Journal of Chromatography, 388 (1987) 441–447
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 167

ANALYSIS OF ASPARTIC ACID RACEMIZATION

EVALUATION OF A CHIRAL CAPILLARY GAS CHROMATOGRAPHIC AND A DIASTEREOMERIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

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(Received September 22nd, 1986)

SUMMARY

A recently developed chiral gas chromatographic method and a diastereomeric high-performance liquid chromatographic method for the analysis of aspartic acid enantiomers in protein hydrolyzates have been evaluated. Although both techniques are fast and convenient, the latter is preferred because of its higher reproducibility and shorter analysis time. Furthermore, this method offers the possibility of on-line derivatization and analysis.

INTRODUCTION

Aspartic acid racemization is of interest for two reasons. First, it has been recognized as a geochronological tool for dating fossils[1]. Secondly, it has been identified as a post-translational protein modification[2–4]. *In vivo* racemization of aspartic acid in proteins is a rather slow non-enzymatic process with a rate constant of about $1.3 \cdot 10^{-3}$ year^{-1}. High D/L ratios can therefore be found only in long-living tissues in which the protein turnover is low or absent, such as the lens of the eye[5–8], tooth enamel and dentine[9,10] and white brain matter[11,12]. The effects of the optical isomerization of aspartic acid residues upon protein structure and its rôle in aging have been the subject of much speculation[2–4,9,13] but have still to be elucidated. It has been suggested that aspartic acid racemization is involved in cataract formation[5,6], but this is not accepted by other workers[8]. Recently it has also been shown that D-Asp as well as L-isoAsp residues are substrates for the ubiquitous enzyme protein carboxylmethylase (E.C. 2.1.1.24)[14]. It was postulated that this enzyme might function in a racemization- or isomerization-repair pathway[14–16].

For studies of the *in vivo* racemization of Asp, most investigators have used ion-exchange separation of diastereomeric dipeptides as introduced by Manning and Moore[17]. Since in this procedure the isolation of aspartic acid is a necessity and elution times exceed 1 h, it is very laborious and time-consuming. In a previous

0021-9673/87/$03.50

paper[18] we discussed a more convenient technique based on the direct enantiomeric resolution of a non-purified protein hydrolyzate by chiral capillary gas chromatography (GC).

However, high-performance liquid chromatography (HPLC) often offers even better reproducibility and shorter analysis times. Furthermore, HPLC is becoming a routine technique, and is very well suited to automation. Many enantioselective HPLC techniques have recently been developed[19–22]. The separation is based either on a chiral mobile or stationary phase or a pre-column derivatization with a chiral agent transforming the enantiomers into diastereomers. Chiral stationary phases have recently been reviewed by Däppen *et al.*[19]. Ligand-exchange phases for amino acids have been extensively studied and were reviewed by Davankov[20,21]. The use of chiral eluents has also been reviewed[22]. Most methods are characterized by rather high enantioselectivity which is adequate for samples with few components, but causes peak overlap in such complicated mixtures as a protein hydrolyzate. Recently, less enantioselective HPLC methods for amino acid enantiomers have been described, all using the successful pre-column derivatization with *o*-phthalaldehyde (OPA) in which the traditional coupling agent β-mercaptoethanol has been replaced by chiral thiols such as N-acetyl-L-cysteine (NAC)[23,24] or *tert.*-butyloxycarbonyl-L-cysteine[25]. In this paper we evaluate a pre-column derivatization type HPLC method and a chiral GC technique for the detection of D- and L-Asp in protein hydrolyzates.

MATERIALS AND METHODS

Sample preparation and acidic hydrolysis

The isolation of human eye lens proteins was performed as described[26]. Bovine serum albumin (Sigma, A-8002) was used to determine background racemization. As pointed out before[18], it is essential to remove metal traces from samples and hydrolysis tubes in order to minimize background racemization during acidic hydrolysis. Therefore, protein samples were dialyzed against an EDTA solution and ultrapure water. Hydrolysis tubes were pre-boiled with 6 *M* hydrochloric acid[18]. Acidic hydrolysis was performed for 6 h at 110°C under vacuum. In reproducibility tests, mixtures of D- and L-Asp (Sigma) were used.

