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Note

Isotachophoretic analysis of two synthetic peptides

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The inclusion of tyrosine (Tyr) and glutamine (Gln) in parenteral solutions is a prerequisite for optimum protein economy in chronic uremia[1,2], in childhood[3–5] and in post-injury states[6–8]. The extremely poor solubility of free Tyr and the formation of toxic pyroglutamic acid during heat sterilization of free Gln[9] have hampered the use of these amino acids in such solutions.

In a previous paper the synthesis and characterization of Tyr- and Gln-containing peptides were described[10]. Two peptides, L-alanyl-L-glutamine (Ala-Gln) and N^2-L-tyrosyl-N^6-L-tyrosyl-L-lysine [Tyr-Lys(Tyr)], were synthesized by applying the N-carboxy anhydride method in an aqueous medium[11,12]. The synthetic peptides were highly soluble and were stable during heat sterilization, thus enabling inclusion of Gln and Tyr in solutions intended for parenteral nutrition.

In the present work both these peptides were investigated by using capillary analytical isotachophoresis. This technique has been tried since it has been found to be a rapid and sensitive method for determination of purity[13,14], and has also been shown to be a useful tool in the qualitative and quantitative analysis of peptides[15,16]. We could demonstrate that Ala-Gln was isotachophoretically pure, whereas minor impurities were present in Tyr-Lys(Tyr). Each of the zones separated corresponds to a single synthetic compound and excellent linearity was apparent, allowing estimation of the quantity and recovery of the respective peptides by measuring the zone lengths.

MATERIALS AND METHODS

Materials

Aqueous solutions of the synthetic peptides, in concentrations and amounts given in Figs. 1 and 2, were analyzed. For comparison, solutions of free alanine (Ala), Gln and Tyr were investigated alone or in doping experiments. All free amino acids were purchased from E. Merck (Darmstadt, G.F.R.) and were of analytical grade. The peptide syntheses and the preparative procedures have been described previously[10].

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Analytical isotachophoresis

Isotachophoretic separations were performed on a *LKB 2127 Tachophor*[17]. The instrument was equipped with a *PTFE* capillary (*610 × 0.5 mm I.D.*). *UV* detection was made at 254 nm. A newly developed conductivity detector was used and the linear stepwise conductivity signal was detected. The UV transmission and conductivity were monitored by using a two-channel recorder (Kipp and Zonen, *Delft*, The Netherlands) with a chart speed of 6 cm/min. The separations required about *35* min and the current at detection was 80 *μA*.

Electrolytes

The chemicals used were of analytical grade. The water was glass distilled and ion exchanged, freshly boiled for 15 min and cooled without access of air.

Leading electrolyte solution for rinsing and filling the capillary (100 ml). A mixture of 107 mg MES (2-morpholinoethanesulphonic acid) (Fluka, Buchs, Switzerland), 212 mg Ammediol (2-amino-2-methyl-1,3-propanediol) (Fluka) and 0.4% hydroxypropylmethylcellulose (HPMC; Methocel 90 HG 15000 cps; Dow Chemical, Midland, MI, U.S.A.) was filtered through a 5-*μm* membrane filter (Schleicher & Schüll, Dassel, G.F.R.). The pH of this solution was 9.1.

Leading electrolyte solution for the electrode vessel (100 ml). A mixture of 107 mg MES and 225 mg Ammediol (to achieve pH 9.1 in this system, additional 13 mg of Ammediol have to be added) was filtered through a 0.45-*μm* membrane filter (Schleicher & Schüll).

Terminating electrolyte solution (100 ml). 89 mg *β*-alanine (Serva, Heidelberg, G.F.R.) and 106 mg Ammediol were dissolved in 90 ml of water and brought to pH 10.2 with freshly prepared and filtered $Ba(OH)_2$ solution. The solution was then made up to 100 ml.

RESULTS AND DISCUSSION

Isotachophoretic analyses were performed repeatedly on the two synthetic peptides as exemplified in Figs. 1 and 2.

Satisfactory detection and recovery of the peptides in amounts less than 0.5 nmol (about 0.11–0.24 *μg*) was achieved by using the new conductivity detector.

