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Note

Determination of dipeptides in protein hydrolysates for total parenteral nutrition

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The use of protein hydrolysate or crystalline amino acid solutions as nutritional sources in pathological conditions is increasing in clinical practice[1,2]. The choice of the type of product depends on the way it is to be administered. For instance, for parenteral nutrition a casein hydrolysate with a relatively high percentage of oligopeptides would be the most suitable[3]. On the other hand, direct infusion of crystalline amino acids into the bloodstream is often thought to be more appropriate[4]. From a general point of view, the infusion of oligopeptides is not metabolically useful as they must be hydrolysed to their single amino acid constituents for utilization[5]. The industrial process for the hydrolysis of protein does not always avoid the presence of small peptides that could display uncontrolled biological activity[6]. Hydrolysis may also cause racemization of some amino acids, especially glutamic and aspartic acids[7]. The infusion of racemic amino acids is known to cause acidosis in man[8].

In order to overcome the presence of oligopeptides in casein hydrolyzates several companies follow sophisticated preparative techniques, claiming that their products are completely free of these compounds. As a means of checking this, we describe in this paper a comprehensive analytical approach for the detection of the presence of very small amounts of small peptides in an amino acid mixture of protein hydrolysates.

EXPERIMENTAL

Chelex-100 (Na^+) resin (200–400 mesh) was purchased from Bio-Rad Labs (Richmond, CA, U.S.A.). 5-Dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl), Dns-amino acid standards and diethyldithiocarbamate were obtained from Sigma (St. Louis, MO, U.S.A.). Glycyl-L-tyrosine, glycyl-L-tryptophan and glycyl-

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glycine were obtained from Aldrich Europe (Beerse, Belgium). Different casein hydrolysate batches were kindly donated by Pierrel SpA (Milan, Italy).

The Chelex–copper resin was prepared according to Bellinger and Buist[9] and Rothenbühler *et al.*[10]. A 100-g amount of Chelex-100 resin was hydrated in 1000 ml of saturated copper(II) sulphate solution and kept at −4°C for 24 h. A glass column (300 × 40 mm I.D.) was filled with the resin and washed with distilled water for 10 h until no leakage of copper was evident in the eluate using a 1% diethyldithiocarbamate solution. The analyses were carried on smaller glass columns (150 × 10 mm I.D.) prepared as follows. They were filled to the first 2 cm with Chelex resin free of copper and then Chelex–copper resin was added to form a layer 10 cm high. They were equilibrated with 70 ml of 0.05 *M* sodium chloride solution adjusted to pH 11 with 33% ammonia solution. The column eluates were collected with an LKB (Bromma, Sweden), Uvicord III fraction collector connected to a detector unit operated at 275 nm. Amino acids and peptides were Dns derivatized according to Gray[11]. High-performance liquid chromatographic (HPLC) separation was carried out according to Weiner and Tishbee[12] using a Perkin-Elmer 3B apparatus connected with a Perkin-Elmer Model LC fluorimeter with excitation wavelength 340 nm and emission wavelength 510 nm. The analyses were performed with a 250 × 4.6 mm I.D. LiChrosorb RP-18 (5 μm) (Merck, Darmstadt, G.F.R.) column and the two solvent systems used for the stepwise gradient were buffer A, 0.5 *M* sodium acetate buffer (pH 6.3)–12.5% acetonitrile–5% isopropanol and buffer B, 0.5 *M* sodium acetate buffer (pH 6.5)–50% acetonitrile–1% isopropanol.

RESULTS

Fig. 1 shows the separation on the Chelex–copper column of 40 μg of glycyl-L-tyrosine added to 3 mg of the protein hydrolyzate. Peak 1 corresponds to the dipeptide and peak 2 to the pool of amino acids in the hydrolysate. The continuous line indicates the elution pattern of a blank column. As already reported[10], peptides are

