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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR QUANTITATION OF PROLINE AND HYDROXYPROLINE IN BIOLOGICAL MATERIALS

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SUMMARY

Numerous high-performance liquid chromatography systems have been described for the determination of hydroxyproline (Hyp) and proline (Pro) levels in biological materials. These methods are generally complicated and have shortcomings in applicability due to poor separation, low sensitivity or derivatization-associated problems. The large number of chemical components present in biological samples further complicates the analysis of Hyp which usually occurs in extremely low concentrations.

The present investigation describes the development of a simple highly sensitive derivatization method which results in good separation of peaks and which is capable of quantitating less than 10 pmol of Hyp and Pro in complex test systems. The method is based on removal of *o*-phthalaldehyde (OPA) derivatives of primary amino acids using reversed-phase chromatography, pre-column derivatization with OPA and phenylisothiocyanate, and detection of derivatized Hyp and Pro using a UV detection system. The procedure yields good peaks and a 93% recovery of Hyp and Pro provided that the analysis is initiated within 5 min of completion of OPA derivatization. While a 93% recovery of Pro was obtained up to 100 min post-derivatization with OPA, the recovery of Hyp is decreased to approximately 80% within the same time interval.

INTRODUCTION

The development of an efficient sensitive method for the analysis of hydroxyproline (Hyp) in biological materials is an essential requisite for studying the composition and metabolism of connective tissues. As Hyp is found in extremely low concentrations in other biological matter and fluids, its separation and quantitation is difficullt in complex samples that contain large numbers of chemical components present in disproportionately large amounts.

In recent years considerable effort has been made to apply pre- or post-column derivatization, and spectrophotometric or fluorescence methods of detection of de-

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rivatized amino acids using high-performance liquid chromatography (HPLC)[1–7]. In general some shortcomings have been found with these derivatization methods. For example, in *o*-phthalaldehyde (OPA) derivatization, the quantitation of proline (Pro) and Hyp required special oxidative procedures to cleave the ring of these amino acids[6]; 5-dimethylaminoaphthahalene-1-sulfonyl (dansyl) chloride is more sluggish in reactivity and therefore has not gained wide acceptance[7]; arylation with the sulfonyl halides dimethylaminoazobenzene-4-sulfonyl (dabsyl) chloride and dansyl chloride presents difficulties with quantitation[7].

Recently, derivatization with phenylisothiocyanate (PITC)[7] was developed for quantitative pre-column derivatization of amino acids. In this derivatization method excess PITC, which is volatile, is removed by evaporation. The reported sensitivity of the method for the derivatized amino acids is in the range 1–10 pmoles, and the derivatives are stable for weeks. Furthermore, the more complicated post-column derivatization and fluorescence detector system were not a requirement for this procedure. Because of these advantages, the utility of the PITC method was investigated in combination with the OPA method. As the determination of Hyp in a biological sample is difficult to perform because of its low concentration[3], it was necessary to modify the reported HPLC procedures[1–5] to improve the separation of Hyp from other amino acids. Casini *et al.*[4] employed dabsyl chloride derivatization to detect Hyp. However, their separation of Hyp was not complete as one-third heights of arginate and glutamate peaks interfered. Furthermore they found that the sensitivity to Hyp was low (0.485 nmol), and that the employed dabsyl chloride had the previously mentioned disadvantage. Wiedmeier *et al.*[2] applied a reversed-phase column for determining Hyp using the fluorescent dansyl chloride derivative method. In their system, the glutamate peak interferes with the Hyp peak which was too small in comparison to the other amino acids in the biological sample. Nakazawa *et al.*[5] reported a method using OPA derivatization that needed a special oxidation step. They employed a standard addition method to detect very small amounts of Hyp. However, this method required four different concentrations of Hyp added to test samples to construct a standard addition curve. Their system was further complicated through the use of post-column derivatization and fluorescence detection. Roth[3] separated Hyp from other amino acids on a cation-exchange column using post-column derivatization with 4-chloro-7-nitrobenzolfurazan (NBD-Cl) which had much higher fluorescence intensitities with secondary than with primary amino acids. However, the method lacked sensitivity. Ahnoff *et al.*[1] also employed NBD-Cl derivatization and reversed-phase column separation. While they found that NBD-Hyp was stable in 100% methanol, the agent was not stable when used as a component of the test systems[1]. In applying this method to analyses of [^{14}C]proline uptake by fibroblast cell cultures and a standard amino acid mixture, we found this method to be unsatisfactory for the recovery of ^{14}C-labelled Pro and Hyp. With this method only 55% of the ^{14}C-activity was derivatized (45% underivatized) and a large amount of NBD-Cl by-products was obtained which eluted during later stages of chromatography.

Thus the objective of the present study was to develop a simple highly sensitive method suitable for a good separation of Hyp and Pro free from any interference by other amino acids. In accordance with this objective, OPA derivatization and reversed-phase column chromatography were used first to remove primary amino acids

from the test samples. This was achieved because OPA does not react with secondary amines in absence of oxidative agents. Then the samples were subjected to PITC derivatization which is one of the best methods for the detection of Hyp and Pro.