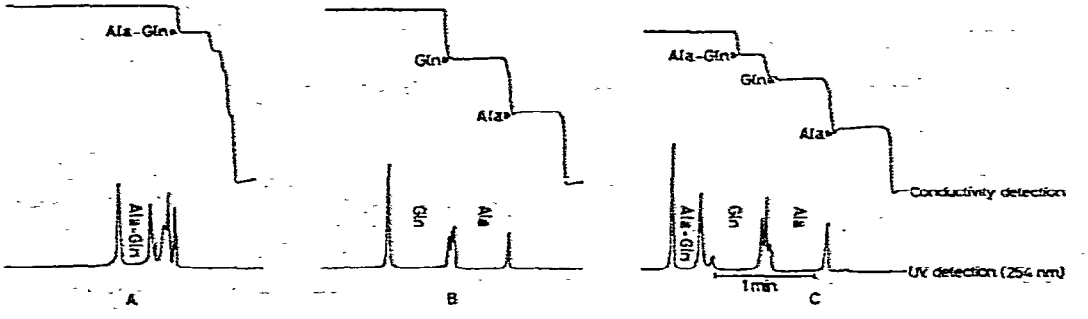

Fig. 1. Isotachophoretic analyses of Ala-Gln, free Ala and free Gln. A, Ala-Gln, 5 *μl* injected corresponding to 2.17 *μg*; B, mixture of free Ala and free Gln, 5 *μl* injected corresponding to 0.89 *μg* Ala and 1.46 *μg* Gln; C, mixture of Ala-Gln, free Ala and free Gln, 5 *μl* injected corresponding to 2.17 *μg* Ala-Gln, 0.89 *μg* Ala and 1.46 *μg* Gln.

Although a direct comparison with the thermal detector was not made in the present study, earlier data indicated that a minimum amount of 10 nmol of peptide was required when using thermometric detection[13,14] and quantification was restricted to the UV detector only. Since Ala-Gln cannot be detected by UV absorption (Fig. 1), the high degree of sensitivity of the conductivity was advantageous in the present investigation.

Analysis of the synthetic peptide Ala-Gln resulted in a single non-UV-absorbing zone, characterized with a conductivity step height of 14.8% (Fig. 1). For comparison, the separation of free Ala and free Gln is also shown. Both free amino acids revealed distinct non-UV-absorbing zones with conductivity step heights of 60.9% and 28.7%, respectively.

The results demonstrate that no free amino acids were present in the purified synthetic peptide Ala-Gln. This was verified by doping experiments, in which an equimolar mixture of Ala-Gln, free Gln and free Ala was analyzed. As illustrated in Fig. 1, no mixed zones were observed, and the three compounds were recovered according to their respective net mobilities. The results of these experiments indicate that the product is isotachophoretically pure.

Analyses of Tyr-Lys(Tyr) showed that this product consists of a major UV-absorbing zone corresponding to the synthetic peptide and two minor UV-absorbing zones which may correspond to overreaction products as di- or tetrapeptides (Fig. 2). The major zone is characterized by a UV level of 38.7% and a conductivity step height of 32.2%, respectively. In Fig. 2 the resolution of free Tyr is compared with that of the main tripeptide, showing the same conductivity step height (32.2%), but different UV level (43.2%). Free lysine (Lys) could not be analyzed with this system, as also noted by other investigators[13]. However, thin-layer chromatography (TLC) and amino acid analysis of the purified synthetic product showed no evidence of free Lys, while after acid hydrolysis free Lys was fully recovered[10]. In a doping experiment the purified products and free Tyr were mixed in equimolar amounts and analyzed as shown in Fig. 2. This experiment demonstrates that the main characteristic zones for the tripeptide and free Tyr were recovered according to their mobilities.

These results may suggest that further purification of the synthetic tripeptide Tyr-Lys(Tyr) may be required. In the present study the major zone corresponding to the tripeptide amounts to more than 75% of the injected preparation. In preliminary

