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SEPARATION OF PROTEIN AMINO ACIDS AS THEIR N(O)-ACYL ALKYL ESTER DERIVATIVES ON GLASS CAPILLARY COLUMNS

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SUMMARY

A comparison of the elution properties of the major protein amino acids as their N(O)-acyl alkyl ester derivatives (O-*n*-propyl, -*n*-butyl, -isopentyl; N(O)-trifluoroacetyl, -heptafluorobutyryl) on open-tubular glass capillary columns coated with SE-30, OV-17, OV-210 and EGA is described. A single-column separation to the baseline of the protein amino acids as their N(O)-heptafluorobutyryl *n*-butyl ester derivatives in less than 35 min was obtained on the SE-30 column. OV-210 columns have properties complementary to those of SE-30 columns and can be used as an aid to compound identification from retention time data. Separations of the amino acids from beer and dialysate from uremic patients are used to illustrate the practical possibilities of the method.

INTRODUCTION

Until recently, the most popular derivatives for the gas chromatography (GC) of amino acids have been the N(O)-trifluoroacetyl *n*-butyl ester derivatives, which were extensively evaluated by Zumwalt *et al.*[1]. The separation of these derivatives by GC with packed columns is difficult owing to the decomposition of some amino acid derivatives on polar phases and incomplete resolution of others on less polar stationary phases[1–8]. Usually, two packed columns coated with different stationary phases were required for the separation of all protein amino acids[1,8]. Gehrke and Takeda[9] described a single-column separation of the protein amino acids on a packed column of Apiezon M, but the separation obtained was poor and this column has received little attention in the literature.

The obvious advantages to be gained from a single-column separation of the major protein amino acids led several groups of workers to seek alternative derivatives to the N(O)-trifluoroacetyl *n*-butyl ester derivatives that would have better GC separation characteristics, including the N(O)-heptafluorobutyryl *n*-propyl ester[10,11], N(O)-heptafluorobutyryl isobutyl ester[12–16] and N(O)-heptafluorobutyryl isopentyl ester[17,18] derivatives. Analysis of these derivatives on non-polar silicone oil phases allowed a

single-column separation to be achieved but the baseline separation of all amino acids could not be obtained with packed columns.

With the derivatives currently available, complete baseline resolution of the protein amino acids can be achieved only by increasing the efficiency of the columns used for the GC separation. The possibilities of this approach were indicated by Jonsson *et al.*[19], who obtained a good separation of protein amino acids as their N(O)-heptafluorobutyryl *n*-propyl ester derivatives on a "chemically bonded" dimethylsiloxane glass capillary column. During the preparation of this paper, a report by Adams *et al.*[20] appeared, in which protein amino acids were separated as their N(O)-acetyl *n*-propyl ester derivatives on a glass capillary column coated with a mixture of Carbowax 20M and Silar 5CP.

In this paper we describe the separation of six N(O)-acyl alkyl ester amino acid derivatives on glass capillary columns coated with four stationary phases of different polarity.

EXPERIMENTAL

Reagents

Mixture A contained the amino acids alanine, glycine, threonine, valine, serine, leucine, isoleucine, proline and phenylalanine at a concentration of approximately 1 mg · ml^{-1} of each amino acid in 0.05 *M* hydrochloric acid. Mixture H was a commercial preparation obtained from Pierce Eurochemie (Rotterdam, The Netherlands), and contained all of the protein amino acids, except glutamine, asparagine and tryptophan, at a concentration of 2.5 μmole · ml^{-1} (cysteine could be detected as well as cystine in the sample received). Trifluoroacetic anhydride (TFAn) and heptafluorobutyric anhydride (HFBAn) in 1-ml ampoules were obtained from the same supplier.

All alcohols and solvents were dried and distilled before use. Methanol 1.25 *M* in hydrochloric acid and *n*-propanol, *n*-butanol and isopentanol all 3 *M* in hydrochloric acid were prepared by bubbling dry hydrogen chloride gas into the alcohols until the required increase in weight had been obtained.

General procedure for the preparation of derivatives

The N(O)-acyl alkyl ester amino acid derivatives were prepared by published procedures[1–19].

