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GAS CHROMATOGRAPHIC SEPARATION OF DIASTEREOISOMERIC AMINO ACID DERIVATIVES ON GLASS CAPILLARIES

THE USE OF PENTAFLUOROPROPIONYL-AMINO ACID (+)-3-METHYL-2-BUTYL ESTERS

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SUMMARY

The separation of diastereoisomeric pentafluoropropionyl-D,L-amino acid (+)-3-methyl-2-butyl esters, including those derived from histidine, arginine and tryptophan, is achieved on glass capillaries. Separation factors of between 1.03 and 1.09 are observed for the derivatives of protein amino acids. The gas chromatographic separation of a mixture of common amino acids usually takes less than 1 h.

INTRODUCTION

It is preferable to use gas chromatography (GC) for the determination of the configuration of amino acids because of the small amount of sample required for analysis. The high polarity of the amino acid molecules makes it necessary to prepare volatile derivatives, and this derivatization is usually performed in two steps. In the first step the carboxyl functions of the amino acids are esterified and subsequently the amino groups as well as NH groups of heterocyclic side-chains, OH and SH groups are acylated.

The separation of derivatives of D- and L-amino acids can be achieved by two different approaches. The difference in the internal energies of diastereoisomeric molecule complexes and of diastereoisomeric molecules respectively is the reason for the different retention times on a GC column. In the first approach, enantiomeric amino acid derivatives, for example N-trifluoroacetyl-D,L-amino acid isopropyl esters, are separated on a chiral stationary phase. Optically active amino acid and peptide derivatives[1–7] may serve as stationary phases. The separation of enantiomers is brought about by the formation of diastereoisomeric molecule complexes between the D- and L-amino acid derivatives and the chiral liquid phase. Hydrogen bonding, previously thought to be the main force of interaction, does not seem to be of major importance[8].

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In the second approach, a second asymmetric centre is introduced into the amino acid molecule and diastereoisomers are formed, which can be separated on conventional, optically inactive stationary phases[9–13].

In this work, the separation of diastereoisomeric amino acid derivatives on glass capillaries is described. The second asymmetric centre, necessary for the formation of diastereoisomers, is introduced by esterification of the amino acids with (+)-3-methyl-2-butanol. The amino groups and other acylatable functional groups are substituted by pentafluoropropionyl groups. By using this procedure, we succeeded in separating the derivatives of all common amino acids, including histidine, arginine and tryptophan.

EXPERIMENTAL

Gas chromatography and mass spectrometry

A Carlo Erba Model 2101 gas chromatograph equipped with an all-glass injection port, inlet splitter and flame-ionization detector was used with a 25-m glass capillary coated with SE-30 (LKB, Type 2101-210, AmAc). Hydrogen was used as the carrier gas. The number of theoretical plates was 114,000 at 120° for *n*-tetradecane and nitrogen as carrier gas.

For combined gas chromatography–mass spectrometry (GC–MS), an LKB 2091 instrument with the same type of glass capillary was used.

Formation of derivatives

About 100 μg of the D,L-amino acid sample were heated in 150 μl of a 7 *N* solution of hydrogen chloride in (+)-3-methyl-2-butanol of about 99% optical purity (Norse Labs., Santa Barbara, Calif., U.S.A.) for 90 min at 100° (ref. 14). Basic amino acids were esterified before with a solution of hydrogen chloride in methanol (1.25 *N*) for 60 min and 100°. After removal of the excess of reagent under reduced pressure, the sample was acylated in a mixture of 200 μl of dichloromethane and 50 μl of pentafluoropropionic anhydride for 30 min at room temperature. After removing excess of reagent, the sample was dissolved in 100 μl of ethyl acetate and the solution obtained was used for gas chromatography.

