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EMPLOYMENT OF GAS—LIQUID CHROMATOGRAPHY FOR THE ANALYSIS OF COLLAGEN AMINO ACIDS IN BIOPSY TISSUE

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SUMMARY

In this paper, gas—liquid chromatography, adapted for the determination of collagen amino acids, is described. This technique was attractive for its sensitivity in that only a small amount of protein such as in 0.5 mg of tissue, especially as obtained from biopsy tissue, was needed for the separation and determination of proline (Pro), 4-hydroxyproline (4-Hyp), 3-hydroxyproline (3-Hyp), lysine (Lys), hydroxylysine (Hyl) and ε-hydroxynorleucine (ε-OH-Norleu), the characteristic amino acids of collagen. Thus, without purification of collagen, by determining the ratio Hyl/4-Hyp and 4-Hyp/Pro it was possible to determine some anomalies in the collagen content of biopsy tissue (skin or liver). The ratio Hyl/4-Hyp allows an estimation of the lack of hydroxylation of polypeptidic lysine as in the Ehlers-Danlos syndrome type VI; and the ratio 4-Hyp/Pro allows measurement of variations in collagen content in relation to protein, especially in the liver, as in alcoholic cirrhosis.

INTRODUCTION

During the last decade, the gas—liquid chromatography (GLC) of amino acids has been developed as an analytical technique allowing the determination of free amino acids in biological fluids, and of protein amino acids [1]. Thus, it was interesting to examine the possibilities of this technique for the analysis of collagen, especially because of its particular amino acid composition characterized by two amino acids derived from polypeptidic proline (Pro), 4-hydroxypyroline (4-Hyp) and 3-hydroxyproline (3-Hyp) [2], and by two amino acids derived from polypeptidic lysine, 5-hydroxylysine (5-Hyl) and α-amino-δ-semialdehyde adipic acid or lysine aldehyde upon which depends the crosslinking of collagen [3, 4]. The latter amino acids are products of the metabolism of polypeptidic proline and polypeptidic lysine, respectively, during the post-translational step and they thus allow the different steps of collagen metabolism, to be identified.

By utilizing the advantages of GLC (high sensitivity, speed, accuracy), we were able to separate and determine the collagen amino acids, particularly 4-Hyp, 3-Hyp and Hyl. The accuracy of the method as applied to collagen was verified using pure collagen. Its usefulness was emphasized by its very small requirement for biological material, especially as obtained from skin and liver biopsies.

EXPERIMENTAL

Synthesis of ε-hydroxynorleucine

For the identification of ε-hydroxynorleucine, we synthesized this standard according to the method of Gaudry [5]. The structure of the synthesized product was verified by infrared spectrometry, mass spectrometry and the characteristic peak of its N,O-trifluoroacetyl (TFA) butyl ester was identified by GLC [6].

Preparation for biopsy tissue

The size of skin biopsies from adult patients was 0.25 cm^2. After removing fatty tissue from the biopsy material the remaining lipids were extracted with chloroform—methanol (2:1) for 24 h. The samples were then weighed and hydrolysed for 24 h at 100° in 6 *N* HCl in screw-stoppered tubes (2 mg of protein per 1 ml of 6 *N* HCl).

The samples from liver biopsies were hydrolysed directly using the same conditions. The collagen samples were hydrolysed for 6 h under the same conditions since Pro, 4-Hyp and Hyl were liberated after hydrolysis for 6 h.

After hydrolysis the solution was evaporated to dryness in vacuo and the free amino acids were derivatized to their N-TFA butyl esters and analysed by GLC.

N-Trifluoroacetylated butyl ester synthesis

For their separation by GLC, the amino acids were derivatized to the corresponding volatile N-TFA butyl esters, which were synthesized by the method described by Zumwalt et al. [7] and Kaiser et al. [8].

The first step was esterification of the carboxylic moieties in 2 ml of anhydrous *n*-butanol—HCl (1 *N*) at 100° for 2 h (for samples in the range 0.1—10 mg of amino acids). After cooling, the *n*-butanol—HCl was evaporated to dryness under a stream of dry nitrogen at 40°; the second step was acylation with trifluoroacetic anhydride (TFAA) (50 μl in 200 μl of methylene chloride) at 100° for 1 h.