Gas chromatography

Lyophilized hydrolyzates were derivatized with acidic isopropanol and trifluoroacetic acid anhydride as before[18]. Analyses were performed with a fused-silica capillary column (20 m × 0.22 mm I.D.) coated with the chiral polysiloxane XE-60-*S*-α-phenylethylamide (Chrompack, Middelburg, The Netherlands). An Hewlett-Packard 5710A gas chromatograph equipped with a flame ionization detector was operated in split mode with helium as the carrier gas. Aspartic acid enantiomers were chromatographed isothermally at 135°C. Less volatile derivatives were removed by increasing the oven temperature to 180°C for 4 min. The injector and detector temperatures were 250°C, the column pressure was 1.7 bar, vent 80 ml/min and the splitting was set at 1%. Automatic data handling was performed by an Hewlett-Packard 3390A integrator.

High-performance liquid chromatography

The derivatization and analysis were adapted from the description given by Aswad[23]. To 4 mg OPA, dissolved in 300 μl methanol, were added 250 μl of 0.4 *M* Na_3BO_3, pH 9.4, 390 μl water and 60 μl of 1 *M* NAC. The NAC could be dissolved in water by the addition of small amounts of sodium hydroxide. The final pH of the OPA–NAC solution should be between 9 and 10.

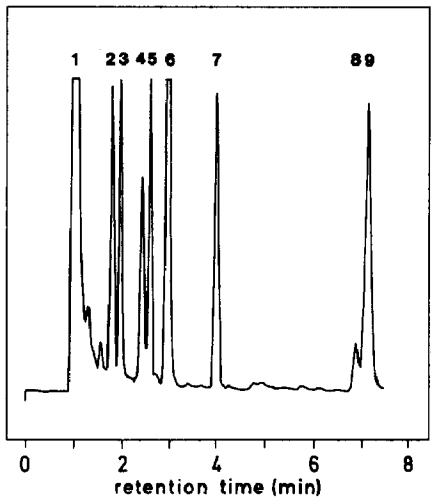
The derivatization and injection were by means of a Gilson autosampler Model 231 equipped with a Gilson dilutor Model 401. A 22-μl volume of the OPA–NAC reagent was mixed with 3 μl of an aqueous hydrolyzate containing about 10–100 nmol Asp. After 2.5 min the reaction was stopped by lowering the pH through the addition of 200 μl of 50 m*M* sodium acetate, pH 5.2. Of the resulting solution, 100 μl were placed on a combined 10-cm C_{18} column and 1-cm guard column (Hypersil-ODS, ChromSep cartridge system, Chrompack). Gradient elution with 50 m*M* sodium acetate, pH 5.7 (eluent A) and a mixture of eluent A with methanol (20:80, eluent B) was performed with two Beckman Model 100A pumps controlled by a Beckman Model 420 programmer. After 3.5 min of isocratic elution with 10% eluent B, the percentage of eluent B was increased to 100% in 0.5 min and after 3 min reduced to 10% in 0.5 min. The flow-rate was 0.8 ml/min. A Perkin-Elmer Model 204-A fluorimeter was used to monitor the fluorescence at 445 nm. Excitation was at 340 nm, and spectral bandwidths were 10 nm. Peak areas were integrated automatically by an Hewlett-Packard 3353 LAB-DATA system equipped with a 18652A A/D-converter.

RESULTS AND DISCUSSION

We have evaluated two chromatographic methods for the analysis of D- and L-Asp in protein hydrolyzates. In the GC procedure a capillary column was used, coated with a chiral liquid phase, as developed by König *et al.*[27,28]. The chiral phase enables direct separation of the aspartic acid enantiomers. For the HPLC analysis, the amino acid enantiomers have to be converted into diastereomers. For this purpose they were derivatized with OPA and NAC as described by Aswad[23] and Nimura and Kinoshita[24] to form highly fluorescent diastereomeric isoindols[24,29] that can be separated on a regular reversed-phase column.

The enantiomeric separations of hydrolyzates of human lens protein fractions by these GC and HPLC methods are shown in Figs. 1 and 2. In both methods, the D-Asp derivative is eluted before that of L-Asp, thus enabling the detection of small amounts of D-Asp in the presence of an excess of its optical antipode. Although both techniques are capable of resolving D- and L-aspartic acid, the enantioselectivity of the GC method is a little higher, having a resolution factor of 1.4 *vs.* 1.0 for the HPLC method (Table I). However, the HPLC technique is inferior to the GC method only in respect of the resolution. In all other aspects it is superior. Table I shows that the total analysis, including derivatization and column regeneration, is about three times faster. The reproducibility of the determination of the racemization percentage, defined as $D \cdot 100/D+L$, is much higher for the HPLC method. The detection limit differs by two orders of magnitude (Table I).