An aliquot (50–200 μl) of mixture A or H was added to a PTFE-lined screw-capped culture tube (10 cm × 1.5 cm O.D.; Sovirel, Paris, France) and evaporated to dryness at 100° in an aluminium heating block with the aid of a stream of nitrogen. Remaining traces of water were removed by re-evaporation with dichloromethane and the amino acids were esterified by the addition of 2 ml of acidic alcohol reagent, vortex mixing and heating at 100° for 20 min. After cooling, the reagents were removed with nitrogen at 70° and the last traces of alcohol removed by re-evaporation with dichloromethane. N(O)-Trifluoroacetyl derivatives were prepared by re-dissolving the residue in 500 μl of dichloromethane and 100 μl of TFAn with heating at 150° for 5 min in a temperature-controlled oil-bath. N(O)-Heptafluorobutyryl derivatives were prepared by the addition of 500 μl of acetonitrile and 100 μl of HFBAn with heating for 10 min at 150°. After cooling to room temperature, the reagents were removed with a stream of nitrogen and the residue was re-dissolved in fresh solvent–anhydride mixture (2:1, 150

μl) for immediate analysis or storage. In order to obtain the maximum peak height of histidine, on-column acylation was required (see Discussion).

Preparation of capillary columns

Methods currently in use in this laboratory for the preparation of capillary columns were described at the Second International Symposium on Glass Capillary Chromatography, Hindelang, and will be published separately[21]. For the preparation of the SE-30 column, the Pyrex glass was etched with gaseous hydrogen chloride, deactivated with triethanolamine solution and coated with a 0.5% solution of the stationary phase in dichloromethane by the static method[22]. For the preparation of the OV-17, OV-210 and EGA columns, the inner surface of the glass capillary column was first "whiskered" by published procedures[23] and, after deactivation, coated with the stationary phase using the static method for EGA and the dynamic method for OV-210 and OV-17.

The columns were conditioned overnight either at 220° or at their upper temperature limit (if lower), with nitrogen as the carrier gas; amino acid derivatives were separated with hydrogen as the carrier gas as it gives shorter analysis times. The conditions used for the separations are given below and in the legends to the figures.

All chromatograms were obtained with a Varian 2100 gas chromatograph with an injection port temperature of 225° and a detector temperature of 250°. The properties of the injection port splitter[24] and methods for coupling capillary columns to the detector and injector inserts[25,26] with PTFE shrinkable tubing have been described elsewhere.

Sample preparation and ion-exchange clean-up

Prior to GC analysis, samples of beer and dialysate from a uremic patient were purified by ion-exchange chromatography on a 5 × 1 cm column of Amberlite CG-120 (100–200 mesh); for the experimental conditions, see the papers by Otter and Taylor[27] and Zumwalt *et al.*[1].

For the analysis of dialysate, 3 l of dialysis fluid were evaporated to dryness at 40°, de-salted by triturating several times with 80% aqueous ethanol and the ethanolic extract was evaporated to dryness. The residue was re-dissolved in 75 ml of distilled water, 25 ml of 1% picric acid solution were added and the amino acid fraction was separated by ion-exchange chromatography in an identical manner to the beer samples[27].

RESULTS AND DISCUSSION

The general procedure for the formation of N(O)-acyl alkyl ester derivatives was successful for all amino acids included in mixture A.

The formation of the N(O)-heptafluorobutyryl butyl ester derivatives was investigated more thoroughly using mixture H. The different solubilities of the amino acids in the derivatizing reagents, their different hydrolytic stabilities and the presence of functional groups other than the amino acid function led to different rates of reaction under any set of experimental conditions for each individual amino acid. At the microgram level, few difficulties were encountered and the conditions given under Experimental represent the best compromise for the maximum peak area of each

amino acid on GC. For larger amounts of sample, problems arose owing to the poor solubility of cysteine, arginine, histidine, glutamic acid and lysine in the acidic *n*-butanol reagent. A the 1.0-mg level of these amino acids (except histidine) and using 2.0 ml of *n*-butanol 3 *M* in hydrochloric acid, the amino acids can be dissolved by intermittent heating at 100° and vigorous mixing with a vortex mixer. Once completely dissolved, these amino acids can be derivatized as indicated. With histidine, at this concentration it is necessary to use the longer trans-esterification procedure described by Zumwalt *et al.*[1].