RESULTS AND DISCUSSION

Comparison of the range of applicability and experimental convenience of enantiomer separation and separation of diastereoisomers indicates that both procedures have different advantages and disadvantages. There are no special problems associated with the formation of derivatives for enantiomer separation, but this technique requires the use of special stationary phases and their coating on capillary columns. The separation of most protein amino acids can be obtained in short analysis times[5], but some amino acid enantiomers, such as histidine, arginine and tryptophan, have so far not been separated or have given only very poor results. The separation of derivatives of proline and aspartic acid is difficult, and is not possible on some of the stationary phases described in the literature. The precision of the quantitative determination of the degree of racemization, however, is usually very high[15].

The separation of diastereoisomeric, acylated amino acid esters is compara-

tively simple, because conventional capillaries with thermostable stationary phases can be used. These columns are commercially available. The quantitative esterification of amino acids with higher alcohols is difficult however, because of poor solubility of amino acids in the alcohols. The use of high acid concentrations (7 *N* HCl)[14,16] or acetyl chloride[17] as catalyst may be necessary in order to obtain good yields. Interesterification of the methyl esters is another means of improving the esterification[18,19].

Furthermore, a decrease in accuracy may be caused by different reaction kinetics in the formation of diastereoisomers, as the reaction may proceed through transition states of different energy levels. A serious source of error may also result from the optical impurity of the chiral alcohol. As the enantiomers L-amino acid (+)-alkyl ester and D-amino acid (−)-alkyl ester, and L-amino acid (−)-alkyl ester and D-amino acid (+)-alkyl ester, cannot be separated on achiral stationary phases, the presence of any amount of the antipode of the chiral alcohol in particular will lower the accuracy of the results when very different amounts of enantiomers are used, as in racemization tests. Both methods can be combined as a means of separating diastereoisomers on a chiral stationary phase[16,17]. This procedure allows all stereoisomers to be completely separated and the error due to the optical impurity of the chiral alcohol can be eliminated.

The separation of diastereoisomer derivatives on symmetric stationary phases can be applied preferentially when precise measurement of the degree of racemization is not important and a decision has to be made as to whether an amino acid present in a hydrolyzate has an L- or D-configuration; this problem may occur in the structural

TABLE I

SEPARATION FACTORS, α, FOR THE SEPARATION OF N-PENTAFLUOROPROPIONYL-L-AMINO ACID (+)-3-METHYL-2-BUTYL ESTERS (LONGER RETENTION TIME) *versus* N-PENTAFLUOROPROPIONYL-D-AMINO ACID (+)-3-METHYL-2-BUTYL ESTERS (SHORTER RETENTION TIME)

Amino acid (in order of emergence)	α	*Column temperature (°C)*
Ala	1.09	100
Gly	—	100
α-Aminobutyric acid	1.09	100
Thr	1.03	100
Val	1.08	100
Ser	1.05	100
Leu	1.09	100
a-Ile	1.08	100
Ile	1.09	100
Cys	1.07	100
Pro	1.06	100
Diaminopropionic acid	1.04	140
Met	1.05	140
Orn	1.06	140
Phe	1.04	140
Asp	1.04	140
His	1.03	140
Lys	1.05	140
Tyr	1.04	140
Glu	1.05	140
Arg	1.05	140
Trp	1.04	200