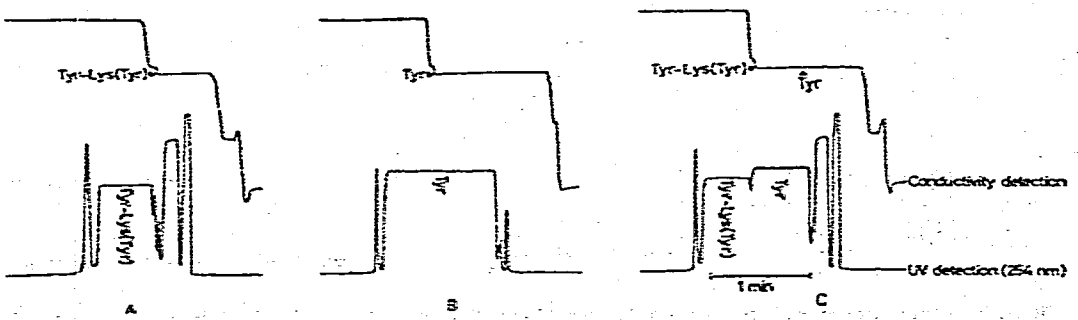

Fig. 2. Isotachophoretic analyses of Tyr-Lys(Tyr) and free Tyr. A, Tyr-Lys(Tyr), 5 μl injected corresponding to 4.73 μg; B, free Tyr, 5 μl injected corresponding to 1.81 μg; C, mixture of Tyr-Lys(Tyr) and free Tyr, 5 μl injected corresponding to 4.73 μg Tyr-Lys(Tyr) and 0.91 μg Tyr.

unpublished studies, a purity of more than 90% could be achieved by changing the conditions for the gel chromatography procedure. Minor amounts of overreaction products were, however, still present. The exact structure of these minor compounds remains to be defined. Their presence may not necessarily invalidate the material for nutritional applications since di- and tetrapeptides are similarly transported and utilized as the tripeptide[18,19].

The characteristic values for the two synthetic peptides and the free amino acids analyzed are summarized in Table I.

TABLE I

CHARACTERISTIC VALUES FOR THE PEPTIDES AND THE FREE AMINO ACIDS

Compound	*Conductivity step height (%)**	*UV level (%)***
Ala-Gln	14.8	
Glutamine	28.7	
α-Alanine	60.9	
Tyr-Lys(Tyr)	32.2	38.7
Tyrosine	32.2	43.2

* As a percentage of the mobility of the terminator.
** As a percentage of full scale.

The conductivity step height of substances expressed as per cent of the conductivity step from the leading ion to the terminating ion is inversely proportional to the net mobility[20]. The relative step heights are characteristic for each compound and the step lengths are proportional to the amount of each compound. Similarly the UV zone length is directly proportional to the amount of a compound in equilibrium with the leading electrolyte, according to the Kohlrausch formula[21]. By applying the above principles, we determined the amounts of peptides by measuring the respective zone lengths and by calculating from the absolute amounts applied. When evaluating single zones at different concentrations, an excellent linearity was found allowing estimation of the quantity of peptides by measuring the zone lengths. The zone lengths detected and measured for Ala-Gln and Tyr-Lys(Tyr) are recorded in Fig. 3. It was possible to inject up to 20 μl, corresponding to 40 nmol, without the occurrence of mixed zones.

As pointed out earlier[13], even the purest commercially available chemicals contain small amounts of UV-absorbing impurities. Figs. 1 and 2 show that such impurities were present with the terminating β-alanine. When analyzing the synthetic peptide Ala-Gln the above impurities occurred after the zone, but between the zones when analyzing the free amino acids Ala and Gln. It has been claimed that such impurities with different net mobilities serve as markers indicating the zone boundaries between two non-UV-absorbing sample zones[13]. In our hands, however, the presence of a "separation peak" between two non-UV-absorbing zones was found to be independent of the occurrence of impurity peaks. This may suggest that the "separation peak" is not necessarily related to a UV-absorbing material.

In an earlier study the two synthetic peptides were characterized by TLC, amino acid analyses and PMR[10]. The previous data are in good agreement with those

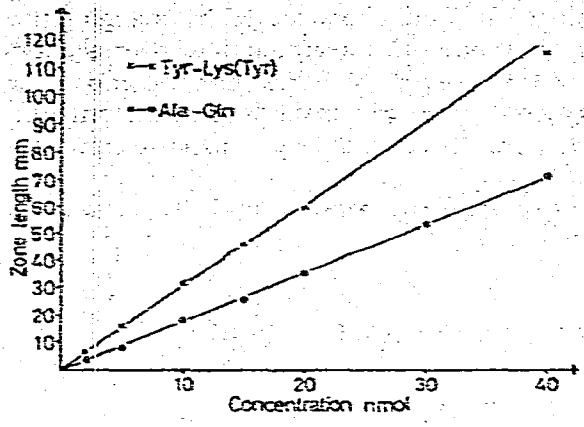

Fig. 3. Quantification by measuring the zone length.

reported in the present paper. However, analytical isotachophoresis was found to be more rapid and sensitive compared to the above methods. A further obvious advantage of the present method is the possible concomitant quantitative evaluation of the purified peptides.

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