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Note

Determination of hydroxyproline in tissue collagen hydrolysate by derivatization and isocratic reversed-phase high-performance liquid chromatography

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The determination of tissue hydroxyproline (Hyp) is currently used to estimate tissue collagen content. Recently, there has been an increased interest in the use of high-performance liquid chromatography (HPLC) for Hyp and other amino acid assays [1–8]. Ahnoff et al. [1] described the isocratic separation and fluorescence detection of 4-chloro-7-nitrobenzofurazan derivatives of amino acids from tissue collagen hydrolysates. Though the method produced useful results, the derivatives were light-sensitive and not particularly stable in solution, thus necessitating immediate analysis. Also, the mobile phase was adjusted to pH 1.9 which corresponds to the lower pH limit of most bonded-phase columns. Another method [2] involves the use of two columns in series at 50°C, a lengthy derivatization procedure and collection of fractions. More recently, gradient methods have been published for the analysis of phenylthiohydantoin (PTH) derivatives [3] and phenylthiocarbamyl (PTC) derivatives [4] of amino acids, first described by Knoop et al. [5]. These methods are useful, but require a gradient controller system and, in some cases, careful column temperature control. The method described by Macek and Adam [6] is isocratic, but involves the use of an expensive post-column reactor and requires column back-flushing. The methods of Tarr [7] and Black and Coon [8] may be applicable, but PTH-Hyp is not mentioned in either article.

This paper describes a simple and reliable method for the quantitative analysis of Hyp in tissue collagen hydrolysate. The PTC derivatives of amino acids from tissue collagen hydrolysate are formed according to the method of Bidlingmeyer et al. [3]. The resultant mixture is subjected to isocratic HPLC analysis at ambient temperature using a bonded-phase (C_{18}) column and spectrophotometric detec-

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tion. The Hyp derivative elutes within 3 min. The remainder of the amino acid derivatives elute from the column within 20 min. The method can be performed manually or fully automated.

EXPERIMENTAL

Chemicals

Reagent-grade sodium acetate, glacial acetic acid, concentrated hydrochloric acid, triethylamine (HPLC grade) and acetonitrile (HPLC grade) were purchased from Fisher (Cleveland, OH, U.S.A.). Phenylisothiocyanate (PITC) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Hydroxyproline, collagen hydrolysate standard and other amino acids were purchased from Sigma (St. Louis, MO, U.S.A.). Ethanol was obtained from U.S. Industrial Chemical (New York, NY, U.S.A.). All chemicals were used without further purification.

Apparatus

The chromatographic system was composed of the following: Model 590 pump, Model 710B WISP autosampler, lambda-max 481 variable-wavelength UV detector (set at 254 nm) and Model 730 data module integrator–recorder, all from Waters Assoc. (Milford, MA, U.S.A.).

Chromatography

The PTC derivatives of the amino acids were separated using a 10 cm × 4.6 mm I.D. octadecylsilane (ODS) column with 5-μm packing (Nova-Pak C_{18}, Waters Assoc.); a precolumn (5 cm × 4.6 mm I.D.) containing 40-μm Pelligard C_{18} packing (Supelco, Bellefonte, PA, U.S.A.) was placed in-line just prior to the analytical column. The mobile phase was composed of acetonitrile–water–140 m*M* acetate buffer, pH 6.3 with 0.5 ml/l triethylamine (0.6:0.4:9.0, v/v/v). The column back-pressure was typically about 1900 p.s.i. (131 bar) at a flow-rate of 1.5 ml/min. The pre-column inlet filter frit was changed after approximately every 50 sample injections. The column was equilibrated with mobile phase for 30 min prior to the first injection. If the fully automated system was employed, the WISP autosampler was used to automatically start and stop the recording integrator.

Sample preparation

Samples of tissue from polytetrafluoroethylene implants recovered from mice after two to fourteen days were hydrolyzed in 6 *M* hydrochloric acid for 16 h at 116°C [9]. The hydrolyzed samples were evaporated at 55°C over sodium hydroxide–potassium hydroxide pellets under vacuum. The dried samples were then converted to PTC derivatives [5]. The final residue was diluted to a total volume of 2 ml with mobile phase and mixed well prior to injection. The injection volume was 10 μl.

