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Short communication

Liquid chromatographic determination of hydroxyproline in tissue samples

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Abstract

We describe a reversed-phase assay of hydroxyproline in rat lung tissue using sarcosine for the internal standard and pre-injection reaction with both o-phthalaldehyde (OPA) and 9-fluorenylmethylchloroformate (FMOC). Intra-assay variability in the concentration range of 25–500 μM hydroxyproline was less than 1%. Normal rat (left) lung was found to have a hydroxyproline content of 1.08 ± 0.18 mg/lung. This ability to measure minute amounts of hydroxyproline is being applied to the measure of collagen and pathological fibrosis.

Keywords: Hydroxyproline; Amino acids

1. Introduction

This method was developed to satisfy a need to measure small changes in the amount of fibrosis in rat lung tissue used as a model for human asthma. Hydroxyproline is one of three predominant amino acids constituting collagen. We sought to develop an assay that could quantify changes in pulmonary fibrosis by measuring increases in pulmonary hydroxyproline content.

The use of fluorescent derivatives is common for the measurement of amino acids. An initial reaction with *o*-phthalaldehyde (OPA) is currently used to react with the primary amines, leaving secondary amines such as proline and hydroxyproline to react with other fluors [1]. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) [2], phenylisothiocyanate (PITC) [3], and 4-dimethylaminoazobenzene-4′-sulfonyl chloride (dabsyl chloride, DABS-Cl) [4] have all been used in the secondary pre-column derivatization of hydroxyproline. HPLC has been tried in the measurement of the hydroxyproline-NBD-Cl derivative but yielded suboptimal sensitivity in the aqueous mobile phases [5]. Monboisse et al. have demonstrated the specificity and sensitivity of an HPLC assay combining sequential precolumn derivatization with OPA and 9-fluorenylmethylchloroformate (FMOC) [6].

Einarsson and separately Mazzi et al. reported the application of this OPA/FMOC process to the HPLC analysis of the hydroxyproline in seawater, urine, and serum [7,8]. The FMOC derivative was measured by fluorescence detection and yielded an extremely sensitive assay for hydroxyproline. Our

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method reported below adapts these prior works to provide a technique suitable for measuring hydroxyproline in rat lung tissue.

2. Experimental

2.1. Materials

Hydroxyproline, sarcosine, sodium acetate, sodium monophosphate, *o*-phthalaldehyde, iodoacetamide, and FMOC were all reagent grade and were purchased from Aldrich (Milwaukee, WI, USA). Chemical grade β-mercaptoethanol, ethyl ether, boric acid, and sodium hydroxide were obtained from Fisher Scientific, as were the HPLC-grade acetonitrile and glacial acetic acid. Reverse osmosis purified water was passed through a Barnstead water filtering system (Barnstead International, Dubuque, IA, USA). The isocratic mobile phase was prepared by combining 3% glacial acetic acid buffered with sodium acetate to a pH of 4.3 (650 ml) with acetonitrile 350 ml, followed by vacuum filtration/degassing.

2.2. Instrumentation

The instrumentation included a Shimadzu LC-10AD HPLC pump, SIL-10A auto injector and a SCL-10A system controller (Columbia, MD, USA). A McPherson Model FL-750 spectrofluorometer (Chelmsford, MA, USA) was used with the optional high-sensitivity module, an excitation wavelength of 265 nm and without an emission cut-off filter. Separation was obtained using a Lichrosphere 5 RP18e 250 mm×4 mm, 5-μm column from Phenomenex, Torrance, CA, USA. The mobile phase was pumped at a constant rate of 1 ml/min.

