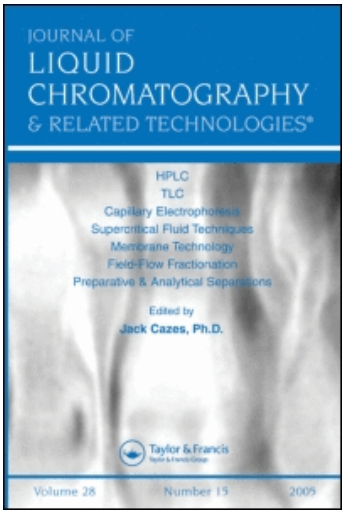

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ANALYSIS of Acid-Soluble Hydroxy-Proline, Free Proline and Collagen-Bound Hydroxyproline in Rat Liver by High Performance Liquid Chromatography With Pre-Column Derivatization

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ANALYSIS OF ACID-SOLUBLE HYDROXY-PROLINE, FREE PROLINE AND COLLAGEN-BOUND HYDROXYPROLINE IN RAT LIVER BY HIGH PERFORMANCE LIQUID CHROMATO-GRAPHY WITH PRE-COLUMN DERIVATIZATION

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ABSTRACT

A High Performance Liquid Chromatographic analysis of acid-soluble hydroxyproline, free proline and collagen-bound hydroxyproline from rat liver is described. A pre-column derivatisation with the fluorogenic reagent 7-chloro-4-nitrobenz-2-oxa-1,3-diazole was adopted. The chromatographic assay was performed by using a Spherisorb ODS2 reversed phase column, with fluorometric detection. Elution was carried out isocratically with acetonitrile-0.1 M sodium phosphate buffer, pH 7.2 (9:91, v/v). The derivatives of standard imino acids and of internal standard (3,4-dehydro-L-proline) can be separated in less than 15 min and quantitated with high sensitivity (1 in-

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jected pmole of hydroxyproline and 5 injected pmoles of proline). Key steps in the approach with the biological sample include initial extraction of acid-soluble hydroxyproline, free proline and collagen; acid hydrolysis of collagen and of hydroxyproline-containing peptides; selective derivatisation of imino acids with the fluorogenic reagent, after a previous reaction of the sample with o-phthalaldehyde; finally, chromatographic analysis of the derivatives. The assay of acid-soluble hydroxyproline requires a clean-up step on a Sep-Pak C_{18} cartridge prior to the analytical chromatography. Owing to its high sensitivity and reliability, the presented procedure can be used in studies on collagen metabolism and it should be preferred over the time-consuming and less sensitive colorimetric assays previously described.

INTRODUCTION

The determination of collagen-bound hydroxyproline is widely used as a measurement of the collagen content in tissues. The rationale of this assay is that the imino acid is synthesised in animals by an enzymatic post-translational modification of proline, almost unique to procollagen (1). Moreover, the evaluation of hydroxyproline in either the dialyzable (2) or the acid-soluble (3) fraction of tissues, can be taken as a convenient index of collagenolysis.

Many procedures have been described for hydroxyproline analysis, either employing ion-exchange chromatography based on the work by Moore and Stein (4), or using a colorimetric method involving the Chloramine T oxidation procedure (5,6). Although precise, these methods are time-consuming, tedious and of low sensitivity. More recently, various methods using High Performance Liquid Chromatography with fluorometric detection have been de-

veloped for the determination of hydroxyproline, as well as of proline and other secondary amines. For this purpose, post-column derivatisation with o-phthalaldehyde (OPA)-hypochlorite (7) or with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl)(8) and pre-column derivatisation with dansyl chloride (9), dabsyl chloride (10), NBD-Cl (11-13) have been reported. These techniques have been used for the analysis of imino acids in standard solutions, in collagen hydrolysates and in blood plasma.

During our studies, dealing with the CCl_4-induced liver fibrosis in the rat (14), we needed of a rapid, accurate and sensitive method both for the evaluation of the acid-soluble hydroxyproline and free proline pools in rat liver and for the quantification of collagen-bound hydroxyproline. The expression acid-soluble hydroxyproline includes the free imino acid as well as the one in small acid-soluble peptides, both originated from collagen degradation. For this purpose a suitable HPLC method was developed and this paper deals with its experimental details. The method adopts a sample clean-up, a pre-column NBD-Cl derivatisation, an isocratic separation of the derivatives on a reversed phase column and a fluorometric detection of the analytes. Satisfactory results are obtained as sample clean-up, derivative separation, analysis time and method reliability are concerned.

