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IMPROVED GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PROTEIN AMINO ACIDS

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SUMMARY

The gas–liquid chromatographic method previously reported by us was improved with regard to the gas chromatographic columns and their operating conditions. It became possible to perform more accurate quantification of the 22 protein amino acids, including asparagine and glutamine, in less than 25 min on a dual set of columns. At the same time, the method allowed determination of the amino acids in the range 0.5–10 μg of each by eliminating the contaminant peaks derived from solvents and reaction vials. A single sample could be analysed in an overall time of 1 h.

INTRODUCTION

We have previously reported a simple and convenient derivatization method for gas–liquid chromatographic (GLC) determination of the protein amino acids[1], in which the volatile derivatives, N(O,S)-isobutyloxycarbonyl (isoBOC) methyl esters, of amino acids except arginine were prepared by two-step procedures involving isobutyloxycarbonylation with isobutyl chloroformate (isoBCF) in aqueous alkaline medium, followed by esterification with diazomethane. Prior to derivatization, arginine, by treatment with arginase, was converted into ornithine, which could be determined as its isoBOC methyl ester. Both asparagine and glutamine could also be derivatized by this method and separated by GLC in the presence of the other protein amino acids[2]. The derivatives could be easily and rapidly prepared and were stable, unlike most derivatives previously proposed for GLC determination of amino acids. However, our previous method was relatively time-consuming since it necessitated the operation of two columns under different thermal conditions for the separation of the 22 protein amino acids including asparagine and glutamine, and it has been subsequently shown that separation of some of the amino acid pairs was not entirely satisfactory especially when one of the pair was present in large excess. For these reasons, an investigation aimed at improving the previous method with regard to GLC columns and their operating conditions has been made. Furhtermore, by eliminating the contaminant peaks caused by solvents and reaction vials, we have been able to carry out the determination of all the protein amino acids in sub-microgram amounts.

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EXPERIMENTAL

Materials and reagents

All amino acids were obtained from Nakarai Chemicals (Kyoto, Japan); *p*-hydroxyphenylacetic acid, used as an internal standard, was supplied by Sigma (St. Louis, MO, U.S.A.). Two standard stock solutions (each 100 μg/ml), one containing the 22 protein amino acids and the other containing the 22 protein amino acids plus ornithine, were prepared in 0.1 *M* hydrochloric acid, and aliquots were taken and diluted with water to make the test mixtures as required. Arginase solution was prepared with some modifications as follows: prior to use, 10 mg of arginase (40 units/mg) (Sigma) were activated in 0.4 ml of 1.25 *M* ammonium acetate and 0.1 ml of 0.05 *M* manganese(II) sulphate at 37°C for 4 h. After the solution had been centrifuged for 1 min at 3000 rpm, the supernatant was separated, to which 0.5 ml of water was added. This solution was stored frozen when not in use.

IsoBCF stabilized with calcium carbonate (Tokyo Kasei Kogyo, Tokyo, Japan), was used without further purification and stored at 4°C. N-Methyl-N-nitroso-*p*-toluenesulphonamide and diethylene glycol monoethyl ether for the generation of diazomethane[3] were obtained from Wako (Osaka, Japan). Purified diethyl ether was prepared as previously reported[1]. The water which was used was treated as follows. Deionized water was distilled in an all-glass system after addition of several pellets of sodium hydroxide to remove the acidic contaminants. Sodium sulphate and sodium chloride were washed with methanol, then with purified diethyl ether and dried at 100°C. All other chemicals and solvents were the purest grades available from standard commercial sources. Materials for GLC were as follows: Poly-I-110, Poly-A-101A and FFAP (Applied Science Labs., State College, PA, U.S.A.) and 100–120 mesh Uniport HP (silanized) (Gasukuro Kogyo, Tokyo, Japan). Prior to coating, the support was treated as follows to attain maximum column efficiency. The support was floated on concentrated hydrochloric acid and complete contact with the liquid was ensured by gentle swirling. The grey and black particles which were precipitated and the acid were removed by pipette. This procedure was repeated three times. The support was again floated on deionized water and a similar procedure to that described above was carried out to neutrality to remove further grey and black particles as well as the remaining acid. The support precipitated by the addition of methanol was then washed four times with the same solvent, and dried at 100°C. The dry support was silanized with 5% dimethyldichlorosilane (DMCS) in toluene[4,5].