2.3. Tissue preparation

Sample rat lungs stored at −80 °C were thawed and the wet weight of each lung was recorded. Only the left lung of each rat was used, since other, unrelated tests were performed on the right lung. Extrapulmonary blood vessels and airways were trimmed away before weighing and homogenization. The lung was homogenized in 1 ml of 6 *M* HCl with a motorized homogenizer (Tissue Tearor, Biospec Products, Bartlesville, OK, USA). Then, 200 μl of homogenate were placed in a screw-top glass test tube (Kimax) and additional 3.8 ml 6 *M* HCl were added. 100 μl of 2 m*M* sarcosine standard in water were added to each tube, after which the tubes were tightly capped and placed in a 110 °C heating block for 18 h. The hydrolysates were allowed to cool to room temperature and neutralized with 4 ml of 6 *M* NaOH. Each sample was brought to a pH of 9.5±1.0 with 6 *M* NaOH. Aliquots of 900 μl of this solution were removed for the subsequent derivatization process.

Hydroxyproline standards of 25, 50, 100, 250, 500, 750, and 1000 μ*M* in water were prepared from a 2 m*M* stock standard. Then, 11.3 μl of 2 m*M* sarcosine were added to each 900-μl sample of hydroxyproline standard. Incubation of the hydroxyproline-sarcosine standards at 110 °C over 18 h did not affect the results (data not shown), so this step was omitted for the standards. Standards and samples were run in triplicate.

2.4. Derivatization

Aliquots (900 μl) of homogenate supernatant or standard were transferred to 5-ml Reactivials (Supelco, St. Louis, MO, USA), followed by addition of 200 μl of borate buffer (0.7 *M* boric acid, pH 9.5, with NaOH). Next, 100 μl of OPA solution were added (50 mg *o*-phthalaldehyde dissolved in 1 ml acetonitrile containing 26 μl of β-mercaptoethanol), followed 60 s later by 100 μl of iodoacetamide reagent (140 mg/ml of iodoacetamide in acetonitrile). One minute later, 300 μl of 5 m*M* FMOC in acetone were added. The reaction vial was capped and vortexed between each addition of reagent. One minute after the addition of FMOC reagent, 2 ml of ethyl ether were added to each reaction vial. The vial was shaken vigorously for 30 s to wash the contents of the vial. The organic layer was discarded and the wash was repeated twice for a total of three washes. Then, 50 μl of the remaining aqueous phase were injected into the HPLC. Sample injections were made every 30 min without an intervening wash.

3. Results

The derivatized hydroxyproline peak eluted at 4.7 min, with the sarcosine standard, proline, and FMOC eluting at 11.7, 16.5, and 23 min, respectively (Fig. 1). The assay provided excellent reproducibility in the range of 25–500 μ*M* hydroxyproline, and the coefficients of variation at all concentrations of the standards used for the curve were under 0.93%. Linear regression yielded an equation of HYP (μ*M*)=32.29×(Peak Area)−9.5 (r^2= 0.999).

The average amount of hydroxyproline in five normal rat (left) lungs was 1.09±0.18 mg (Mean±SD), which represents approximately 7.7 mg collagen/(left) lung. This is equivalent to a hydroxyproline concentration of 145 μ*M* in the 900-μl aliquot taken from the alkalinized hydrolysate and processed with OPA and FMOC. The mean coefficients of variation of the triplicate analyses were 2% for the five left lungs taken from normal rats. The signal-to-noise ratio for the lowest standard (25 μ*M*) was 250, giving an extrapolated detection limit of less than 500 n*M*, or about 14 pmol on the column. This suggests that the assay has adequate sensitivity to measure hydroxyproline concentrations in a sample of lung as low as 10 mg (wet weight).

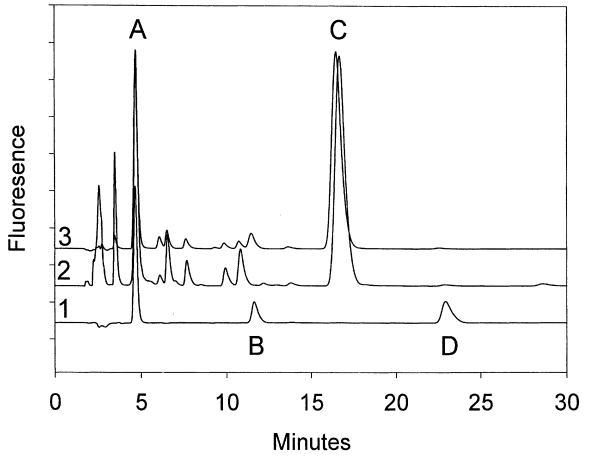

Fig. 1. Sample chromatograms illustrating elutions of hydroxyproline (A), internal standard (sarcosine, B), proline (C), and FMOC (D). Chromatograms are: (1) water spiked with hydroxyproline and internal standard; (2) rat lung without internal standard; (3) separate rat lung with added internal standard.

