 35% after 30 min and 67% after 60 min of the injected (heat-sterilized) Gln had been converted into pGlu. When analyzing these preparations with the electrolyte system 2 the decrease in the Gln concentration could be followed (Fig. 4B). In comparison to the untreated solution (Fig. 4B, 1), a 27% and a 70% decrease in the original amount

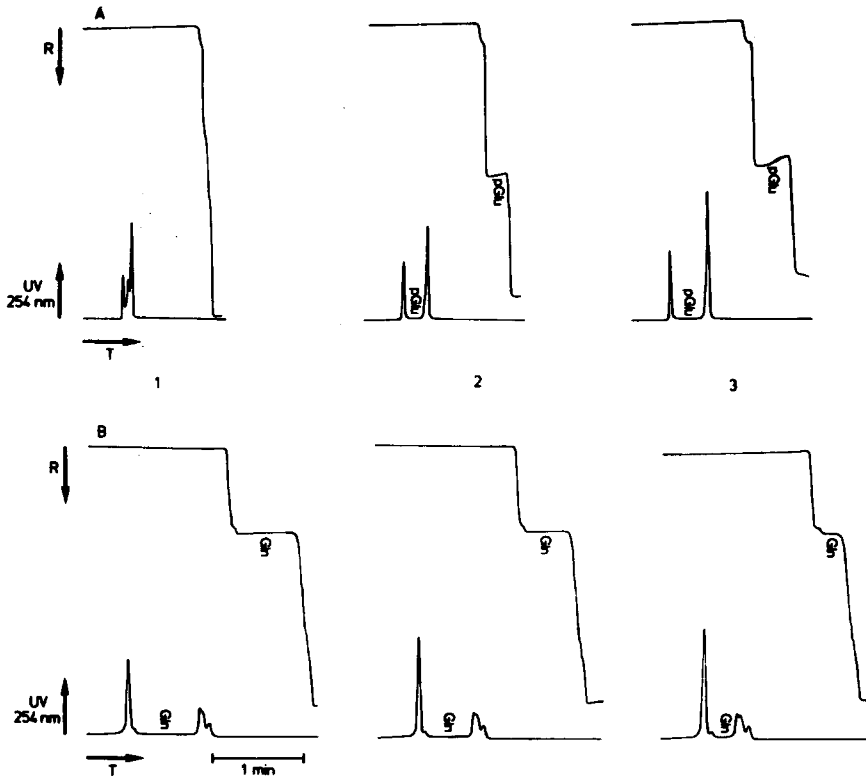

Fig. 4. Isotachophoretic analyses of free Gln before and after the sterilization procedure using electrolyte systems 1 (A) and 2 (B) (Table I). Key as in Fig. 3. For each separation, 5 μl were injected corresponding to 1.46 μg Gln in the untreated sample.

of Gln could be observed after heat sterilization for 30 and 60 min respectively (Fig. 4B, 2 and 4B, 3). These values compare very favourably with those obtained when the extent of degradation of Gln was estimated on the basis of the amount of pGlu formed (Fig. 4A).

In conclusion, the present investigation indicates that the synthesized peptide Ala-Gln is stable during heat sterilization. The results also show that free Gln forms toxic pGlu. The explanation for these findings may depend on the fact that the carboxamide group of free Gln is hydrolyzed via a concerted mechanism by the neighbouring α-amino group, whereas the latter group with its lone electron pair cannot take part in this reaction when engaged in peptide linkage[17,18].

Analytical isotachophoresis enables direct and simultaneous determination of free Glu and pGlu within 20 min. This was highly appreciated in the present work, because pGlu is hardly detectable by alternative methods such as amino acid analysis or thin-layer chromatography with ninhydrin and/or fluorescence detection.