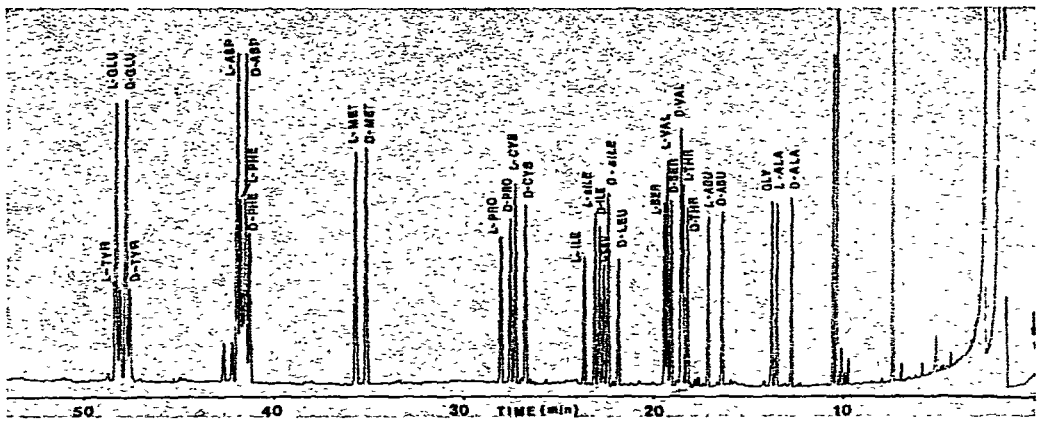

Fig. 1. Gas chromatogram of diastereoisomeric pentafluoropropionyl-amino acid (+)-3-methyl-2-butyl esters on a 25-m glass capillary column (SE-30 AmAc, LKB 2101-210). Column temperature: 85°, temperature program: 2°/min to 220°. Carrier gas: hydrogen, split ratio 1:30.

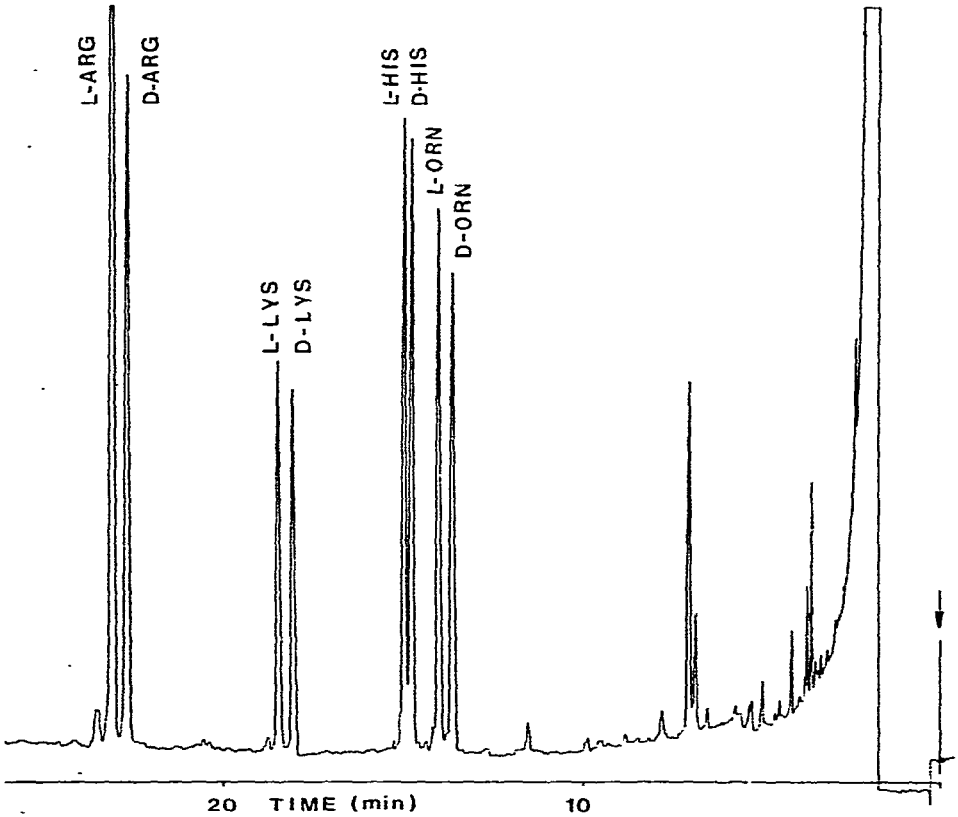

Fig. 2. Gas chromatogram of diastereoisomeric derivatives of basic amino acids. Column and carrier gas as in Fig. 1. Column temperature: 150°, temperature program: 2°/min to 220°.

identification of peptide antibiotics and other natural peptides containing D-enantiomers.

