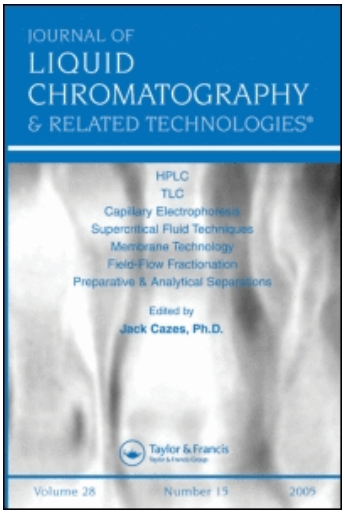

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Hydroxyproline Measurement by High Performance Liquid Chromatography: An Improved Method of Derivatization

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HYDROXYPROLINE MEASUREMENT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: AN IMPROVED METHOD OF DERIVATIZATION

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ABSTRACT

Hydroxyproline is a postsynthetic derivative of proline which is commonly used to estimate the collagen content of tissues. Values (weight %) of hydroxyproline range from ten to twenty percent in most collagen types, with Type I having 11.4 percent hydroxyproline. This fact is useful for estimating the collagen content in various acid hydrolyzed tissue samples. In this paper, the authors describe a technique which produces linear hydrolysis of collagen coupled with a sensitive ultraviolet detection scheme for the 9-Fluorenylmethoxycarbonyl Chloride(FMOC-Cl) derivative of hydroxyproline. The separation itself employs reverse phase chromatography with a sensitivity of approximately 0.12 nmoles of hydroxyproline or 0.14 μg Type I collagen.

INTRODUCTION

The determination of hydroxyproline concentration in tissue samples is a widely accepted method of quantifying and classifying the collagen content of tissues (1,2,3). The biological relevance of measuring collagen content encompasses many fields which includes connective tissue and wound healing research. Current HPLC methods for precolumn derivatization of hydroxyproline includes NDB-Chloride, Dansyl-Chloride, and FMOC-Cl derivatization. All of these methods have been successfully used by various investigators yet we wanted a more stable derivative than was formed by NDB-Chloride or Dansyl-Chloride and we also wanted to avoid the extraction procedure used with previously developed FMOC-Cl techniques (8). Initial attempts focused on adapting a recommended manufacturer's automated technique for FMOC-Cl derivatization utilizing an autosampler. This method yields stable FMOC-Cl derivatives and circumvents extraction steps which result in possible yield losses (6). To monitor the percent derivatization of hydroxyproline we used FMOC-proline as a standard since commercial FMOC-hydroxyproline was not available. Proline and hydroxyproline, which have the same secondary amine group, were assumed to react in a similar manner with FMOC-Cl (4,5). When commercially prepared FMOC-proline and proline derivatized by the automated method were compared we found that only up to 27 percent of the injected amino acid was derivatized by the automated procedure. This low yield prompted us to develop an alternative means of FMOC-Cl derivatization.

MATERIALS AND METHODS

HPLC grade water, acetonitrile, and tetrahydrafuran (non-stablized against peroxide formation), and sodium acetate trihydrate, analytical grade, were purchased from Fisher Scientific, Minneapolis, MN. Disodium borate decahydrate, boric acid, hydrochloric acid, reagent grade, were purchased from Mallickrodt, St. Louis, MO. FMOC-Cl

(F0378), FMOC-l-proline (F0634), l-proline (P0380), hydroxy-l-proline (H6002) and the amino acid standard (A9531, a simulated collagen hydrolysate), were purchased from Sigma Chemical Company, St. Louis, MO. Collagen, Type I (Vitrogen 100) was obtained from Collagen Corporation, Palo Alto, CA. A Pierce Reacti-Therm and hydrolysis tubes were purchased from the Pierce Chemical Corporation (Rockford, ILL).

HPLC System

A Hewlett-Packard 1090 Chemstation equipped with a ternary gradient system, a diode array detector, and an autosampler, was used to analyze the FMOC derivatives. We monitored absorbance at 263 nm using a 4nm band width and a time constant of 160 milliseconds. The reference wavelength was 490 nm and encompassed a 20 nm band width. We used a Hypersil ODS, 5μ, Hewlett-Packard, (5061-3330), column which measured 2.1mm x 200mm. A similar type of guard column was used. This chromatographic system was operated at 35°C during the analytical procedure.