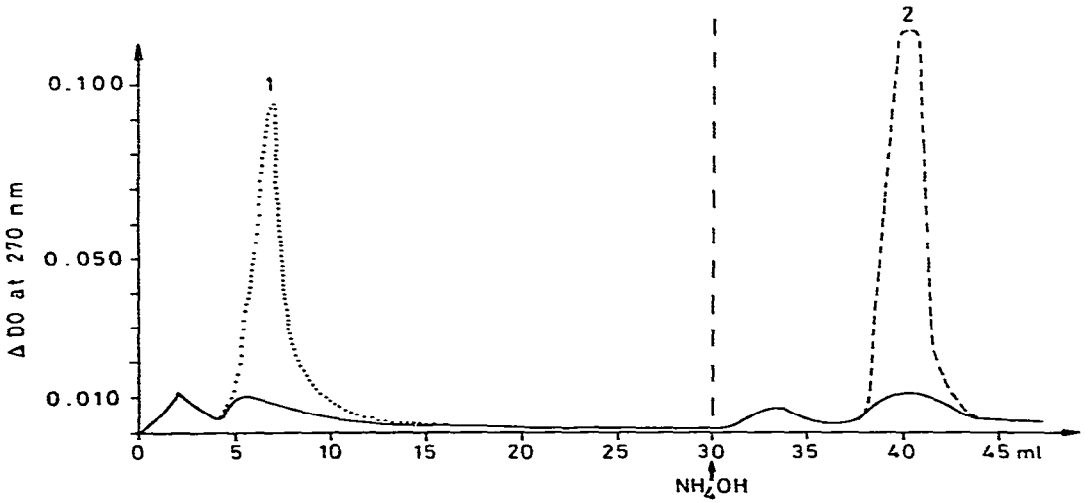

Fig. 1. Separation on a Chelex–copper column of 3 mg of an amino acid mixture containing 40 mg of glycyl-L-tyrosine. DO = optical density.

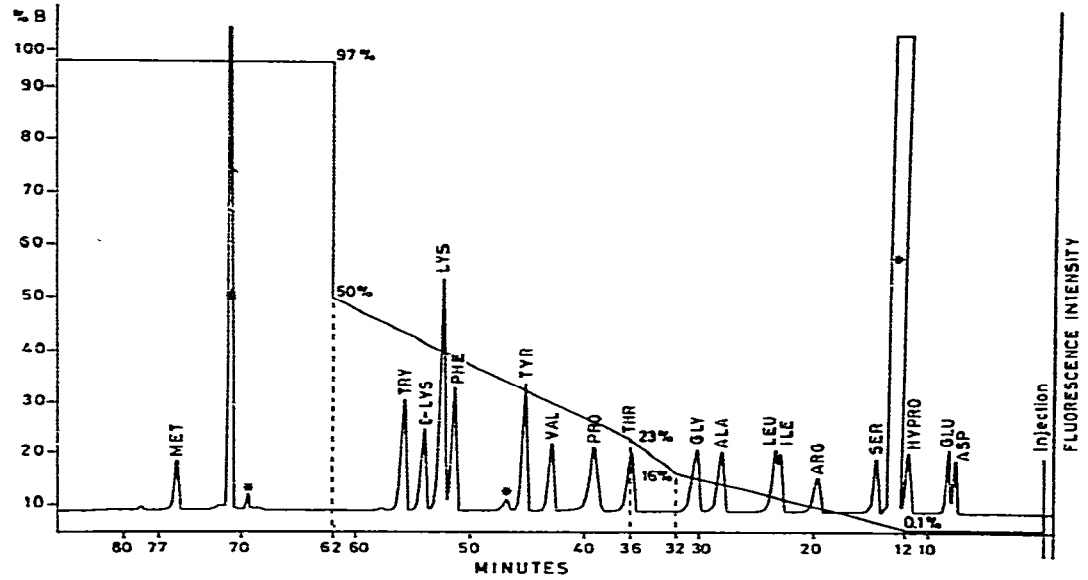

Fig. 2. HPLC separation of a mixture of pure amino acids after derivatization with Dns-Cl. The peaks marked with asterisks are Dns hydrolysis products.

collected in the first 20 ml of eluate and the amino acids are eluted only after application of 1 *M* ammonia solution on the column. The same was observed for glycyl-L-tryptophan and glycylglycine. The elution pattern of the latter dipeptide was monitored by measuring its content in the eluate by HPLC as described above. As a result