4. Discussion

Our initial attempts to apply the derivatization reported by Mazzi and Einarsson to hydrolyzed tissue were promising. One change from the previously published methods was the addition of a slightly supra-stoichiometric amount of NaOH to the acid hydrolysate to decrease the volume of borate buffer required to establish a pH of 9.5 for the derivatization steps. Another modification was the use of three ether washes to minimize the amount of unreacted FMOC in the HPLC sample. Although the washes add to the preparation time of the sample, Fig. 1 shows that it was effective, and we determined that the extra washes did not significantly affect the recovery of the derivatized hydroxyproline. Finally, homogenization of the rat lung was extended to 18 h to ensure thorough maceration of the sample, since hydrolysis at 100 °C for only 12 h left visible pieces of tissue.

The lowest hydroxyproline concentration of 25 μ*M* incorporated in our standard curve represents 92.2 ng of hydroxyproline on the column, or approximately 660 ng of collagen. In contrast to the HPLC assay, a Sirius Red-based assay produced by Biocolor (Newtownabbey, N. Ireland) to measure soluble collagen has a sensitivity limit of 2.5 μg [9]. The signal-to-noise ratio of 250 at this concentration suggests that much smaller, more focal sections of tissue can be accurately assayed. Alternatively, other spectrofluorometers without high-sensitivity modifications such as ours should be well suited for this assay. In such cases, an emission wavelength of 316 nm should be used [7].

Based only upon its availability in our laboratory, we utilized a longer (250 mm) analytical column than did previous reports. The excellent resolution of the shorter columns with the correspondingly shorter elution times reported by others [6–9] supports the use of shorter columns and run times than are reported here. Our assay includes a modest amount of sarcosine as internal standard, as evidenced by the relative sizes of the hydroxyproline and sarcosine peaks in Fig. 2. It can be argued that the precision of the assay could be improved further by increasing the amount of sarcosine added to the tissue prior to hydrolysis.

These modifications to the methods of earlier

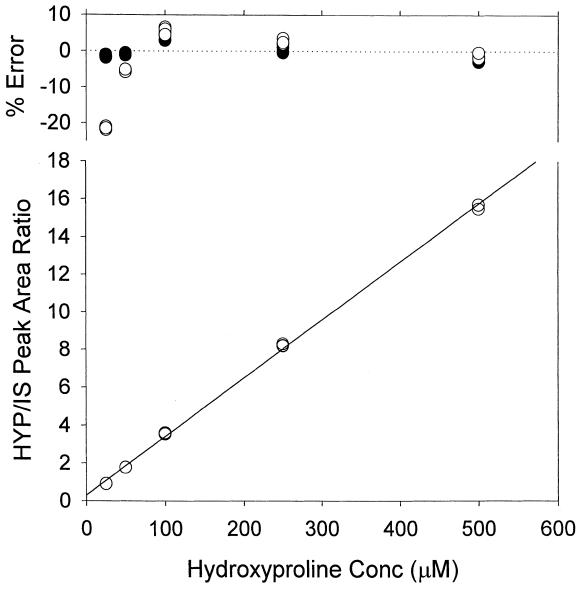

Fig. 2. Hydroxyproline standard curve showing linear regression of hydroxyproline concentration versus ratio of hydroxyproline/sarcosine (internal standard) peak areas. Upper panel shows percent error of measured versus concentration points expected from the linear regression line. Solid circles indicate identical points.

innovators of the FMOC-HPLC analysis of hydroxy proline in water or biological fluids have yielded a sensitive, precise, and robust assay of hydroxyproline that is now being applied to the analysis of fibrotic changes in rat lung models of asthma.

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