Elution was performed using a binary gradient. Buffer A consisted of 100mM sodium acetate trihydrate with 0.5% tetrahydrafuran. The solution was filtered (0.45 μm) before the addition of the tetrahydrafuran. After the addition of tetrahydrafuran, the solution was adjusted to a pH of 7.4 with 10N NaOH. Buffer B consisted of 80% acetonitrile and 20% 100mM sodium acetate trihydrate and was similarly adjusted to a pH of 7.4 with 10N NaOH and filtered through a 0.45um filter. Both buffers were briefly degassed with helium prior to use. The gradient timetable is shown in Table 1.

Collagen hydrolysis procedure

Collagen standards (0.0, 145.0, 290.0, 580.0, 1160.0 μg) and wound samples were hydrolyzed with 6N HCl, 2ml, at 110°C for 20 hr under vacuum (7). The amino acid standards were not subjected to hydrolysis. Samples were evaporated under vacuum, resuspended with 0.5 ml 0.1N HCl, and centrifuged for 5 minutes in a Beckman microcentrifuge. One hundred microliters of this mixture were used in the derivatization procedure.

Table 1

Time	Percent B	Flow Rate (ml/min.)
0.0	0.0	0.45
9.0	70.0	0.45
13.01	50.0	0.45
18.01	50.0	0.45
18.2	50.0	0.80
18.9	50.0	0.80
19.0	50.0	0.45
19.1	100.0	0.45
21.0	100.0	0.45
22.0	0.0	0.45
30.0	0.0	0.45

FMOC-Cl Derivatization

Borate buffer (0.125M) was prepared by mixing boric acid (0.125M) and disodium borate decahydrate (0.125M) 50:50 (v:v) and adjusting the pH with 10N NaOH to 9.5. FMOC-Cl (10mg/ml), dissolved in tetrahydrafuran, was prepared fresh daily.

The following were added to a glass tube in the order listed with constant vortexing; 100 µl hydrolysate or amino acid standard, 400 µl borate buffer, 400 µl tetrahydrafuran, and 100 µl FMOC-Cl. FMOC-Cl was added dropwise in 25 µl fractions allowing 15 seconds between each fraction.

The derivatization mixture was then transferred to microcentrifuge tubes and centrifuged for 5 min. This centrifugation step clears the sample of any borate which may have precipitated during the reaction. Twenty µl of this mixture was then injected onto the column.

RESULTS

Table 2 shows a high degree of derivatization of the amino acids by this technique. Our results are 95.1% of the value for commercially

TABLE 2
THE DEGREE OF PROLINE AND HYDROXYPROLINE DERIVATIZATION BY FMOC-Cl

	mAU/nmole (n=3) mean(SD)	% Deriv. mean(SD)
Commercial		
FMOC-Pro	178.9 (8.7)	100.0
Improved Technique		
Pro	170.2 (1.4)	95.3 (4.2)
Hypro	167.9 (7.0)	93.5 (1.5)

obtained FMOC-proline, while derivatized hydroxyproline showed a 93.5% yield of purchased FMOC-proline. The reproducibility of the derivatization procedure was also quite good as seen by the standard deviation values.

Figure 1 shows a sensitivity of approximately 0.12 nmole of hydroxyproline, with a linear correlation coefficient of 0.99 over a range of 0 to 5 nmoles. A typical chromatogram of the amino acid mixture used, Figure 2, shows a baseline separation of hydroxyproline from the other 19 amino acids in the mixture. This injection contained approximately 25 nmoles of various primary and secondary amino acids. Despite this high concentration of derivatized amino acids the FMOC-Cl peak (19.75 min.) was still very large, indicating that there was still an excess of reagent present.

The yield of hydroxyproline from varying collagen hydrolysates, Figures 3 and 4, was linear (r=0.99) over a range of 0.0 to 4.54 μg. The clearly defined hydroxyproline peak can also be seen in the chromatogram of a wound sample hydrolysate (Figure 5). Although a large number and concentration of other amino acid derivatives were present, the reagent peak was still present, indicating an excess of