The chromatography of histidine is difficult owing to the facile deacylation of the imidazoyl nitrogen derivative, which can occur at both the evaporation step for the removal of solvents and reagents and also in the injection port of the gas chromatograph[1,7]. Direct injection of the derivatized amino acid mixture produces no peak or a small peak for the monoacyl histidine derivative. To overcome this problem, on-column acylation as described by Roach *et al.*[7], in which a double injection of first the sample followed by a plug of fresh acylating reagent (equal to twice the volume of the original sample) a few seconds later, is made, allows a single peak for the diacyl-histidine derivative to be obtained. This can be used for the qualitative identification of histidine, but the peak height to molar ratio would indicate that this reaction is not quantitative for capillary columns with split injection.

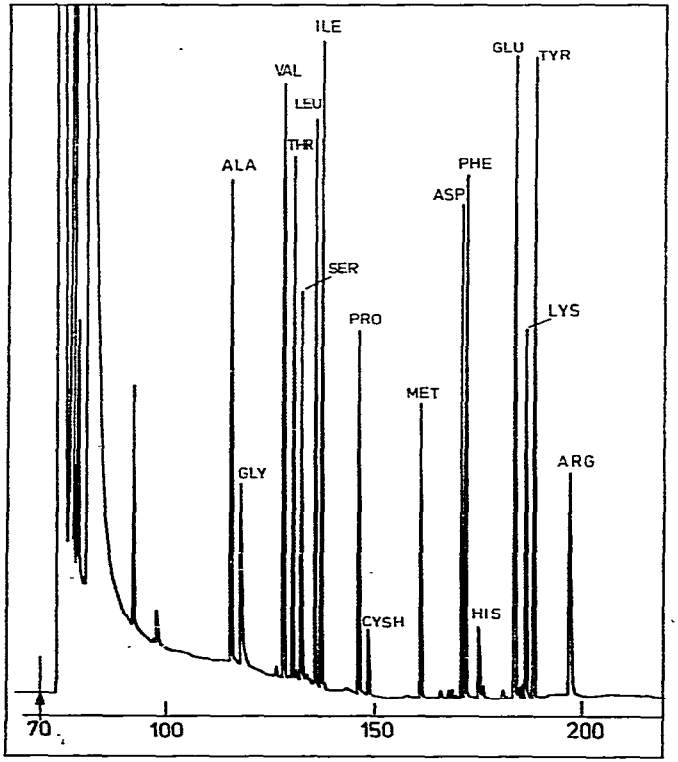

Fig. 1. Separation of the N(O)-heptafluorobutyryl *n*-butyl ester derivatives of amino acid mixture H using a 30 m × 0.25 mm I.D. glass capillary column coated with SE-30. Operating conditions: hydrogen carrier gas, flow-rate 3.3 ml·min^{-1}; temperature programme, 70° to 220° at 4°·min^{-1}.

Separation of amino acid derivatives on glass capillary columns

The best separation of amino acids was obtained with the N(O)-heptafluorobutyryl butyl ester derivatives on the SE-30 capillary column. The complete separation to the baseline of 17 protein amino acids in less than 35 min is illustrated in Fig. 1. Analysis of the same mixture on a capillary column coated with OV-17 (20 m × 0.25 mm I.D., carrier gas hydrogen at a flow-rate of 4.0 ml · min^{-1} and temperature programmed from 70 to 220° at 4° · min^{-1}) indicated a similar order of elution of amino acids but a poorer separation between peaks (*e.g.*, leucine–isoleucine not separated to the baseline; alanine–glycine–valine eluted as two peaks; similarly for glutamic acid–lysine–tyrosine).

