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Note

High-performance liquid chromatographic determination of hydroxyproline after derivatization with 4-dimethylaminoazobenzene-4′-sulphonyl chloride

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Human fibroblast cultures can provide a good means for studying collagen metabolism and its alterations in numerous diseases Various methods have been reported for specifically measuring collagen in fibroblast cultures and in other biological samples, based on the presence of hydroxyproline (Hyp) which is characteristic of the collagen molecule[1]. Some of these methods are based on spectrophotometric determination of Hyp after hydrolysis, oxidation and extraction of the sample[2,3] Other procedures utilize the incorporation of [^{3}H]- or [^{14}C]-Hyp after a hydrolysis and a long series of oxidations and extractions[4–6], or measure the soluble ^{14}C-labelled proline and Hyp obtained by collagenase digestion of the sample[7,8].

Unfortunately, these procedures are often tedious and time-consuming and most of them utilize radioactive materials

The present paper reports a simple and very rapid method for Hyp assay in fibroblast cultures, using high-performance liquid chromatographic (HPLC) separation and determination of the 4-dimethylaminoazobenzene-4′-sulphonyl (dabsyl) derivatives of amino acids in the hydrolyzed samples.

MATERIALS AND METHODS

Apparatus

The HPLC system (Perkin-Elmer) consisted of a Model Series 3 solvent-delivery system, a Rheodyne 7120 injection valve with a 10-μl injection loop, a Model LC 100 column oven and a Model LC-55 B variable wavelength UV detector operat-

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ing at 486 nm. A reversed-phase RP-8 column (Brownlee; 25 cm × 4.6 mm I.D., particle size 10 μm) was used. All chromatograms were recorded on a Perkin-Elmer Model 56 recorder. The mobile phases were. A, acetonitrile, 1 66 m*M* acetic acid and 6 99 m*M* orthophosphoric acid, pH 3.5; B, 10 m*M* sodium acetate, 1.66 m*M* acetic acid, pH 3 00 adjusted with orthophosphoric acid[9]. Other conditions were: flow-rate, 1.5 ml/min; pressure, 3.5–4 MPa; column temperature, 40°C. Solvent program. T_1 = 10 min (30–34% solvent A), gradient curve 1; T_2 = 10 min (34–60% solvent A), gradient curve 0 3 (Fig. 1).

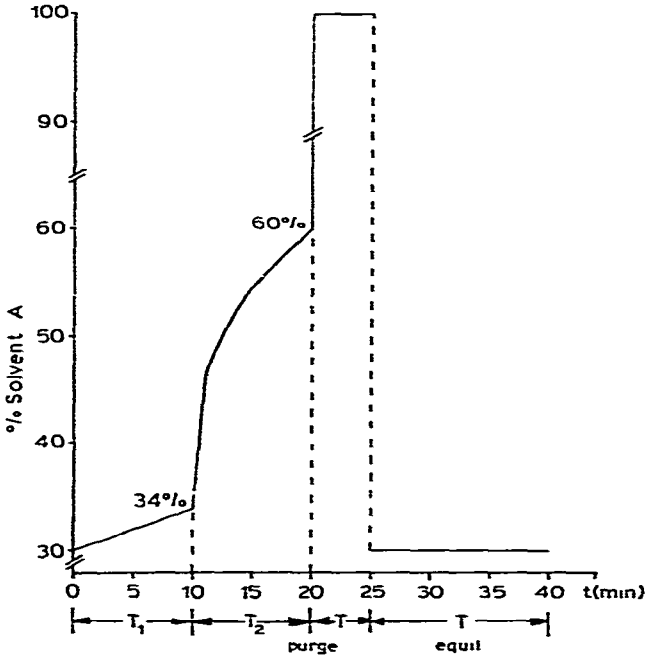

Fig. 1 Solvent program used for the column elution. Solvent program T_1 curve 1, T_2 curve 0 3

Reagents

L-4-[^{3}H(G)]Hydroxyproline ([^{3}H]Hyp), specific activity 5 4 Ci/mmol, was purchased from New England Nuclear (Boston, MA, U S A.). Dabsyl chloride, 3 3 mg/ml in acetone, was obtained from Pierce (Rockford, IL, U S A). N-Methyltaurine (99% purity according to a liquid chromatographic assay), kindly supplied by Professor Adembri, was used as an internal standard. Acetonitrile LiChrosolv, highly purified Hyp and other standard amino acids were obtained from E Merck (Darmstadt, G.F.R.). Solvents and other chemicals were of AR grade.

Procedure

Fibroblast cultures. Human fibroblasts, obtained from normal donor skin explants, were cultured as previously described[10]. Supernatants and cell monolayers of early confluent fibroblast cultures were used as samples for the HPLC procedure.