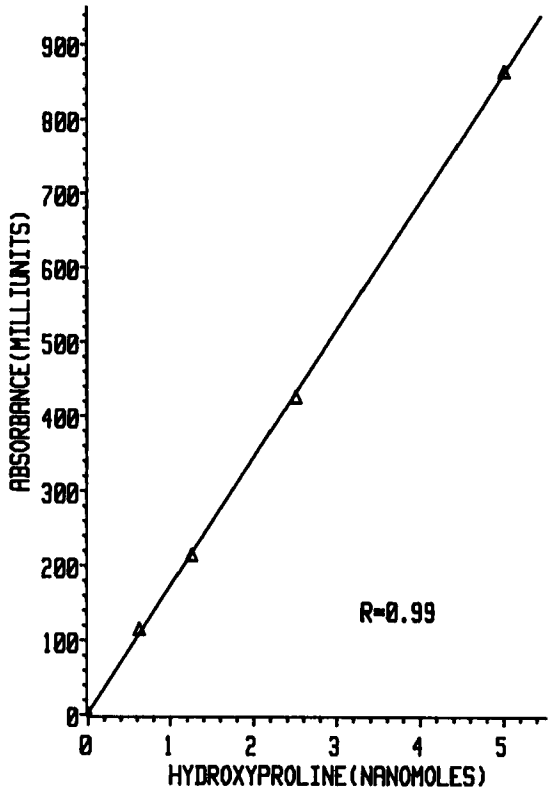

FIGURE 1. Standard curve exhibiting linearity of peak height verses nmoles hydroxyproline in a simulated collagen hydrolysate standard solution which was reacted with FMOC-Cl.

reagent. Figure 5 shows that the hydroxyproline in this sample was well within the limit of detection with only 0.4 percent of the total sample having been injected.

DISCUSSION

The results presented in this paper show an accurate and reproducible method of quantifying hydroxyproline in tissue samples. This was demonstrated by the linear and complete hydrolysis of the

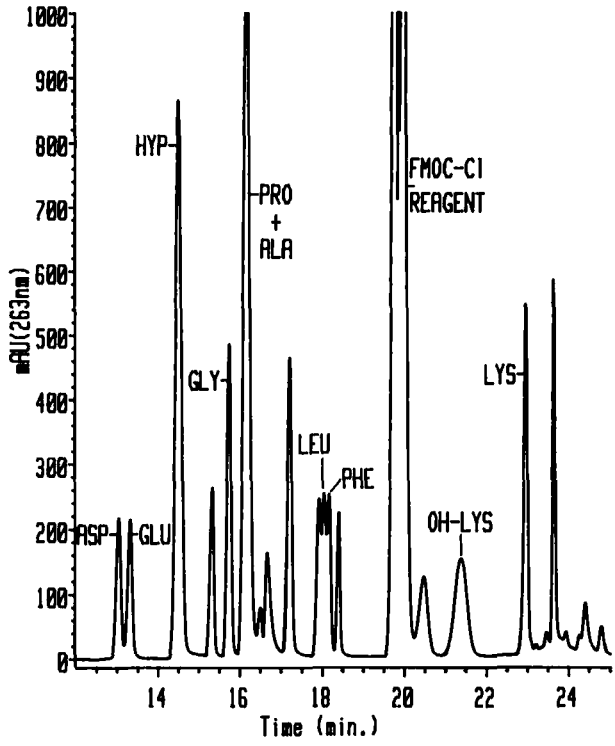

FIGURE 2. Chromatogram of a FMOC-Cl derivatives in a simulated collagen hydrolysate containing five nmoles of hydroxyproline and various amounts of other amino acids.

collagen standards plus baseline resolution of the FMOC-hydroxyproline derivatives made from them. The near one hundred percent derivatization of hydroxyproline into the very stable FMOC form lends assurance that all the hydroxyproline in the sample is being quantitated. With this technique we have been able to repetitively measure hydroxyproline with a sensitivity of approximately 0.12 nmoles and collagen to a level of 0.14 μg in actual wound samples.

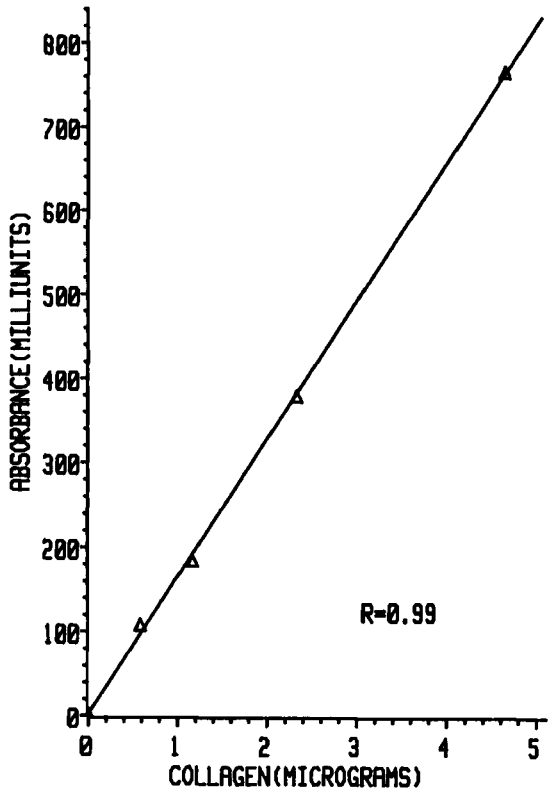

FIGURE 3. Standard curve of FMOC-hydroxyproline peak verses micrograms of hydrolyzed collagen standard which was derivitized with FMOC-Cl.