Gas—liquid chromatography

The separation of the derivatized samples was performed in a gas chromatograph (GV Erba Sciences) equipped with a glass column (2 m × 3 mm I.D.). The column was silanized and filled with Gas-Chrom P 100—120 mesh coated with 3% QF-1 mixed with Gas-Chrom P 100—120 mesh coated with 1% SE-30, in the ratio 3:2 (w/w). Nitrogen was used as carrier gas at a flow-rate of 40 ml/min. The column oven temperature was programmed from 90 to 250° at a rate of 4°/min. The injection port was kept at 230° and the detection port at 270°.

Calibration curves were established for each amino acid by using the ratio of amino acid peak height over internal standard (*n*-hexadecane; Merck, Darmstadt, G.F.R.) peak height used as reference [9]. The amount of *n*-hexadecane used as internal standard was 3.45 μmoles per 250 μl of methylene chloride.

RESULTS

Specificity of the GLC separation

We have previously reported [6, 10] the separation of Pro, 4-Hyp and ε-hydroxynorleucine (ε-OH-Norleu). Fig. 1 depicts the chromatogram obtained after separation of standard amino acids. Each derivative gave only one peak. A double peak was obtained for Hyl (Koch Light Labs., Colnbrook, Great Britain), one peak for the isomers D,L-hydroxylysine and another peak for the isomers D,L-*allo*-hydroxylysine. We have verified that in pure collagen and in biopsy tissue there was only one peak for Hyl, since this amino acid was a product of the enzymatic hydroxylation of polypeptidic lysine [11].

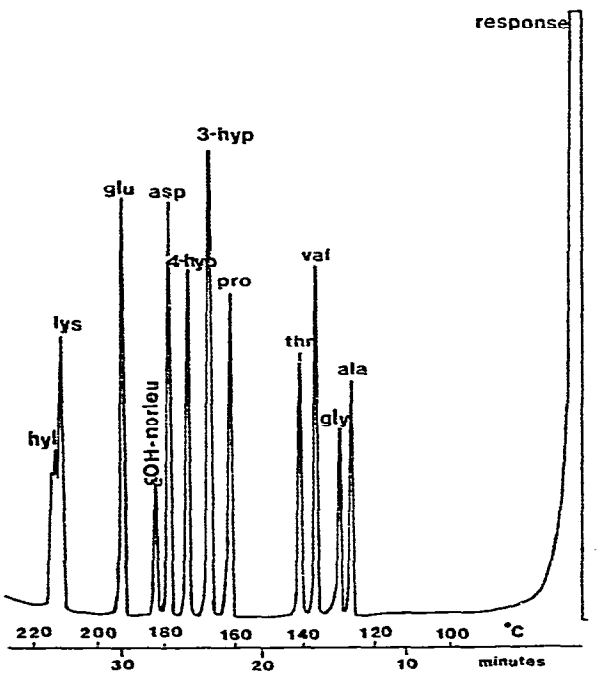

Fig. 1. GLC separation of N-TFA butyl esters of standard amino acids. Column packing: mixture of Gas-Chrom P, 100—120 mesh, coated with 3% QF-1 and coated with 1% SE-30 in the ratio 3:2 (w/w). Carrier gas: nitrogen 40 ml/min. Temperature injection port: 230°. Temperature detection port: 270°. Temperature programme: 4°/min from 90 to 250°.

Use of GLC for the separation of 3-hydroxyproline

The products of hydroxylation of polypeptidic Pro are 4-Hyp, which represents about 12% of the collagen, and 3-Hyp, which exists only in very small quantities compared to the other amino acids except in collagen from

basement membranes. 3-Hyp was purified by Dr. Szymanovicz (Reims, France) and he and his colleagues have described some properties of this amino acid [12]. 3-Hyp particularly was destroyed by oxidation with chloramine T and it was impossible to characterize this amino acid by the colorimetric methods used for the determination of 4-Hyp.

The separation obtained for 3-Hyp and 4-Hyp is shown in Fig. 1; the volatile derivatives of these amino acids gave only one peak of which the homogeneity was determined by mass spectrometry. The mass spectra were obtained on a VG 305 apparatus (Centre de Spectrométrie de Masse, Domaine Universitaire Rockfeller, Lyon, France). The fragmentation observed was different for 4-Hyp and 3-Hyp.

The ions for these two isomeric amino acids are identical, but the relative intensities of the peaks were different for the N,O-TFA butyl esters of 3-Hyp (Fig. 2A) and 4-Hyp (Fig. 2B).