Fibroblasts (2 · 10^6 cells per flask) were cultured for 72 h at 37°C (pH 7.4) to reach visual confluency with and without ascorbic acid (100 μg/ml) in the medium. Then

supernatants were collected; cell monolayers were solubilized with a 0 5 *M* NaOH solution, washed three times with warm Hank's balanced salt solution and collected Supernatants and solubilized cell monolayers were then hydrolyzed (3 *N* HCl, overnight at 130°C) The hydrolyzates were neutralized at pH 8 9 with 4 *M* K_2CO_3

Dabsylation The dabsylation reaction was performed as described previously[11]. The reaction mixture consisted of 20 μl of the neutralized sample hydrolyzates, 20 μl of 0 1 *M* Na_2CO_3–$NaHCO_3$ buffer solution (pH 8 9), containing 6 2 μmol/ml of internal standard and 20 μl of dabsyl chloride solution The reaction was allowed to proceed at 70°C for 6 min with constant shaking A 10-μl volume of the reaction mixture was injected into the chromatograph

Calibration curve. Standard solutions of Hyp, ranging from 0 19 μmol/ml to 3 3 μmol/ml, were processed as described above The calibration curve was obtained by calculating the ratio between the peak heights of Hyp and the internal standard and plotting these ratios against the concentrations This curve was used to convert peak height ratios of unknown samples into Hyp concentration

RESULTS

Separation of dabsyl-Hyp

Fig 2 shows a typical chromatogram of biological samples (supernatants + cell monolayers from fibroblast cultures) processed by the method described It can be seen that a good separation of dabsyl chloride, N-methyltaurine and Hyp was obtained The retention times of the internal standard and Hyp were 8 and 15 2 min respectively Dabsylated standard Hyp gave a single peak

The Hyp peak was identified on the basis of its absolute and relative retention time and by adding known amounts of standard Hyp to the samples. By the same procedure it was possible to identify the peaks immediately before and after Hyp as shown in Fig 2

In order to better identify the Hyp peak [^{3}H]Hyp was also added to the samples at various concentrations and processed by the method described Throughout this procedure a linear correlation (r = 0 997) was obtained between the single amounts of [^{3}H]Hyp added to the samples and the radioactivity found in the fractions corresponding to the Hyp peak collected from the column

Furthermore, no radioactivity was recovered in the fractions collected before and after the one corresponding to the Hyp peak This confirmed that Hyp gave a single derivative under the analysis conditions used.

Some experiments were performed using a Perkin-Elmer LC85-Autocontrol variable wavelength detector (equipped with a 2.4-μl flow-cell) which could record and analyze spectra of the single peak eluted from the column The UV spectrum of the fraction corresponding to the Hyp peak overlapped (absorbance maximum at 194 5 nm) that of standard dabsylated Hyp injected into the column under the same experimental conditions.

Further evidence that no other dabsyl-positive compounds might interfere with Hyp was obtained by using a thin-layer chromatographic (TLC) procedure The fraction eluted from the column between 15 and 20 min after injection of the sample (corresponding to the last five peaks including Hyp in Fig. 2) was separated on silica gel G plates (Merck, 20 × 20 cm) by two-dimensional TLC according to Lin and

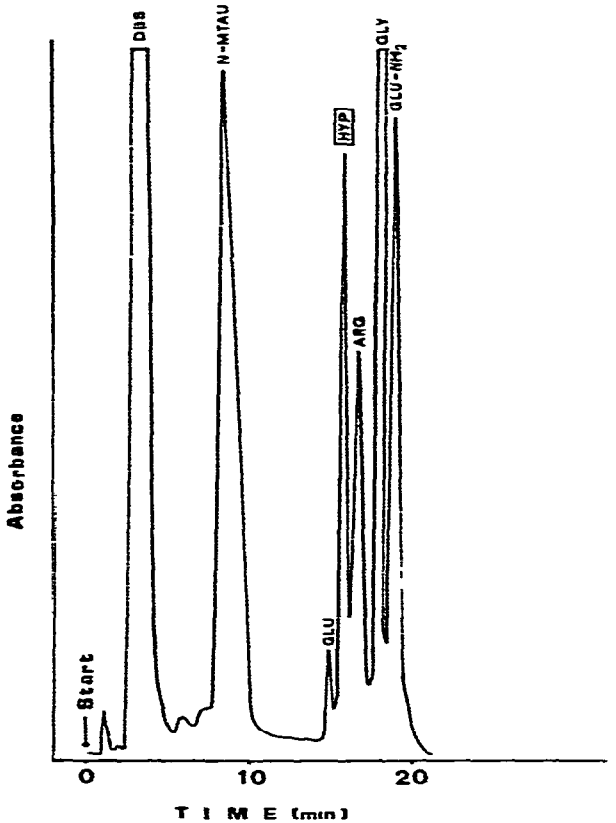

Fig. 2. Representative chromatogram of dabsyl derivative of the biological sample DBS = Dabsyl chloride; N-MTAU = N-methyltaurine, GLU = glutamic acid, HYP = hydroxyproline, ARG = arginine; GLY = glycine; GLU-NH_2 = glutamine.