MATERIALS AND METHODS

Materials

4-hydroxy-L-proline, L-proline, 3,4-dehydro-L-proline, OPA and NBD-Cl were purchased from Sigma. Reagent

grade sodium dihydrogen phosphate, trichloroacetic acid and boric acid were purchased from C. Erba (Milan, Italy); methanol and acetonitrile (HPLC grade) were purchased from Violet (Rome, Italy). Water was demineralised and glass distilled. Sep-Pak C_{18} cartridges were purchased from Waters Assoc. and were attached to Eppendorf combi-tips (Eppendorf, Hamburg, F.R.G.), to facilitate sample application and elution. Buffer used for the mobile phase preparation was filtered by using a Pyrex filter holder equipped with 0.45 μm pore diameter filters, purchased from Millipore.

A 25 mM NBD-Cl solution was prepared in methanol. 150 mM OPA reagents were prepared in 0.2 M sodium borate buffer, pH 9.0, or in methanol. A stock standard solution containing 2 mM hydroxyproline and 4 mM proline in 0.01 M HCl was prepared every two weeks and it was stored at 4 °C. A stock solution of 2 mM 3,4-dehydro-L-proline was prepared in 0.01 M HCl and used as an internal standard.

Extraction and Derivatisation

Male Sprague Dawley rats, averaging 200 g, were fasted overnight before sacrifice. After decapitation, the liver was promptly excised and a 2 g sample was homogenised (1:3, w/v) in water. Protein was precipitated by adding 2 ml 20% (w/v) perchloric acid to the homogenate. After 10-15 min at 4 °C in an ice bath, the entire homogenate was transferred into a centrifuge tube by rinsing the homogeniser with 2 ml 5% perchloric acid and the mixture was centrifuged at 9,000 xg for 15 min at 4 °C. Supernatant was carefully decanted. The precipitate was

resuspended in 4 ml 5% perchloric acid and centrifuged as before. The precipitate was saved and used for the determination of collagen-bound hydroxyproline. The supernatant was combined with the previous one and its pH was raised to 6-7 by addition of 5 M potassium hydroxide in ice. The potassium perchlorate formed was separated by centrifugation at 9,000 xg for 10 min at 2 °C and the supernatant was brought to 15 ml with water.

In order to determine acid-soluble hydroxyproline pool, a 7.5 ml aliquot of the neutralysed extract was hydrolysed in 6 M HCl for 16 h at 105 °C in a sealed glass ampoule. The hydrolysate was spiked with 225 nmoles of internal standard and dried at 40 °C in a rotavapor. The residue was dissolved in 2.5 ml of 0.4 M sodium borate buffer, pH 9.0 and filtered through a Millipore filter. A 1 ml aliquot was poured into a screw-capped glass tube containing 0.5 ml of OPA reagent in borate buffer. The mixture was left at room temperature for 3 min. Then, 1 ml of it was passed through a Sep-Pak C_{18} cartridge previously conditioned with 10 ml methanol and washed with 10 ml water. The first 0.5 ml of the eluate were discarded and the non-derivatised imino acids were eluted with 0.2 M sodium borate buffer, pH 9.0, containing 10% methanol. A total of 2 ml eluate was collected and 0.2 ml were transferred into a screw-capped glass tube containing 0.1 ml NBD-Cl reagent and 50 µl methanol. The derivatisation was carried out by incubating the mixture at 60 °C for 3 min in the dark. The reaction was stopped by diluting the mixture to 1 ml with

cold mobile phase and 50 µl were injected into the chromatograph.

In order to determine the free proline pool, the neutralised liver extract was spiked with internal standard (60 nmoles/ml) and 0.1 ml were transferred into a screw-capped glass tube containing 0.2 ml of 0.2 M sodium borate buffer, pH 9.0. Next, 50 µl of OPA reagent in methanol were added. After 3 min incubation, 0.1 ml of NBD-Cl reagent were added to the mixture and the derivatisation was carried out at 60 °C for 3 min in the dark. The reaction was quenched by adding to the mixture 0.65 ml of the cold mobile phase and 50 µl of the obtained solution were injected into the chromatograph.