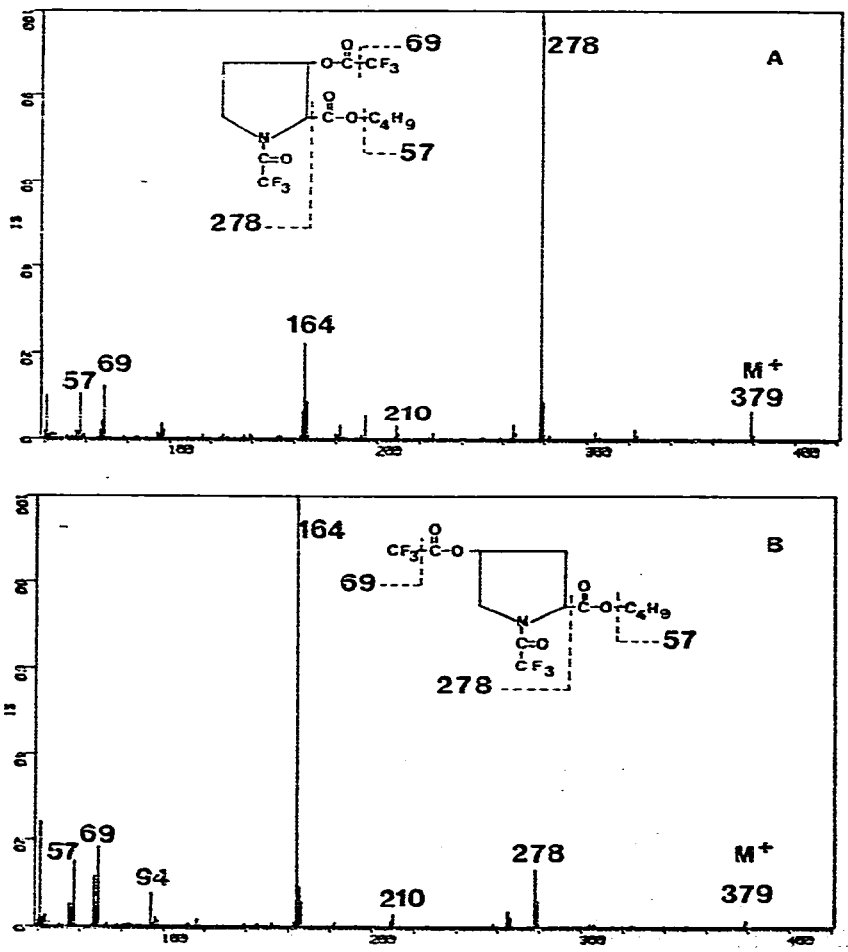

Fig. 2. Mass spectra of the N,O-TFA butyl esters of 3-hydroxyproline (A) and 4-hydroxyproline (B). Ion source energy 70 eV, source temperature 180°.

Use of GLC for the determination of collagen amino acids

The chromatograms obtained from a hydrolysate of collagen type I and type III from human cirrhotic liver (provided by O. Chevalier, C.T.C. Lyon, France) are presented in Fig. 3. Thus it was possible to determine these amino acids by this technique, and we verified that the results obtained with this technique were in good agreement with the results obtained by the usual ion-exchange chromatography. A comparison between the methods is shown in Table I, in which the ratio of each amino acid to 4-Hyp was established for collagen type I and type III.