In this work, a method for the separation of diastereoisomeric derivatives of acylated amino acid esters, which has many variations, was used. The pertinent literature is covered in a recent review by Hušek and Macek[20]. According to Westley *et al.*[21], 3-methyl-2-butanol, when introduced into amino acids, yields diastereoisomers that have a very high separation factor. Both optical isomers of this alcohol are commercially available. By the use of glass capillaries, the separation efficiency and the range of application can be substantially improved compared with known procedures.

Esterification seems to be almost quantitative with all amino acids except basic amino acids (lysine, histidine, ornithine, diaminopropionic acid, arginine) when 7 *N* hydrogen chloride in (+)-3-methyl-2-butanol is used, as judged by gas chromatographic peak areas obtained by injection of an amount of sample corresponding to 10^{-7} g of underivatized amino acid. With basic amino acids, inter-esterification of the methyl esters certainly improves the yield, although we did not optimize the procedure.

Glass capillaries 25 m long are sufficient to give almost complete separation of diastereoisomeric pairs of amino acids commonly present in proteins and natural peptides (Figs. 1 and 2). One has to cope with partial overlapping of pairs of peaks when aspartic acid and histidine or arginine and tryptophan are present together in a sample. For mixtures of these amino acids, the trifluoroacetyl derivatives, which are almost completely separated, are to be preferred.

The pentafluoropropionyl group has the advantage of giving excellent rates of conversion of the amino acid esters into fully acylated and highly volatile derivatives. Only histidine, arginine and tryptophan are incompletely acylated at room temper-

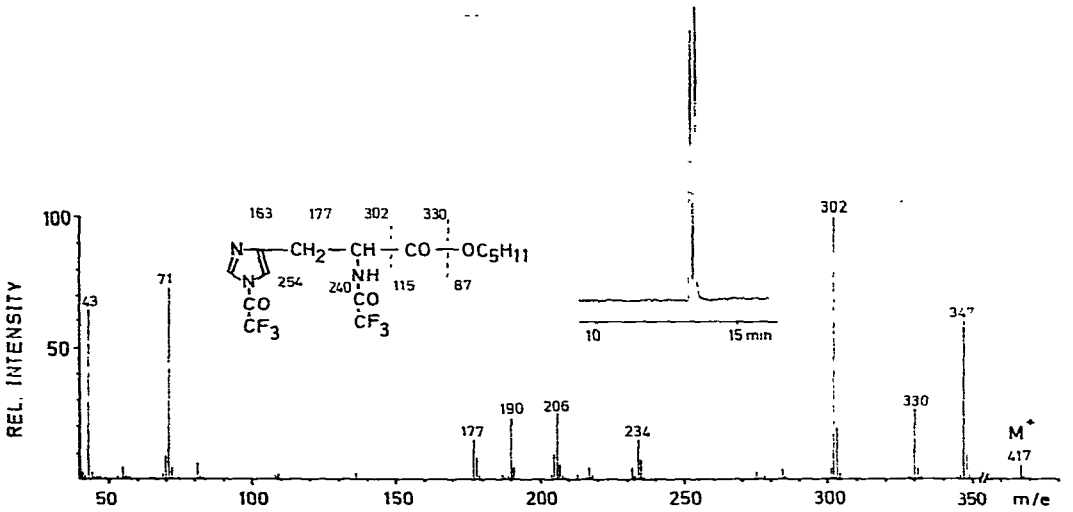

Fig. 3. Gas chromatogram and mass spectrum of bis-trifluoroacetyl-D,L-histidine (±)-3-methyl-2-butyl ester on a 25-m glass capillary column, which was connected to an LKB 2091 mass spectrometer. Electron energy: 70 eV, column temperature: 150°, temperature program: 5°/min to 220°. Carrier gas: helium.