Gas–liquid chromatography

Analyses were performed on a dual set of columns using a Shimadzu 4CM gas chromatograph with two electrometers, two hydrogen flame detectors, on-column injection ports and a temperature programmer. Each electrometer was individually connected to a one-pen recorder. The glass columns (1 m × 3 mm I.D.) and quartz wool (used as plugs at each end of the columns) were treated with DMCS–toluene (5:95). The mixed-phase column packings, 1.605% Poly-I-110–Poly-A-101A–FFAP (1200:300:105, w/w/w) and 1.0% Poly-A-101A–FFAP (1:1, w/w), were prepared by the filtration technique[4,5] using chloroform–*n*-butanol (1:1, v/v) as coating solvents. In the case of the former packing, the solution obtained after dissolving the liquid phases in the coating solvents by vigorous shaking for 1 h was filtered to remove

insoluble materials. The column packings were packed into the columns with gentle tapping under suction by an aspirator. The packed columns were preconditioned at 280°C for 20 h with a nitrogen flow-rate of 30 ml/min after being programmed to 280°C at 2°C/min at the same nitrogen flow-rate. Nitrogen (> 99.99%) was passed through a tube (20 × 3 cm I.D.) containing molecular sieve 5A. Other GLC conditions are given in Fig. 1. A 1.0% Poly-A-101A–FFAP column was used for the determination of leucine, isoleucine and arginine, and a 1.605% Poly-I-110–Poly-A-101A–FFAP column for the determination of the other amino acids, as well as ornithine which was present in the original samples.