Collagen-bound hydroxyproline was determined according to the following procedure. The saved perchloric acid precipitate was washed with 10 ml cold 5% (w/v) trichloroacetic acid and the collagen was extracted in its denaturated form with 4 ml 5% trichloroacetic acid at 75 °C for 90 min, according to Rojkind (15). The extract was dialysed overnight against 250 volumes of water by using a Spectra/Por 1 dialysis tubing. The retentate was collected and the dialysis tubing was rinsed with water. A 10 ml final volume was obtained and a 1 ml aliquot of this solution was hydrolysed in 6 M HCl at 105 °C for 16 h in a sealed glass ampoule. The hydrolysate was spiked with 300 nmoles of internal standard and evaporated to dryness in a rotavapor at 40 °C. The residue was dissolved in 5 ml 0.2 M sodium borate buffer, pH 9.0 and the solution was filtered through a Millipore filter. Next, a 0.1 ml sample was processed for derivatisation

under the experimental conditions described for free proline assay.

Reversed Phase Chromatography

The liquid chromatograph used was assembled with modules purchased from various firms. The pumping system consisted of a Model Clar 002 Violet constant-flow pump (Violet, Rome, Italy). Samples were loaded by a Rheodyne Model 7125 injection valve provided with a 100 µl sample loop. The detector was a Shimadzu Model FC 530 fluorescence spectromonitor (Shimadzu Co., Kyoto, Japan), equipped with a 12 µl quartz flow-cell and operating at an emission wavelength of 530 nm and an excitation wavelength of 470 nm. Chromatographic profiles were displayed on a Model 730 Data Module (Waters Assoc.) recorder with integration. Chromatographic separations were performed on a 15 x 0.4 I.D. cm Spherisorb ODS2 (5 µm particle size) reversed phase column. A Guard-Pak (Waters Assoc.) pre-column module, equipped with a C_{18} cartridge, was used for on-line analytical column protection.

The elution was carried out isocratically with a mixture of acetonitrile-0.1 M sodium phosphate buffer, pH 7.2 (9:91, v/v). The mobile phase was delivered to the column at a flow rate of 1.3 ml/min at room temperature (20 ± 2 °C).

At the end of each working day, the column was washed with acetonitrile-water (60:40, v/v) for 25 min and maintained overnight in this solvent. Before starting a new analysis, the column was conditioned with the mobile phase for 30 min.

Quantitation

The concentration of the analytes was determined from calibration curves and quantitation was aided by the addition of the internal standard to the samples. Calibration curves were drawn by analysing standard solutions containing 5, 10, 20, 50 μM hydroxyproline and 10, 20, 40, 100 μM proline, plus internal standard at 60 μM constant concentration. Standard solutions were prepared by diluting the stock solutions with 0.2 M sodium borate buffer, pH 9.0 and 0.1 ml aliquots were submitted to the derivatisation procedure described above. Ratios of the peak areas (analyte/internal standard) were plotted against concentration of analytes. The unknown concentration of samples was determined from the calculated peak area ratios by interpolation from the calibration curves. The standard curves were analysed by a linear regression method.

Precision

To determine between-day precision, coefficients of variation during five consecutive days were determined by analysing acid-soluble hydroxyproline, free proline and collagen-bound hydroxyproline from rat liver samples. To assess the within-day coefficients of variation of the assay, five replicate analyses of each biological sample were performed on the same day. The samples were stored at -20 °C in glass vials between analyses.

RESULTS AND DISCUSSION

Pre-Column Derivatisation

A pre-column derivatisation of imino acids with NBD-Cl was adopted, since this technique allows an easy sample handling and it shows satisfactory sensitivity and selectivity. The reaction conditions used for the derivatisation were those described by Ahnoff (10). These conditions allow to achieve a direct chromatographic determination of hydroxyproline in standard solutions or in purified collagen hydrolysates. On the contrary, severe troubles arise when the HPLC analysis of imino acids in a derivatised sample of liver extract has to be performed. In fact, although the fluorescence intensities of NBD-imino acids are higher than those of NBD-amino acids neverthless the interference of amino acids cannot be avoided, owing to their quantitative predominance in the sample. In the case of collagen-bound hydroxyproline and free proline assays, this drawback was overcome by making an OPA derivatisation of primary amines precede the NBD-Cl reaction, in agreement with the procedure for the thin layer chromatographic assay of urinary hydroxyproline reported by Bellon et al. (16). Besides the reaction with OPA, the determination of acid-soluble hydroxyproline in liver extracts requires a further clean-up step, which was achieved by purifying the OPA-treated sample on a Sep-Pak C_{18} cartridge. This experimental protocol allows to obtain interference-free chromatographic profiles and an accurate quantitation of hydroxyproline and proline, even in very complex matrix samples, can be performed.