The best resolution of amino acids on OV-210 was again obtained for the N(O)-heptafluorobutyryl *n*-butyl ester derivatives and the separation obtained for the latter derivatives of amino acid mixture H is illustrated in Fig. 2. Under no set of experimental conditions could a separation of the amino acids leucine and isoleucine be achieved on this phase. By changing the flow-rate of the carrier or by using a slower temperature programme, it is possible to obtain a baseline separation of proline and cysteine, but only at the expense of long analysis times for tyrosine, lysine and arginine.

Although the separation of amino acids is incomplete on this column, its

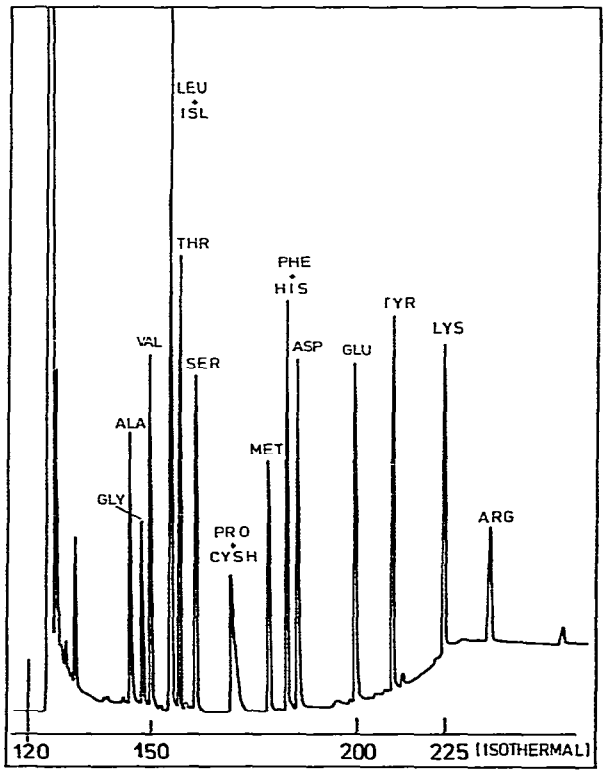

Fig. 2. Separation of the N(O)-heptafluorobutyryl *n*-butyl ester derivatives of amino acid mixture H using a 25 m × 0.25 mm I.D. glass capillary column coated with OV-210. Operating conditions: hydrogen carrier gas, flow-rate 3.0 ml·min^{-1}; temperature programme, 120° to 225° at 4°·min^{-1}.

separation characteristics are complementary to those of the SE-30 column, and as the same derivatives can be used on both columns it should prove useful for identification purposes.

On polyethyleneglycol adipate (EGA) as stationary phase, the results were generally less satisfactory. In contrast to the less polar capillary columns, the heptafluorobutyryl derivatives were more volatile and their separation properties were not as good as those of the trifluoroacetyl derivatives. The N(O)-trifluoroacetyl isopentyl esters gave the best separation, but the resolution was not to the baseline for all amino acids.

Applications

Analysis of the protein amino acids after ion-exchange column clean-up and conversion into the N(O)-heptafluorobutyryl *n*-butyl ester derivatives for GC separation on an SE-30 glass capillary column is reported here for two different problems currently of interest to this laboratory.

(a) The production of a consistent beer requires careful control of the amino acid composition. All of the amino acids common to protein structure are expected to be present, as well as some other related acidic substances[27]. For quality control there is a need for a simple and rapid method of amino acid analysis that does not