We also investigated the optimum conditions for the HPLC separation. Its performance is only satisfactory within sharply defined limits of pH, methanol con-



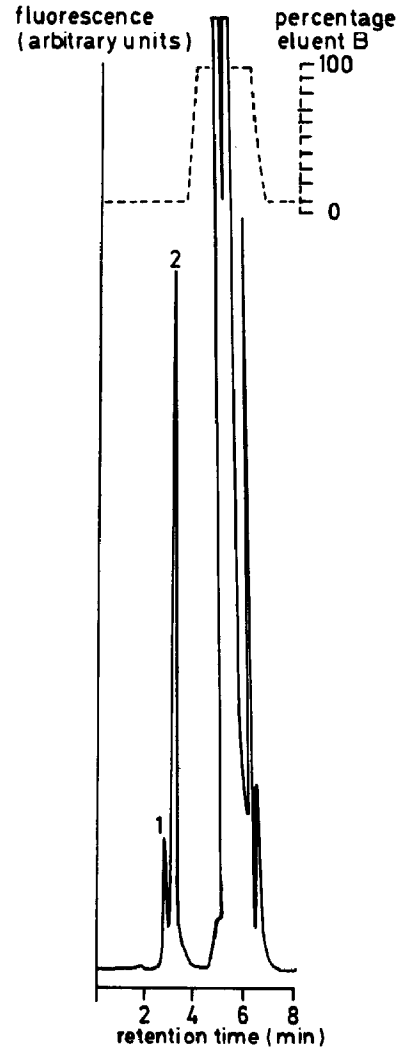


Fig. 1. Chiral GC of a protein hydrolyzate. The hydrolyzed urea-insoluble protein fraction of the cortex of a 60-year-old human lens was analyzed on a 20-m chiral capillary column, revealing a racemization percentage, D · 100/D+L, of 13.7%, not corrected for background racemization. Identified peaks: 1 = dichloromethane; 2 = L-Ala; 3 = L-Val; 4 = L-Thr; 5 = L-Ile; 6 = L-Leu; 7 = L-Pro; 8 = D-Asp; 9 = L-Asp.

Fig. 2. Diastereomeric HPLC separation of a protein hydrolyzate. The hydrolyzed urea-soluble protein fraction of the nucleus of an 86-year-old human lens was derivatized for 2.5 min with the OPA–NAC reagent. After the diastereomeric derivatives of D-Asp (1) and L-Asp (2) had eluted, the percentage of eluent B was increased to 100% in order to elute all other amino acids. The uncorrected D · 100/D+L ratio was 18.5%. The gradient profile is indicated by the dashed line.

centration and eluent flow-rate. The effects of these parameters on the resolution are shown in Fig. 3. Especially the pH of the eluent is very critical (Fig. 3A). The sharpness of the curve in Fig. 3A is explained by the fact that the elution behaviour as a function of pH is nearly identical for both diastereomeric aspartic acid derivatives

TABLE I

CHARACTERISTICS OF THE HPLC AND GC SEPARATIONS OF D- AND L-ASPARTIC ACID

	HPLC	*GC*
Retention time (min)	3.1 (D), 3.3 (L)	7.2 (D), 7.5 (L)
Column regeneration (min)	4	7
Sample derivatization (min)	2.5	15*
Total analysis time (min)**	10	30
Resolution***	1.0	1.4
Coefficient of variation (%)§	2	8
Detection limit (pmol)	1	250§§

* The batchwise derivatization of twelve samples takes 180 min.

** This includes sample derivatization, L-Asp elution time and column regeneration.

*** Calculated as $(t_L - t_D)/(W_{L,0.625} + W_{D,0.625})$ where $W_{L,0.625}$ is the peak width of L-Asp at 0.625 height.

§ Only the racemization percentage, D · 100/D+L, was verified.

§§ At a splitting ratio of 1%.