ature. In order to obtain fully acylated derivatives, the samples of these amino acids were injected together with 0.5 μl of pentafluoropropionic anhydride into the hot injection port (250°) of the gas chromatograph, as proposed for trifluoroacetyl derivatives by Gehrke and Stalling[22]. By using this procedure, only the fully acylated derivatives could be detected in the chromatograms. Acylation at all possible sites could be proved in the case of the trifluoroacetyl derivatives of these amino acids by combined GC–MS. Fig. 3 shows the mass spectrum of the histidine derivative, which illustrates that both the α-amino group and the heterocyclic imidazole ring are trifluoroacetylated. Trifluoroacetylation was replaced by using the pentafluoropropionyl group because of higher volatilities and less overlapping of the peaks in amino acid mixtures.

The fully acylated derivative can be formed from the incompletely acylated histidine derivative even after storage of the sample for several weeks in dichloromethane solution, while the fully acylated derivatives of arginine and tryptophan are very labile and should be chromatographed immediately after their preparation. This is necessary in order to avoid partial deacylation of the arginine derivative and decomposition of tryptophan.

An alternative and more accurate procedure for the determination of the degree of racemization of histidine in peptides, which involves the conversion of histidine into aspartic acid by reaction with ozone, has been suggested recently[16].

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REFERENCES

1 E. Gil-Av and B. Feibush, *Tetrahedron Lett.*, (1967) 3345.
2 B. Feibush and E. Gil-Av, *J. Gas Chromatogr.*, 5 (1967) 257.
3 W. A. König, W. Parr, H. A. Lichtenstein, E. Bayer and J. Oró, *J Chromatogr. Sci.*, 8 (1970) 183.
4 B. Feibush, *Chem. Commun.*, (1971) 544.
5 W. A. König and G. J. Nicholson, *Anal. Chem.*, 47 (1975) 951.
6 R. Charles, U. Beitler, B. Feibush and E. Gil-Av, *J. Chromatogr.*, 112 (1975) 121.
7 R. Brazell, W. Parr, F. Andrawes and A. Zlatkis, *Chromatographia*, 9 (1976) 57.
8 K. Stölting and W. A. König, *Chromatographia*, 9 (1976) 331.
9 E. Gil-Av, R. Charles-Sigler, G. Fischer and D. Nurok, *J. Gas Chromatogr.*, 4 (1966) 51.
10 G. E. Pollock and V. I. Oyama, *J. Gas Chromatogr.*, 4 (1966) 126.
11 G. E. Pollock, V. I. Oyama and R. D. Johnson, *J. Gas Chromatogr.*, 3 (1965) 174.
12 G. E. Pollock and H. A. Kawauchi, *Anal. Chem.*, 40 (1968) 18.
13 F. Raulin and B. N. Khare, *J. Chromatogr.*, 75 (1973) 13.
14 W. Rahn, *Diplom Thesis*, Univ. Hamburg, 1975.
15 W. A. Bonner, M. A. van Dort and J. J. Flores, *Anal. Chem.*, 46 (1974) 2104.
16 W. Rahn and W. A. König, *Hoppe-Seyler's Z. Physiol. Chem.*, 357 (1976) 1223.
17 W. A. König, *Chromatographia*, 9 (1976) 72.
18 W. M. Lamkin and C. W. Gehrke, *Anal. Chem.*, 34 (1965) 383.
19 C. W. Gehrke, R. W. Zumwalt and K. Kuo, *J. Agr. Food Chem.*, 19 (1971) 605.
20 P. Hušek and K. Macek, *J. Chromatogr.*, 113 (1975) 139.
21 J. W. Westley, B. Halpern and B. L. Karger, *Anal. Chem.*, 40 (1968) 2046.
22 C. W. Gehrke and D. L. Stalling, *Separ. Sci.*, 2 (1967) 101.