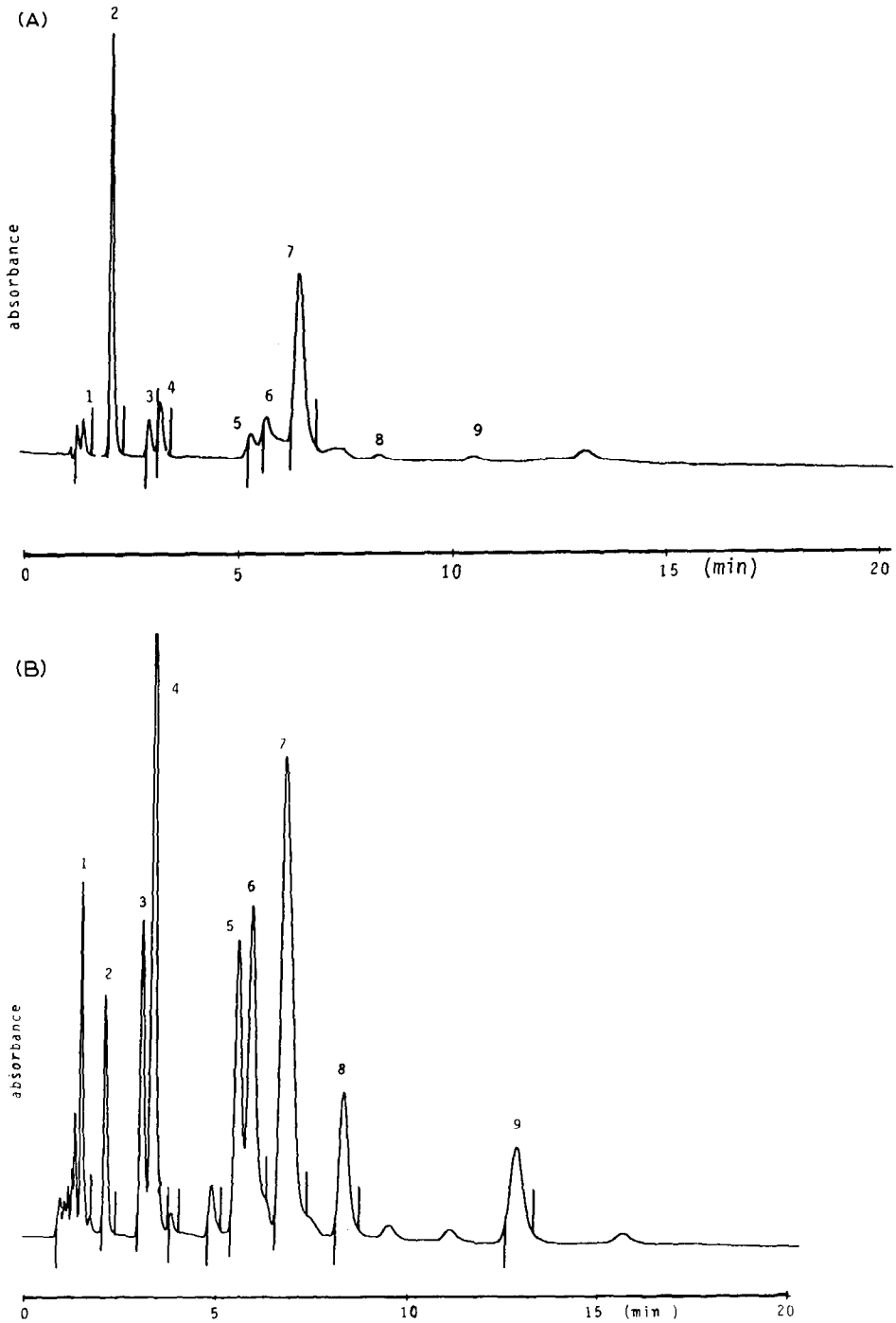

Fig. 1. (A) Chromatogram of derivatized collagen hydrolysate standard. Some peak assignments: 1 = glutamic acid; 2 = hydroxyproline; 3 = serine; 4 = glycine; 7 = proline. The hydroxyproline peak corresponds to 500 pmol on-column. (B) Chromatogram of derivatized tissue collagen hydrolysate. Some peak assignments: 1 = glutamic acid; 2 = hydroxyproline; 3 = serine; 4 = glycine; 7 = proline. The hydroxyproline peak corresponds to 330 pmol calculated from the standard curve.

RESULTS AND DISCUSSION

Chromatograms of PTC derivatives of a collagen hydrolysate standard and a tissue sample hydrolysate are shown in Fig. 1. The PTC-Hyp elutes at 2.1 min with baseline resolution from the other PTC-amino acids shown.

Linearity, accuracy, precision and sensitivity

The system will detect 5 pmol of derivatized Hyp on-column (signal-to-noise ratio of 10:1 at 0.005 a.u.f.s.), however, most of our analyses were performed at 0.10 a.u.f.s. At this detector setting, 20 pmol on-column could be accurately and reproducibly quantitated (signal-to-noise ratio of 5:1).

The linearity of the method was assessed over the range 70–1250 pmol of PTC-Hyp injected (10 μl) from a 2-ml total sample volume of derivatized collagen hydrolysate standard. A plot of absolute peak area versus pmol injected for five concentrations in triplicate produced a good line (slope: 0.42, correlation coefficient: 0.9998 from linear regression analysis). Reproducibility of the 150-pmol standard ($n=5$) was 2.6% and for the 30-pmol standard ($n=5$) 4.4%. The accuracy of the assay for the working control (50 and 200 pmol injected) was 3.7 and 4.2%, respectively. The reproducibility of the injection system was tested by repetitive injection of 100 pmol of a PTC-Hyp standard ($n=10$). The area of the peak was reproducible within 0.5%.

CONCLUSIONS

The method described is suitable for the routine analysis of tissue collagen hydrolysate Hyp content. The system is simple to operate, reliable and adaptable to full automation. The methodology is inexpensive and requires no gradient equipment or temperature-controlling devices. The method could also be extended to the analysis of other early-eluting individual amino acids, but modifications would be necessary for sensitive quantitiation of the more retained derivatives.

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