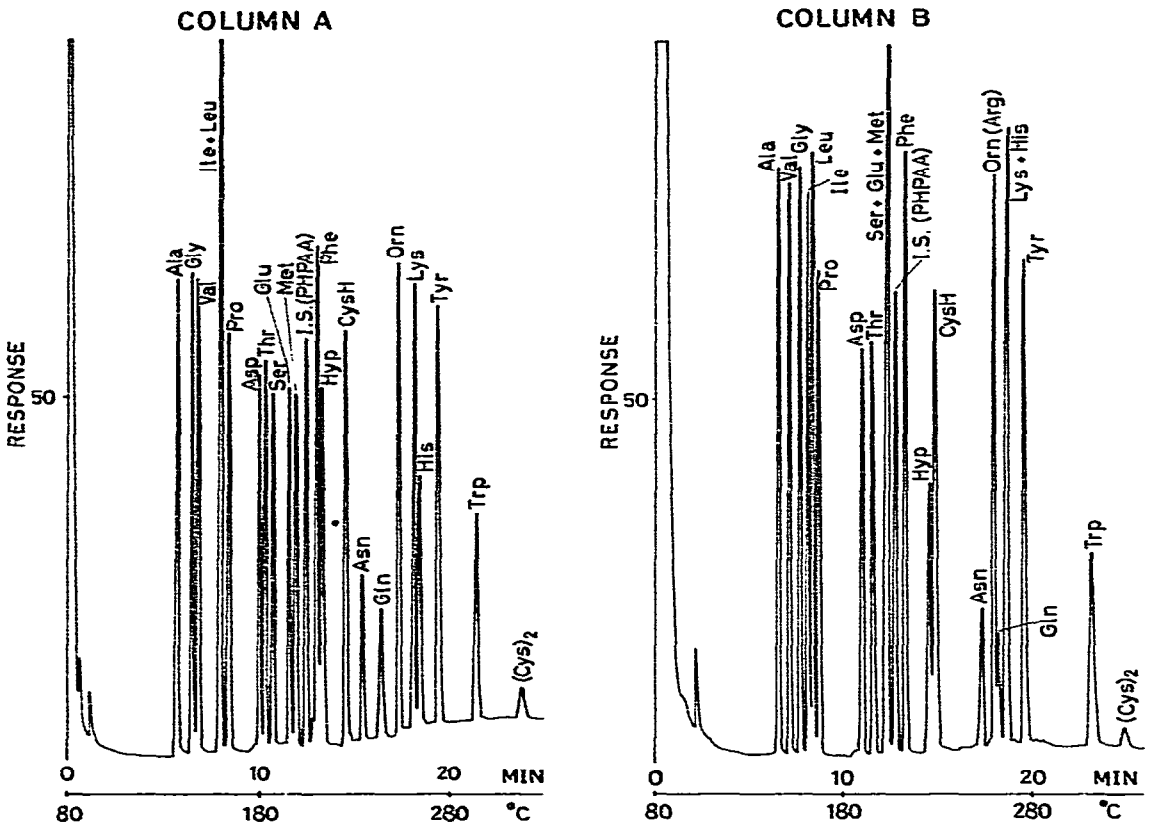

Fig. 1. Gas chromatographic separation of 22 amino acids as N-isobutyloxycarbonyl methyl esters on a dual set of columns. Column A: 1.605% Poly-I-110–Poly-A-101A–FFAP (1200:300:105, w/w/w) on 100–120 mesh Uniport HP, 1 m × 3 mm I.D. glass. Column B: 1.0% Poly-A-101A–FFAP (1:1, w/w) on 100–120 mesh Uniport HP, 1 m × 3 mm I.D. glass. Conditions: injection port and detector temperatures, 280°C; nitrogen flow-rate, 35 ml/min; hydrogen flow-rate, 50 ml/min; air flow-rate, 0.8 l/min; temperature program, linear rise at 10° per min from 80 to 280°C, then held for 5 min. Arginine was separated as the ornithine derivative. Internal standard (I.S.), *p*-hydroxyphenylacetic acid.

In order to obtain a stable baseline in every analysis, the columns were kept overnight at 150°C with a nitrogen flow-rate of 20 ml/min before use. The columns were maintained at 280°C until a suitably low rise in baseline was obtained when they were reconnected to the instrument for the temperature-programmed analysis.

Preparation of derivatives

The details of the principle of the derivatization reactions have been presented previously[1]. The derivatization procedure was modified to make it possible to be performed at sub-microgram levels. Isobutyloxycarbonylation was conducted in a glass screw-top culture tube (Corning No. 9826) whose cap was fitted with a thin PTFE gasket to avoid any contamination which might show as interference peaks on the chromatogram. Test mixtures were used to prepare calibration graphs and to evaluate the reproducibility throughout the overall procedure. Two series of test mixtures (each 1 ml) in the range 0.5–10 μg and 0.5 ml of the internal standard solution (10 μg/ml) were transferred into the vials by pipette. One, not containing ornithine, was treated with arginase according to the procedure of ref. 1 except that 20 μl of arginase solution were added and the resulting solution was centrifuged after incubation for 10 min. The supernatant, after having been subjected to the derivatization procedure, was employed for the assay of arginine, leucine and isoleucine. The other, containing ornithine, was immediately subjected to the derivatization procedure. A 0.5-ml portion of 10% sodium carbonate and 20 μl of isoBCF were added to each vial containing the standard solution or that which had been treated with arginase, and the mixtures were treated as previously reported[1]. It was found that this amount of reagent was enough to perform quantitative isobutyloxycarbonylation of up to 2 mg of a mixture of amino acids. The resulting isoBOC derivatives were extracted five times with 2 ml of diethyl ether from the acidic solution saturated with sodium chloride. Saturation with sodium chloride and extraction five times improved extraction efficiency, especially for the derivatives of threonine, serine, hydroxyproline, asparagine and glutamine. Methyl esterification of the combined ethereal extract was carried out by bubblying through diazomethane without addition of methanol. Evaporation of solvent was accomplished at 40–45°C in a water-bath without use of a current of nitrogen. The residue was dissolved in 20 μl of ethyl acetate and the solution was dried over anhydrous sodium sulphate. A 1–5-μl aliquot was injected on to the gas chromatograph. Peak heights for the amino acids and the internal standard were measured and the peak height ratios were calculated for the construction of calibration graphs.

RESULTS AND DISCUSSION

The 1.605% Poly-I-110–Poly-A-101A–FFAP column provided the desired separation of all the protein amino acids except leucine and isoleucine. Compared to the columns previously developed, separation was better and analysis time shorter, although there still remained the inability to separate the leucine–isoleucine pair. On the other hand, the 1.0% Poly-A-101A–FFAP column did give a good enough separation of this pair when operated under the same thermal conditions as employed for the 1.605% Poly-I-110–Poly-A-101A–FFAP column. This method required a separate analysis if quantitation of ornithine was required at the same time as arginine was being assayed, and it was found that the quantitative determination of methionine, cysteine and tryptophan was interfered with when test mixtures were subjected to the arginase treatment. Therefore, in view of the problems mentioned above, we decided to operate both columns simultaneously and to assay leucine, isoleucine and arginine on the 1.0% Poly-A-101A–FFAP column. In practice, in a sample contain-

ing both arginine and ornithine, the amount of arginine could be determined by subtracting the amount of ornithine determined without arginase treatment using 1.605% Poly-I-110–Poly-A-101A–FFAP column from that determined with arginase treatment using 1.0% Poly-A-101A–FFAP column, and then by converting the remaining ornithine into arginine. Separation of the 22 protein amino acids could be achieved using two columns in a single programmed temperature cycle as shown in Fig. 1, and was complete in less than 25 min. *p*-Hydroxyphenylacetic acid was the most suitable internal standard of the compounds tested. Its O-isoBOC methyl ester was efficiently separated from the other amino acids on both columns, as shown in Fig. 1.