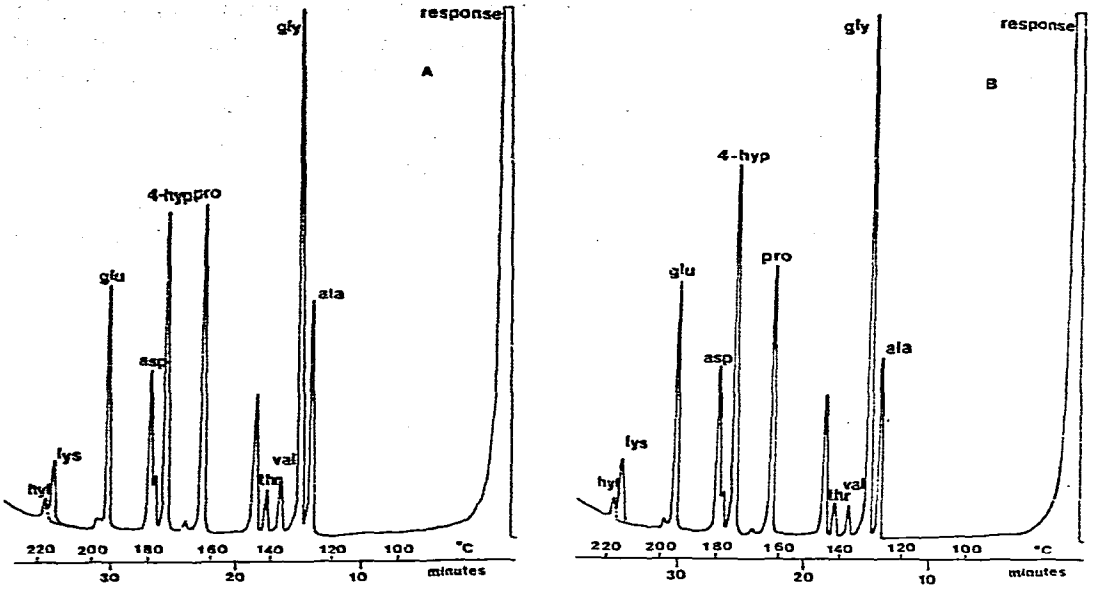

Fig. 3. Separation by GLC of N-TFA butyl esters of amino acids from a hydrolysate of cirrhotic liver collagen type I (A) and type III (B) under the same experimental procedure as in Fig. 1.

TABLE I

COMPARISON OF AMINO ACID COMPOSITION OF HUMAN CIRRHOTIC LIVER COLLAGEN BY GLC AND ION-EXCHANGE CHROMATOGRAPHY WITH REFERENCE TO 4-HYDROXYPROLINE

Amino acid	Collagen type I		Collagen type III	
	GLC	IEC*	GLC	IEC*
Glycine	3.38	3.45	2.81	2.78
Threonine	0.18	0.17	0.13	0.13
Proline	1.13	1.15	0.80	0.86
4-Hydroxyproline	1	1	1	1
Aspartic acid	0.44	0.46	0.39	0.40
Glutamic acid	0.64	0.79	0.58	0.63
Lysine	0.22	0.25	0.20	0.22
Hydroxylysine	0.07	0.09	0.065	0.08

*IEC = ion-exchange chromatography.

Use of GLC for the determination of collagen amino acids in biopsy tissue

The amount of biopsy tissue was small and it was impossible to extract collagen quantitatively from such biological samples. The method of determining the amount of collagen in the tissue studied was to determine the collagen amino acids. The analytical techniques that have been used for determining collagen amino acids in skin biopsy material involved direct colorimetric estimation without separation of the amino acids [13—15]. However, these techniques could not be used for determinations in liver biopsy material since the amount of collagen was very small. We proposed GLC, a useful tool for estimating Hyl/4-Hyp and 4-Hyp/Pro ratios, by determining these amino acids after their separation from skin or liver biopsy material. Thus the chromatograms presented in Fig. 4 were obtained from a hydrolysate of 3 mg of skin biopsy tissue (Fig. 4A) and from a hydrolysate of 0.375 mg of cirrhotic liver biopsy tissue (Fig. 4B). We verified the homogeneity of each peak by GLC—mass spectrometry (MS). In Table II are presented the results obtained for variations of the ratios 4-Hyp/Pro and Hyl/4-Hyp in skin biopsy tissue and liver biopsy tissue without microscopic anomalies. Analysis of these results