Analytical Chromatography

Ion-pair reversed phase (11) and reversed phase (12, 13) chromatography can be used for the quantitation of NBD-imino acids by HPLC. We have selected a new reversed phase chromatographic system able to ensure, under isocratic elution conditions, the complete separation of the derivatives of interest in less than 15 min.

A typical chromatogram of NBD-derivatives of standard hydroxyproline, standard proline and internal standard is shown in Fig. 1. The capacity factor (k') for NBD-derivatives are little influenced by the mobile phase pH in the range 6-7.5. On the contrary, a remarkable effect on k' is shown even by a small variation in the mobile phase composition with respect to the organic modifier percentage. Thus, the acetonitrile percentage was optimized to achieve a rapid and satisfactory chromatographic separation, without need of column washing and regeneration between successive analyses.

The analyte/internal standard peak area ratios determined from the analyses of derivatised standards were linearly related to analyte concentrations over the range investigated. The calibration curves were represented by the equations $y = 0.031x + 0.0058$ for hydroxyproline and $y = 0.053x + 0.051$ for proline, where y is the peak area ratio of analyte/internal standard and x the concentration of the analyte. The coefficient of correlation for both lines was 0.999. The results of precision for the determination of acid-soluble hydroxyproline, free proline and collagen-bound hydroxyproline in rat liver extracts are presented in Table 1.

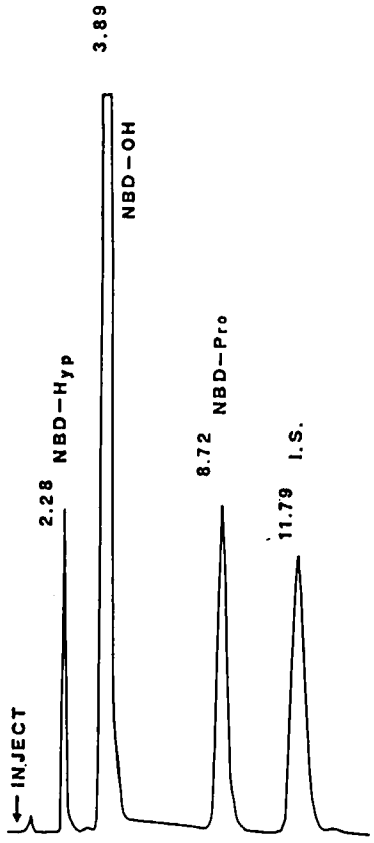

FIGURE 1

Chromatogram of nitrobenzofurazan-derivatives of hydroxyproline (NBD-Hyp), proline (NBD-Pro) and internal standard (I.S.). Peaks of analytes correspond to injected amounts of 100 pmoles hydroxyproline, 200 pmoles proline and 300 pmoles internal standard, from a calibration mixture prepared as described. Chromatographic conditions: column, 15 cm x 4 mm I.D. Spherisorb ODS2; mobile phase, acetonitrile-0.1 M sodium phosphate buffer, pH 7.2 9:91, v/v); flow rate, 1.3 ml/min; fluorimeter wavelengths, 470/530 nm; attenuation detector sensitivity, x2.

TABLE 1

Within-day and Between-day Reproducibility of Hydroxyproline (Hyp) and Proline (Pro) Quantitation in Rat Liver Extracts.

Analyte	Within-day mean concn.	C.V.%	Between-day mean concn.	C.V.%
Acid-soluble Hyp	31.8± 1.3	4.0	35.0± 1.8	5.1
Free Pro	210.5± 6.5	3.1	180.0± 9.0	5.0
Collagen-bound Hyp	1150.0±33.0	2.8	1210.0±42.0	3.5

Figures from five analyses are given in nmol/g of liver (wet wt.). C.V. = Coefficient of Variation.