Another favorable aspect of the method is that one can avoid extraction of the derivatization mixture. Aside from the time savings aspect, this lets the operator monitor the amount of excess reagent and thus maintains assurance of the reaction's completeness. It also circumvents the possibility of losing derivatives in the extraction procedure since the total reaction mixture is injected.

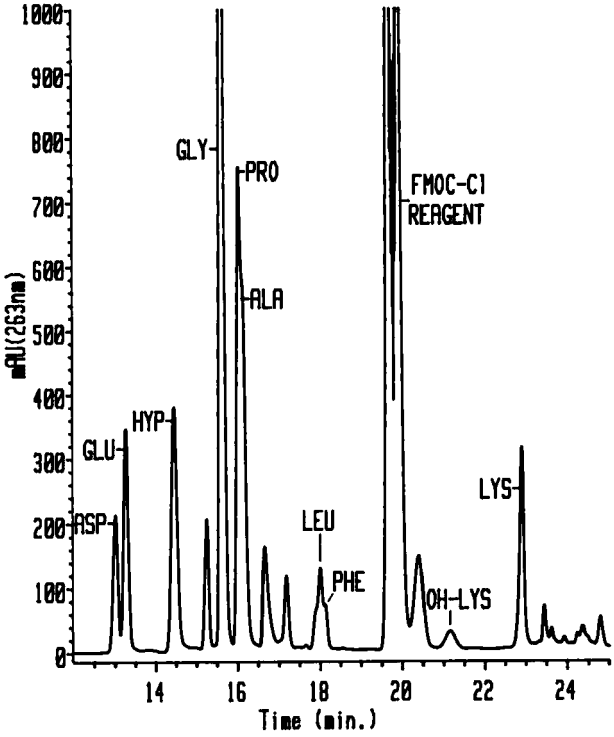

FIGURE 4. Chromatogram of derivatized amino acids in a hydrolyzed collagen standard (2.32 μg).

This technique has been used extensively in this laboratory to quantitate the collagen content in wound samples. Utilizing this technique is a measure of wound repair (9,10,11). Analysis of this type should help various wound healing and connective tissue researchers in understanding the biological repair process.

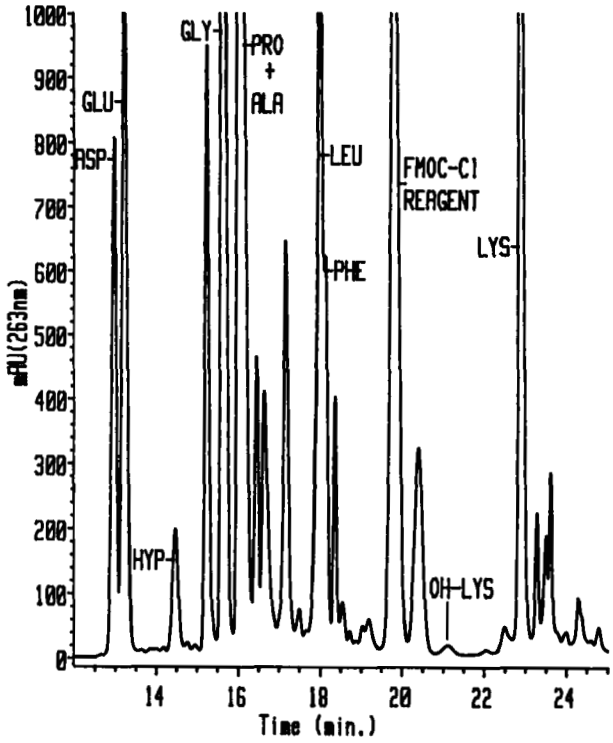

FIGURE 5. Chromatogram of FMOC-Cl amino acid derivatives in a wound sample hydrolysate. This HPLC profile represents 0.4% of the total wound sample hydrolysate.

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