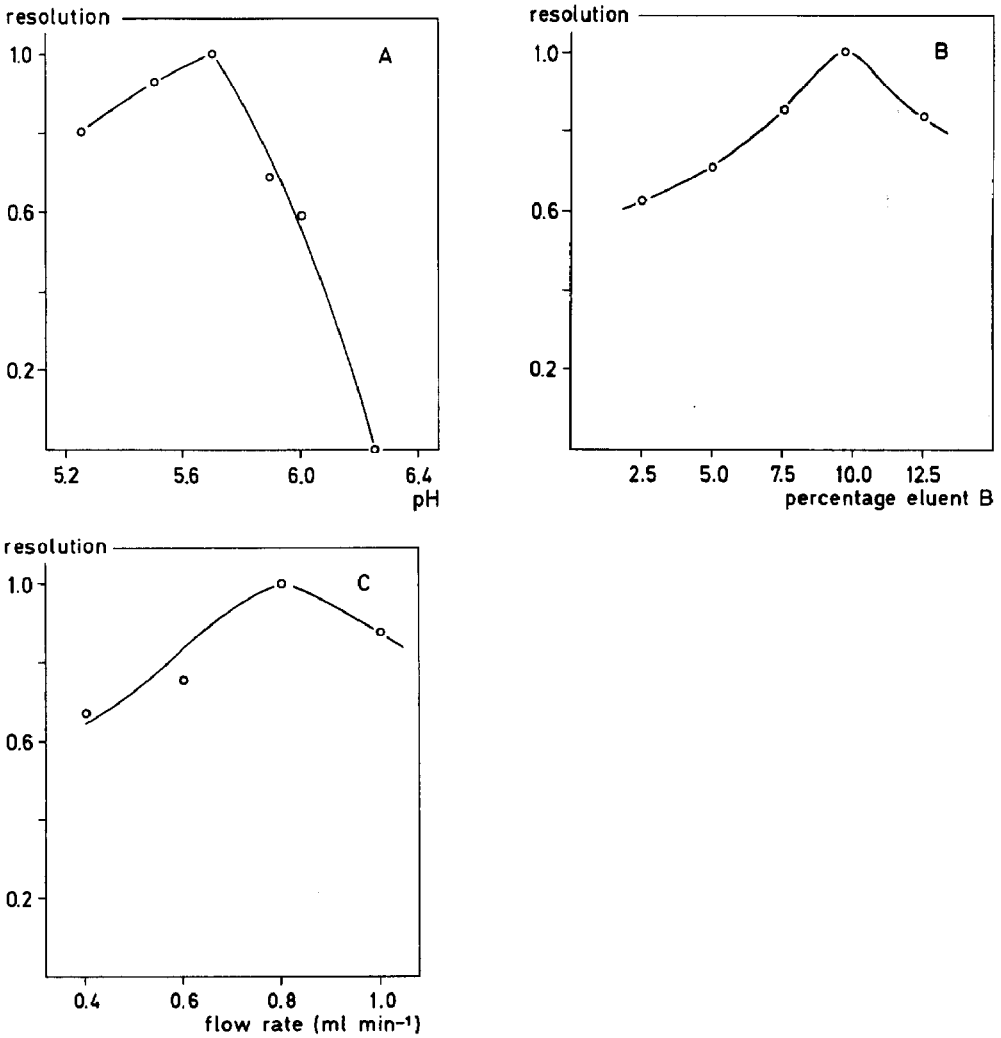


Fig. 3. Optimum conditions for the HPLC separation of D- and L-Asp. Racemic mixtures of aspartic acid were chromatographed under the following conditions: (A) flow-rate 0.8 ml/min, 10% eluent B; (B) flow-rate 0.8 ml/min, pH 5.7; (C) pH 5.7, 10% eluent B.

and only differs for a very narrow pH region. The optimum pH of 5.7 in our system differs from that of 5.9 as found by Aswad[23]. This may be due to the different characteristics of the reversed-phase matrix in the respective set-ups. Nimura and Kinoshita[24] developed a similar method but did not mention the pH of the elution buffer. For an optimum resolution, the HPLC separation was performed with a flow-rate of 0.8 ml/min and 10% eluent B, pH 5.7.

CONCLUSION

In comparison to the diastereomeric dipeptide technique[17] which is generally used to study aspartic acid racemization *in vivo*[3,8], both the GC and HPLC methods described here offer much shorter analysis times and higher reproducibilities. In spite of a lower resolution, the HPLC technique is preferable because of its short total analysis time and low coefficient of variation. Furthermore, since the HPLC derivatization can be carried out at room temperature in aqueous solutions, this method can be fully automated by means of an "intelligent" autoinjector.

ACKNOWLEDGEMENTS

The authors are indebted to Drs. T. B. Vree and E. van der Klein, Department of Clinical Pharmacy, for the use of the GC equipment. The Gilson autosampler was made available by Meyvis en Co. b.v., Bergen op Zoom, The Netherlands. Human eye lenses were supplied by the Eye Hospital, Rotterdam and the Interuniversity Ophthalmological Institute, Amsterdam. This paper was published within the scope of the EC Concerted Action on Cellular Aging and Diseases (EURAGE).

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