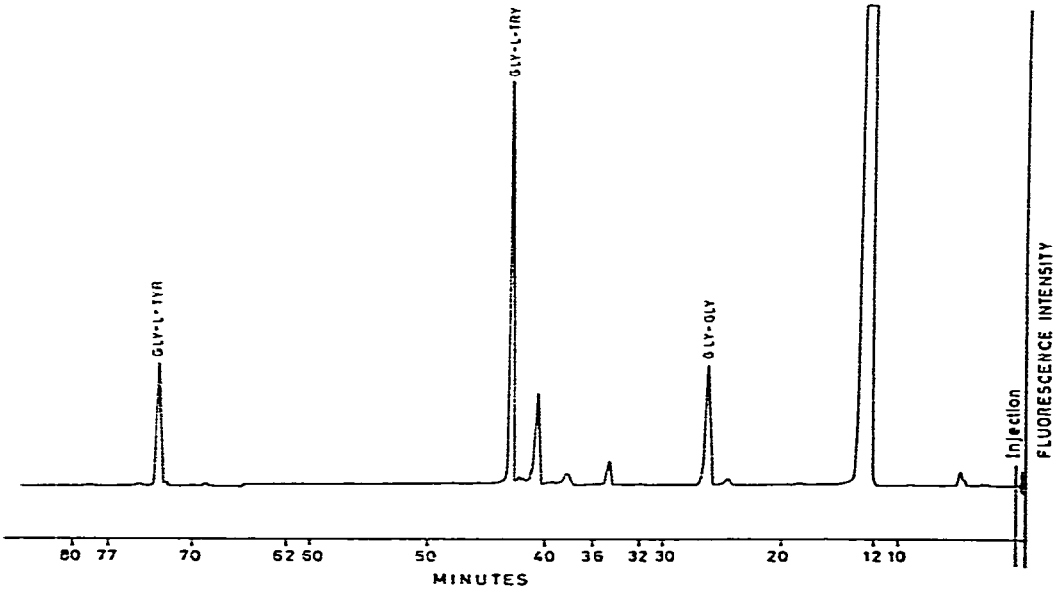

Fig. 3. HPLC separation of glycyl-L-tyrosine, glycyl-L-tryptophan and glycylglycine after derivatization with Dns-Cl.

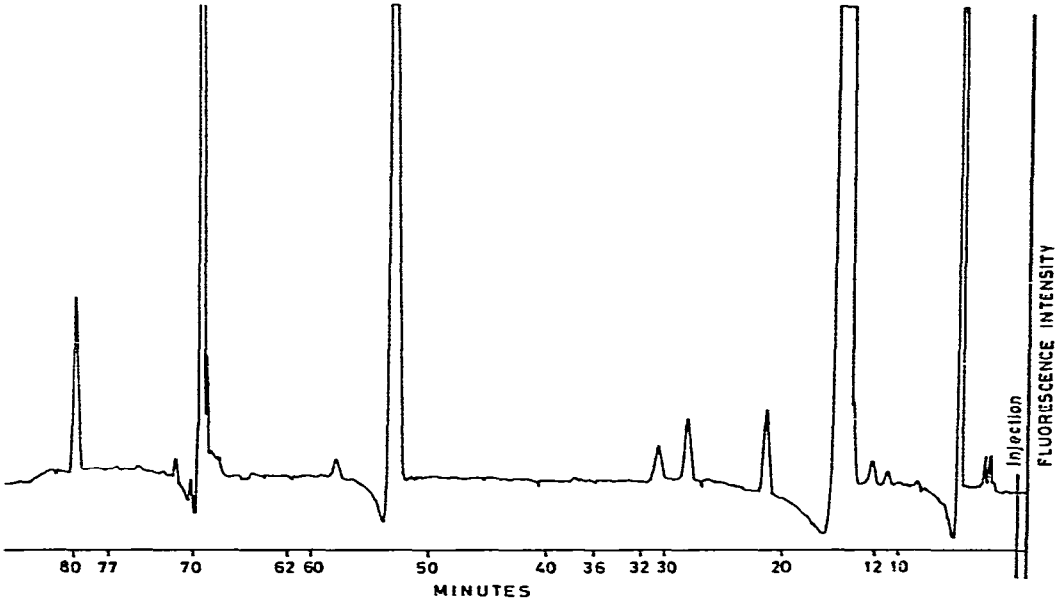

Fig. 4. HPLC separation of a blank sample of the first 30 ml from Chelex–copper column chromatography. The instrumental sensitivity is 100 times that in Figs. 2 and 3.

of these observations, in all subsequent experiments the first 30 ml were always collected and reduced to 2.7 ml under vacuum before performing the reaction with Dns-Cl on 0.5 ml of the concentrated samples.