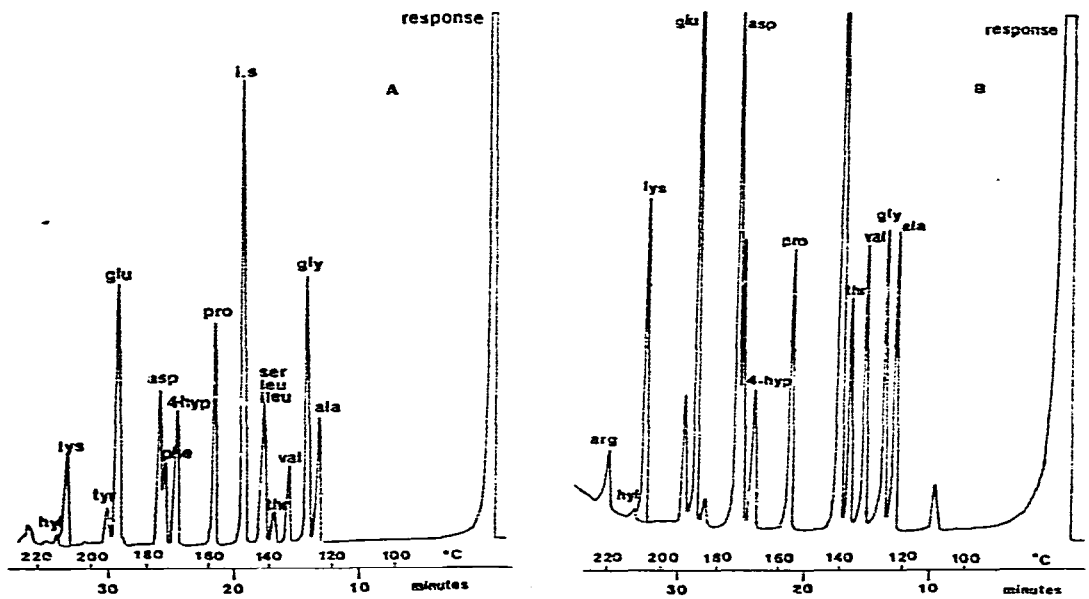

Fig. 4. Separation by GLC of N-TFA butyl esters of amino acids from a hydrolysate of 25 μg of human skin (A) and of 3 μg of human cirrhotic liver biopsy (B) in 2 μl of methylene chloride; experimental procedure as in Fig. 1.

showed that the variations observed for tissue biopsies from different patients were small for both skin and liver for 4-Hyp/Pro and Hyl/4-Hyp. These results were in good agreement with those reported elsewhere [16] for skin biopsy material.

In addition, Hyl/4-Hyp was greater for liver collagen than for skin collagen; and this ratio in liver biopsy tissue was higher than that for purified collagen

TABLE II

DISTRIBUTION OF 4-Hyp/Pro AND Hyl/4-Hyp RATIOS IN SKIN BIOPSIES AND IN LIVER BIOPSIES WITHOUT MICROSCOPIC ANOMALIES

Tissue	4-Hyp/Pro	Hyl/4-Hyp
Human adult skin	$\overline{m}$ = 0.56	$\overline{m}$ = 0.041
(8 biopsies)	σ = 0.028	σ = 0.0087
Human adult liver	$\overline{m}$ = 0.19	$\overline{m}$ = 0.080
(14 biopsies)	σ = 0.027	σ = 0.013

type I and type III (see Table I). This difference could be due to the fact that the Hyl content was determined without separating each type of collagen from liver as collagen type I and type III and basement membrane collagen type A, type B and type E. Indeed, the Hyl content of collagen from basement membranes was higher than that of interstitial collagen type I and type III [17].

DISCUSSION

The results presented in this paper show that GLC is an analytical tool for the separation and determination of collagen amino acids. The advantages of this technique — sensitivity, accuracy, speed — enable its use for determining Pro, 4-Hyp, 3-Hyp and Hyl in a single operation; elsewhere [13, 14], these amino acids have been determined individually in the total hydrolysates by direct colorimetric estimation. In addition, GLC is more sensitive and thus it was possible to repeat these determinations many times from 0.375 mg of liver biopsy tissue since for a chromatographic run only 2 μl from 250 μl of the initial preparation were injected into the column. This possibility was particularly convenient for the determination of Hyl.

Our results, presented in Table II, demonstrate that for skin or liver, these determinations carried out on different samples from different patients yielded a relative constancy for the ratios 4-Hyp/Pro and Hyl/4-Hyp. Indeed, we have verified the constancy of these ratios in duplicate experiments from identical biological samples (skin biopsy). Thus, the values obtained for 4-Hyp/Pro were identical (0.58) and for Hyl/4-Hyp the values lay between 0.037 and 0.039. Thus, under these conditions it was possible to value, in vivo, the lack of hydroxylation of polypeptidic lysine from a skin biopsy as was described by Elsas et al. [18]. In the case reported by these authors the ratio Hyl/4-Hyp was 0.011, and a normal value 0.05 ± 0.009. In addition, it will be possible to determine the variations in collagen content in relation to proteins in a tissue, by measuring 4-Hyp/Pro; this estimation will be particularly suitable for liver biopsies. Indeed, in alcoholic cirrhosis, during which the amount of collagen increases, we have found values for this ratio of between 0.30 and 0.70; thus we could value the extent of hepatic fibrosis. In addition, the ratio 3-Hyp/4-Hyp will allow the measurement of variations in collagen from basement membrane compared to interstitial collagen, since 3-Hyp is a label of collagen from basement membranes.

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