The within-day coefficients of variation ranged from 2.8% at the higher concentration of the analyte (collagen-bound hydroxyproline) to 4.0% at the lower concentration (acid-soluble hydroxyproline). Between-day precision showed coefficients of variation ranging from 5.1% to 3.5%, indicating a good reproducibility of the analyses.

Fig. 2A shows a chromatogram referred to the analysis of hydroxyproline from a hydrolysed sample of purified collagen. As can be seen in the chromatographic profile, the only significant peaks in evidence, besides the one of 7-nitro-4-benzofurazanol (NBD-OH), are those of the imino acids derivatives. This allows an easy quantitation of the imino acids without interference from amino acids, since these last had been converted into undetectable adducts by the previous reaction with OPA. The identity

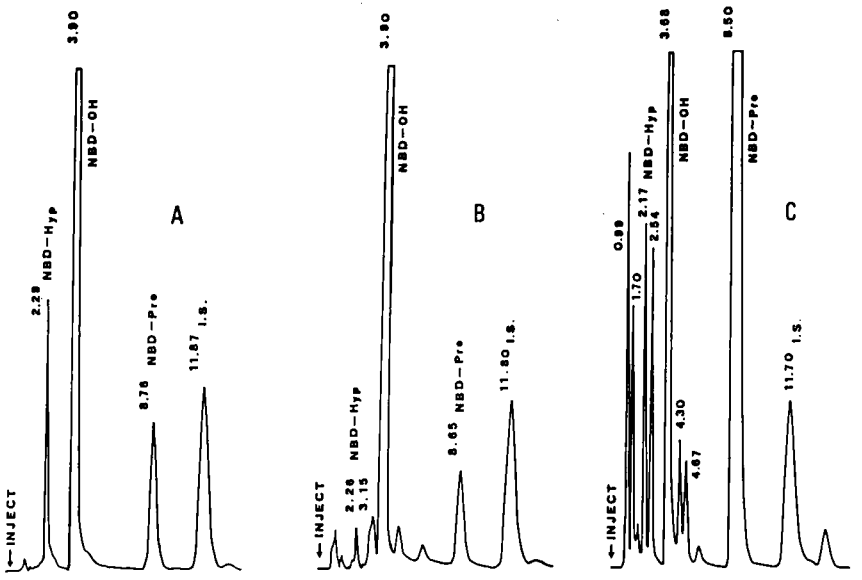

FIGURE 2

Representative chromatograms of imino acid analyses on rat liver samples: (A), collagen-bound hydroxyproline; (B), free proline and (C), acid-soluble hydroxyproline.Chromatographic conditions are as described in Fig.1.

of the peak ascribed to NBD-hydroxyproline was recognized by comparing its retention time with that of the authentic derivative and by addition of known amounts of standard to the sample. Further identification of the hydroxyproline derivative, as well as evidence that no other NBD-derivative had been co-eluted, was gained from a thin layer chromatographic analysis of the material recovered from the eluted peak, performed according to Bellon (16). Under these conditions only one spot, migrating as authentic hydroxyproline derivative, was observed.

In Fig. 2B and 2C are shown the chromatographic profiles for free proline and acid-soluble hydroxyproline analyses from an acid-extract of rat liver. Although some unknown peaks appear in the chromatograms in addition to the NBD-imino acid peaks, interferences are not observed, thus allowing an easy quantitation of the imino acids of interest.

The acid-soluble hydroxyproline, free proline and collagen-bound hydroxyproline concentration in the liver from twenty-one normal rats, measured by the method here reported, was 32±6 nmoles/g wet wt. for acid-soluble hydroxyproline, 195±12 nmoles/g wet wt. for free proline and 1,150±120 nmoles/g wet wt. for collagen-bound hydroxyproline. The limit of detection was found to be 1 injected pmole for hydroxyproline and 5 injected pmoles for proline.

Finally, the procedure has many major advantages over previously described techniques for the determination of hydroxyproline and proline in tissues. The derivatisation is easy to perform, the clean-up procedure, necessary for the acid-soluble hydroxyproline determination, requires a minimal sample manipulation and the HPLC analysis is rapid and precise.

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