The support used was purified and re-silanized, and this allowed us to overcome fluctuations in performance of the support and to obtain quantitative accuracy, most notably with the amides and cystine. Properly prepared columns retained their ability to separate the amino acids for about three months.

Diethyl ether and water purified by distillation were used after confirming that no contaminant peaks were present. Some makes of reaction vials, the caps of which were fitted with thin synthetic resin gaskets, produced interfering peaks when the chromatograph was operated at high sensitivity. A glass tube with a PTFE-lined gasket was the most suitable reaction vial for isobutyloxycarbonylation.

Conversion yields of arginine to ornithine were almost quantitative in the range 0.5–10 μg under the conditions used. Calibration graphs for all the amino acids, obtained by plotting the ratios of their peak heights to that of the internal standard

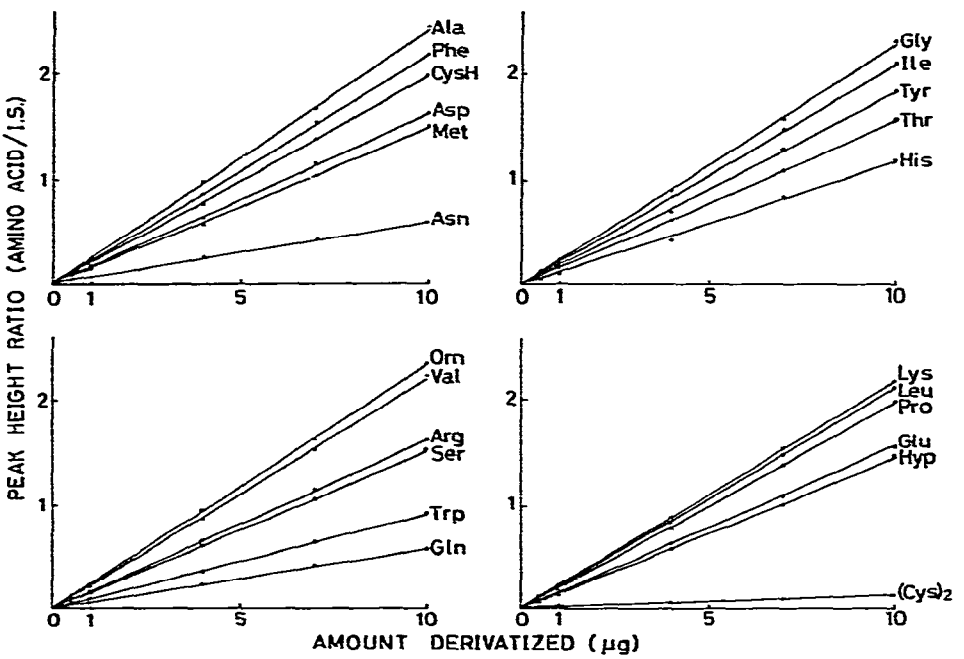

Fig. 2. Calibration graphs for amino acids in the range 0.5–10 μg. Internal standard (I.S.), *p*-hydroxyphenylacetic acid, 5 μg. Arginine was assayed as the ornithine derivative after conversion into ornithine with arginase.

versus the amounts of amino acids (in the range 0.5–10 μg), were linear and passed through the origin, as shown in Fig. 2; reproducibilities were satisfactory. This method permitted detection of even 0.1 μg amounts of each amino acid.

In conclusion, manipulation of these derivatives is easier than the other derivatives which have been proposed, particularly when the operation is carried out on a routine basis. The columns developed in this study gave the desired separation for each amino acid, thus permitting more accurate determinations: the present method allows determination of 0.5 μg amounts of each amino acid. A single analysis, consisting of arginase treatment, derivatization and GLC, could be performed in 1 h. This method has the advantage that both asparagine and glutamine can be determined, unlike N-acetyl or -perfluoroalkyl ester derivatives which require esterification with hydrochloric acid and alcohols under drastic conditions. The method has been applied to biological samples and this will be the subject of a subsequent report.

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