Isotope studies in rats have indicated that the peptide Ala-Gln is easily available and rapidly used for protein synthesis[19,20]. Furthermore, it could be demonstrated that the intravenously administered peptide provides free Gln for maintenance of liver and muscle free intracellular Gln pools in severely catabolic rats re-

ceiving long-term continuous total parenteral nutrition (TPN)[20,21]. These results acquired from animal experiments and the stability of Ala-Gln during heat sterilization as demonstrated in the present study strongly indicate that this synthetic peptide might be a suitable component in parenteral solutions.

ACKNOWLEDGEMENT

We appreciate the financial support of Pfrimmer & Company, Erlangen, F.R.G.

REFERENCES

1 E. Vinnars, J. Bergström and P. Fürst, *Ann. Surg.*, 163 (1975) 665.
2 J. Askanazi, P. Fürst, C. B. Michelsen, D. H. Elwyn, E. Vinnars, F. E. Gump, F. E. Stinchfield and J. M. Kinney, *Ann. Surg.*, 191 (1980) 465.
3 J. Askanazi, Y. A. Carpentier, C. B. Michelsen, D. H. Elwyn, P. Fürst, L. R. Kantrowitz, F. E. Gump and J. M. Kinney, *Ann. Surg.*, 192 (1980) 78.
4 E. Roth, J. Funovics, F. Mühlbacher, M. Schemper and W. Mauritz, *Clin. Nutr.*, 1 (1982) 25.
5 P. J. Milewski, C. J. Threlfall, D. F. Heath, I. B. Holbrook, K. Wilford and M. H. Irving, *Clin. Sci.*, 62 (1982) 83.
6 E. Vinnars, B. Holmström, B. Schildt, A-C. Odebäck and P. Fürst, *Clin. Nutr.*, 2 (1983) 3.
7 L. Heller, A. Becher, A. Beck and F. Müller, *Klin. Wochenschr.*, 45 (1967) 317.
8 P. A. Hamilton, *J. Biol. Chem.*, 158 (1945) 397.
9 A. Meister, *Physiol. Rev.*, 36 (1956) 103.
10 D. M. Matthews and S. A. Adibi, *Gastroenterology*, 71 (1976) 151.
11 S. A. Adibi and Y. A. Kim, in L. R. Johnston (Editor), *Physiology of the Gastrointestinal Tract*, Raven Press, New York, 1981, pp. 1073–1095.
12 P. Stehle, B. Kühne, P. Fürst and P. Pfaender, *J. Appl. Biochem.*, 4 (1982) 280.
13 R. G. Denkewalter, H. Schwam, R. G. Strachan, T. E. Beesley, D. F. Veber, E. F. Schoenewaldt, H. Barkemeyer, W. J. Paleveda, T. A. Jacob and R. Hirschmann, *J. Amer. Chem. Soc.*, 88 (1966) 3163.
14 P. Stehle, B. Kühne, P. Pfaender and P. Fürst, *J. Chromatogr.*, 249 (1982) 408.
15 Th. Graser, H. Godel, P. Stehle, P. Pfaender and P. Fürst, in H. Aigner (Editor), *Königsteiner Chromatographie-Tage*, Waters, Eschborn, pp. 140–155.
16 L. Arlinger, *J. Chromatogr.*, 91 (1974) 785.
17 R. D. Dimarchi, J. P. Tam, S. B. H. Kent and R. B. Merrifield, *Int. J. Pept. Protein Res.*, 19 (1982) 88.
18 E. S. Gould (Editor), in *Mechanismus und Struktur in der Organischen Chemie*, Verlag Chemie, Weinheim, 1964, p. 388.
19 I. Amberger, P. Fürst, H. Godel, Th. Graser, P. Stehle and P. Pfaender, *Hoppe-Seyler's Z. Physiol. Chem.*, 364 (1983) 1253.
20 S. Albers, R. Abele, I. Amberger, J. Mangold, P. Pfaender and P. Fürst, *Aktuel. Ernährungsmed.*, (1984) in press.
21 I. Amberger, S. Albers, P. Pfaender and P. Fürst, *Infusionstherapie*, 11 (1984) 57.