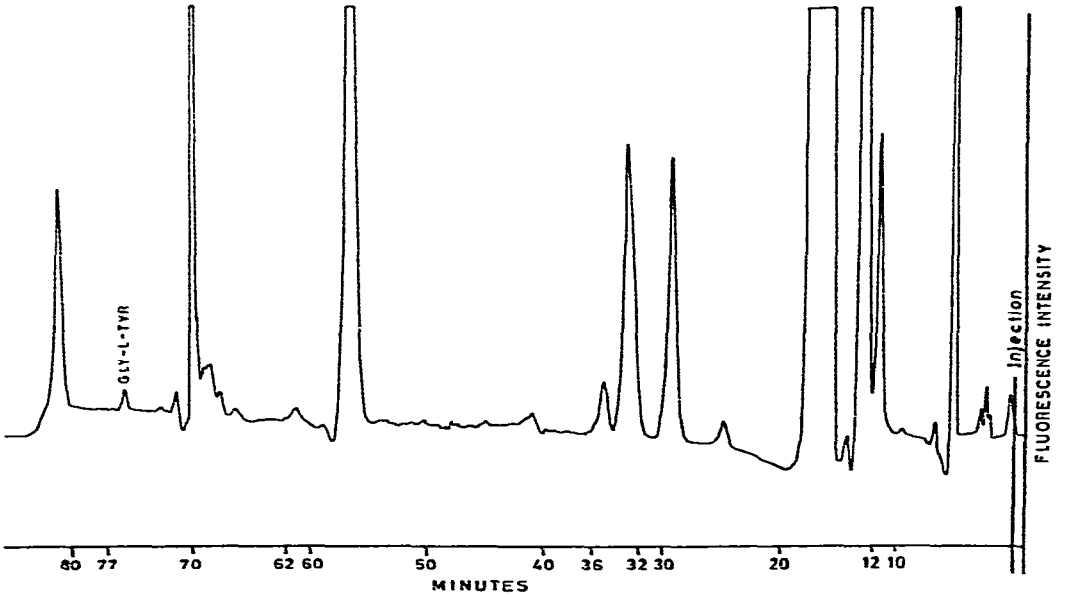

Fig. 5. HPLC identification of 1 μg of glycyl-L-tyrosine added to 3 mg of casein hydrolysate. Analysis was carried out after separation of the peptide from the amino acids by Chelex–copper column chromatography.

The recovery of all three dipeptides eluted and collected separately after their addition to 3 mg of protein hydrolysate in the range 10–100 μg was 88 ± 8%.

Fig. 2 shows the chromatogram for the HPLC separation of Dns-amino acid standards; each peak corresponds to 10 nmole of derivatized amino acids. Fig. 3 shows separation of the dipeptides used as standards following derivatization with Dns-Cl; each peak corresponds to 10 nmole of dipeptide injected.

Fig. 4 shows a chromatogram of the first 30 ml of a blank sample of the eluate from the Chelex–copper column. The sensitivity of the HPLC analyses was increased 100-fold using the highest instrumental amplification. The chromatogram obtained after dansylation of the first 30 ml of eluate from the Chelex–copper column after application of 3 mg of the protein hydrolysate contained no new peaks. Thus, under our experimental conditions, no peak(s) corresponding to unknown peptide(s) were observed.

In order to check the effectiveness of the separation procedure, 1 μg of glycyl-L-tyrosine was added to 3 mg of the amino acid hydrolysate. Fig. 5 shows the resulting chromatogram, clearly containing the peak of the dipeptide marker. The amount of dipeptide injected corresponds to 12.5 pmole and its recovery was calculated to be *ca.* 50%.

DISCUSSION

The technique presented combines, with small modifications, two analytical methods already described[9,12]. The Chelex–copper column enables us to separate quantitatively even very small amounts of peptides from an amino acid mixture, and the HPLC analysis seemed flexible enough to detect a wide variety of peptides[12]. Under our experimental conditions, the Chelex–copper had at the bottom a 2-cm layer of copper-free Chelex resin, in order to elute amino acids and/or peptides not in the complex form, thus overcoming the need to eliminate the copper from the chelates with hydrogen sulphide. The effectiveness of this separation was tested by calculating the recovery (a) of pure amino acids or peptide standards after elution from the Chelex–copper column and (b) of tyrosine after elution of synthetically pure $Cu(Tyr)_2$ from the same type of column (data not reported here).

Dns derivatization of peptides and their separation by HPLC form the basis of a strategy for determining their N-terminal amino acids and their composition. In fact, once the "unknown" peptide is collected from the HPLC column it can be (a) hydrolysed and reinjected and (b) hydrolysed, Dns derivatized and reinjected. The former approach permits the determination of the N-terminal amino acid and the latter the amino acid composition[12].

In the HPLC profile of Dns-amino acids presented in Fig. 1, the retention times of several amino acids differ considerably from those reported by Weiner and Tishbee[12], probably because of the higher concentration of acetate buffer used in our HPLC analysis (0.5 *vs.* 0.05 *M*).

The largest amount of amino acid mixture that could be added to the Chelex–copper column was 3 mg. This could have been increased by changing the column size but under our analytical conditions if any dipeptide contamination existed it was less than 0.03% of the product, as we could easily detect (recovery over 50%) 1 μg of glycyl-L-tyrosine added to 3 mg of casein hydrolysate.

In this paper we focused our attention mainly on dipeptides, as the casein hydrolysate taken into consideration was derived from extensive sulphuric acid hydrolysis. However, under the same analytical conditions the presence of tri- to pentapeptides could also be detected[12].

In conclusion, the procedure described is relatively simple for improving the quality control of protein hydrolysates to be used in total parenteral nutrition, and its use minimizes the risk of products with a composition that is not fully known being given to patients.

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