Chang's method[11]. With this technique (Fig. 3), it was possible to demonstrate that: (1) only five dabsyl-positive substances were present in the fraction collected from the column and (2) these compounds corresponded to the five amino acids shown in Fig. 2. The resulting TLC red spot corresponding to Hyp was submitted to gas chromatography–mass spectrometry which confirmed the purity of this fraction

Analytical variables

Calibration curve and sensitivity. A linear calibration curve was obtained ($y = 0.2794113x - 0.0205341$; $r = 0.995$). The minimum detectable concentration of Hyp in our standard samples was 458 pmoles.

Recovery. The percentage recovery was determined by measuring the levels of Hyp in a biological sample to which known amounts of the standard amino acid had been added. The resulting yield was 93 $\pm$ 1.52% (mean value $\pm$ S.E.; $n = 9$).

Precision. Increasing amounts of Hyp were added to the same sample and processed by the same method. The results, expressed as the coefficient of variation (CV), are shown in Table I.

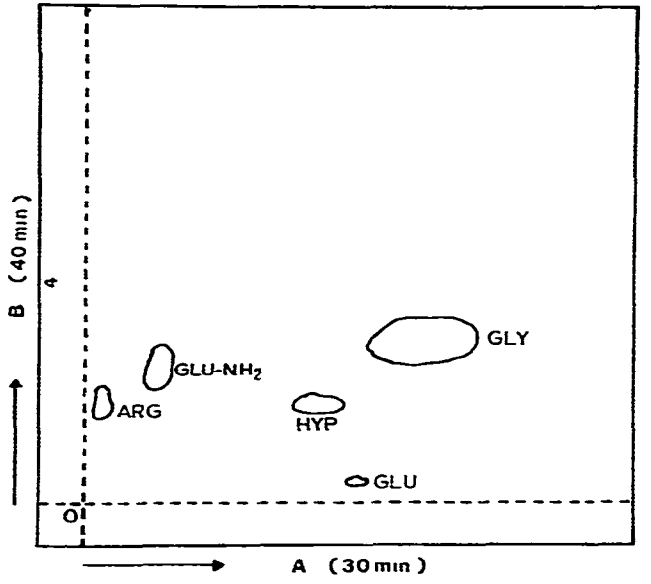

Fig. 3 Two-dimensional TLC of dabsyl amino acids The fraction eluted from the HPLC column (see text) was concentrated to dryness under nitrogen, redissolved in 0 5 ml of 0 1 *M* Na_2CO_3–$NaHCO_3$ buffer and then applied at the origin of the silica gel plate (cut into 10 × 10 cm strips) Solvents[11] A benzene–pyridine–acetic acid (80 20 5 v/v/v), B, toluene–2-chloroethanol–25% aqueous ammonia (100 80 6 7 v/v/v) The resulting five red spots were identified by the same method as the dabsyl derivatives of standard amino acids Abbreviations for amino acids as in Fig 2

Hyp concentration in the fibroblast cultures In order to verify whether the HPLC method was able to measure variations in the imino acid concentration in the cultures, Hyp was assayed in fibroblasts cultured with and without ascorbic acid in the medium[12–14]. The experiments were performed in duplicate during a period of 3 weeks A statistically significant decrease in total Hyp content (from 0 804 ± 0 007 to 0 434 ± 0.008 μmol/ml; $p < 0\ 001$; $n = 5$) was observed when cells were cultured in ascorbic acid-free medium.

TABLE I

PRECISION OF THE HYP HPLC DETERMINATION

A, Data obtained by processing the sample various times during a single day, B day-to-day precision, evaluated during a period of 6 weeks n = Number of assays

Hyp concentration (μmol/ml)	*CV (%)*	
	A (n = 10)	*B (n = 15)*
0 29	5 2	6 2
0 58	1 4	4 1
1 17	4 5	4 1
4 70	4 7	6 1

DISCUSSION

The aim of the present study was to produce a rapid and practical method for determining the concentration of Hyp in fibroblast cultures and other biological samples by HPLC with dabsyl derivatization

The specificity of the method was based on: (1) absolute and relative retention times of the amino acid: (2) collection of standard [^{3}H]Hyp in the fractions corresponding to the Hyp peak; (3) enrichment of biological samples by standard preparation of Hyp.

The sensitivity was higher than the method reported by Prockop and Udenfriend[2], who utilized a spectrophotometric assay. It was not possible to compare our results with those obtained by other groups[4–7] because they used radioisotopic assays and expressed values as dpm per mg of protein.

The yield was almost the same as those reported by Prockop and Udenfriend[2] and Peterkofsky and Diegelmann[7]. On the other hand, it was higher than those achieved in other procedures[4–6].

The present method has the advantage of not requiring an extraction step. In fact, after hydrolysis of samples, only 6 min are needed for dabsyl derivatization and a further 20 min for chromatographic separation Furthermore, it has a higher sensitivity and the same yield as the previously considered procedures Only a small volume (20 μl) is required for the analysis and, what is more, use of radioactive materials is avoided.

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