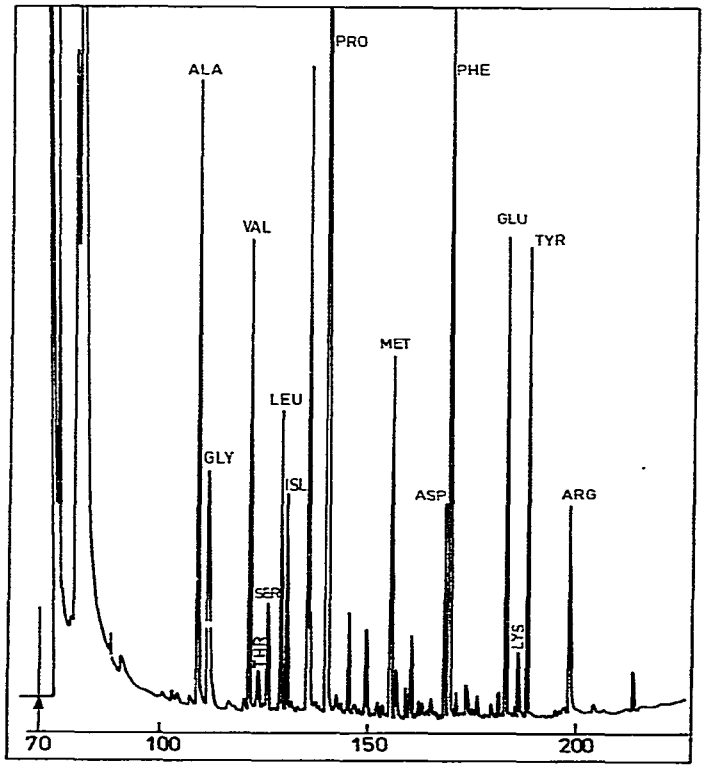

Fig. 3. Determination of the amino acid composition of beer (Belgian lager) after ion-exchange column clean-up. Operating conditions as in Fig. 1.

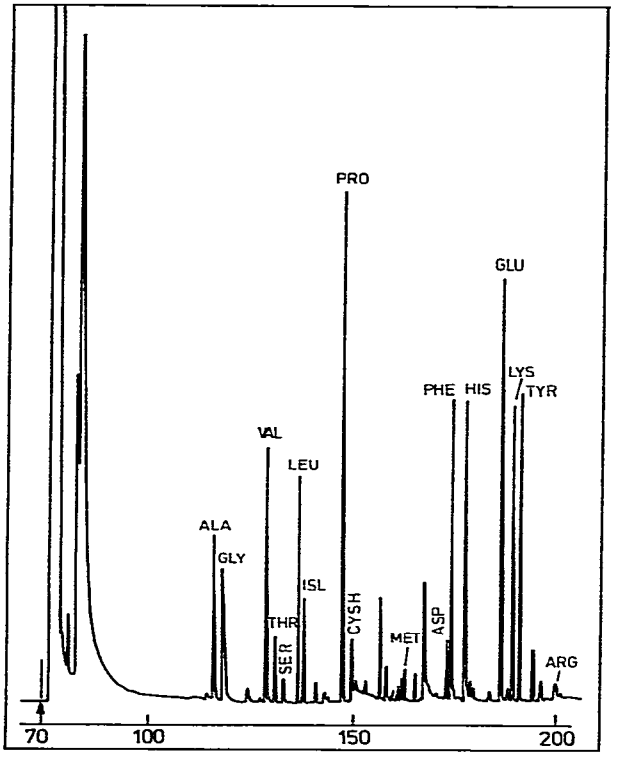

Fig. 4. Determination of the amino acid composition of uremic dialysate after ion-exchange column clean-up. Operating conditions as in Fig. 1.

require expensive or delicate equipment. An analysis of a commercial beer is shown in Fig. 3. Good separation of the amino acids from other samples was obtained.

(b) In connection with a study of the toxic principles present in dialysate obtained from patients undergoing treatment for chronic uremia, a method was required for the analysis of amino acids in this medium and for the identification of the amino acid composition of extracted peptides. An example of the amino acid profile obtained from a sample of uremic dialysate is shown in Fig. 4.

CONCLUSION

Glass capillary columns coated with four stationary phases of different polarity were evaluated for the separation of the protein amino acids as their N(O)-acyl alkyl ester derivatives. The column coated with SE-30 could resolve the N(O)-heptafluorobutyryl *n*-butyl ester protein amino acid derivatives to the baseline. The capillary column coated with OV-210 can be used as an aid in the identification of the same derivatives as a second column confirmation. The latter column does not give a separation of all amino acids. The practical value of the chromatographic system was demonstrated by the analysis of the protein amino acids in beer and in dialysate obtained from a uremic patient.

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