EXPERIMENTAL

Apparatus

All analyses were performed using a fully-automated high-performance liquid chromatography unit (Waters Assoc., Mississauga, Canada) which consisted of a Model 721 programmable system controller, two Model 510 HPLC pumps, a Model 710 WISP, a Model 730 data module and a Model 481 LC UV variable-wavelength spectrophotometer operating at 254 nm. The system was coupled to a Gilson Model 201 fraction collector (Gilson, France) and a 10 cm × 8 mm (5 μm) reversed-phase Nova-Pak C_{18} Radial-Pak column (Waters Assoc.) used with a Z-module radial compression separation system (Waters Assoc.).

^{3}H and ^{14}C radioactivities were detected with a Model PW4700 liquid scintillation counter (Philips, Holland) programmed for dual label counting. For lyophilization, a Model 75040 freeze dryer (Labconco, Kansas City, KS, U.S.A.) was used.

Reagents

L-4-[^{3}H(G)]-Hydroxyproline (^{3}H-Hyp) with a specific activity of 5.9 Ci/mmol, and L-[^{14}C(U)]-proline [^{14}C(U)-Pro] with a specific activity of 250 mCi/mmol, were purchased from DuPont (Boston, MA, U.S.A.) and ICN Pharmaceuticals (CA, U.S.A.). *o*-Phthalaldehyde derivatization solutions were prepared by dissolving 50.0 mg of OPA (BDH, Vancouver, Canada) in 750 μl 0.5 *M* borate buffer (pH 10.4), 200 μl methanol and 20 μl 2-mercaptoethanol. PITC was obtained from Sigma (St. Louis, MO, U.S.A.) and its reagent was composed of 100 μl PITC, 100 μl water, 100 μl triethylamine (TEA) and 700 μl ethanol. Both reagents were freshly prepared for each experiment. Highly purified amino acids (L-alanine, L-asparagine, L-arginine, L-cystine, L-glutamine, glycine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-serine, L-threonine, L-valine, L-proline and L-hydroxyproline) were purchased from Nutritional Biochemicals (OH, U.S.A.).

Acetonitrile, methanol and ethanol were HPLC chemical grade and obtained from BDH. Non-organic water was prepared by passage of 18 MΩ pure water through a Norganic cartridge (Waters Assoc.). Buffer A of the mobile phase was composed of 60 ml acetonitrile and 940 ml of 138 m*M* acetate buffer (pH 6.4) containing 0.05% TEA, while buffer B consisted of 600 ml acetonitrile and 400 ml Norganic water.

Methods

The OPA derivatization[8] was performed to remove primary amino acids from the sample. For this phase of the experiment, 75 μl of sample were filtered through a SJHV004NS filter (Nihon Millipore Kogyo, Japan), mixed with 75 μl of OPA reagent and allowed to react with OPA at ambient temperature for 5 min. Additional analyses were performed up to 4 h after the OPA derivatization to establish the stability of the OPA derivatives of Hyp and Pro.

At designated post-reaction times indicated in Fig. 1, 100-μl aliquots of deri-

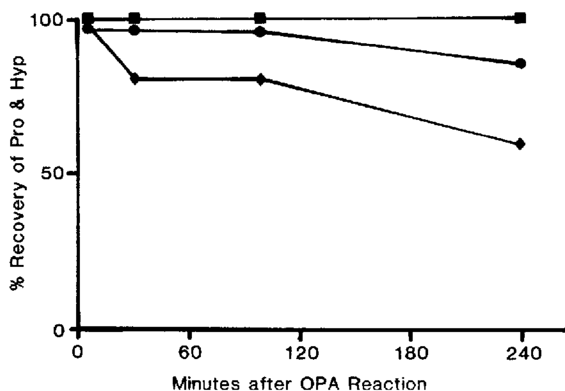

Fig. 1. Recovery of OPA derivatized amino acids as a function of time (■, primary amino acids; ●, Pro; ◆, Hyp). The recovery of Pro was constant up to 100 min, while that of the Hyp derivative gradually decreased after 5 min. The concentration of primary amino acids derivatives remained constant for at least 4 h after reaction with OPA.

vatization solution were applied to a Nova-Pak C_{18} Radial-Pak column. The initial flow-rate of the mobile phase was 1.5 ml/min at 5% B for 3 min, then followed by elution with 100% B buffer for 9 min. The column was then re-equilibrated with A–B (95:5) for 8 min. The secondary amine-containing fractions (0–3 min) were collected, lyophilized, and derivatized again using 150 μl of PITC reagent for 20 min. Following reaction, the derivatives were evaporated to complete dryness.

This is an important step in the procedure as incomplete drying will adversely affect the recovery of derivatized products. The dried samples were then reconstituted in 150-μl volumes of phosphate sample buffer consisting of a 5 m*M* disodium hydrogen phosphate solution adjusted to pH 7.4 with 10% orthophosphoric acid and combined with acetonitrile (95:5, v/v). The samples were then filtered through SJHV004NS filters and 5–50 μl volumes were injected on to the same Nova-Pak C_{18} Radial-Pak column used for the resolution of OPA derivatives.

The mobile phase was programmed at a flow-rate of 1.5 ml/min starting with 100% buffer A, followed by a linear gradient to 50% buffer B for 10 min, then increased to 100% B for 30 s and maintained at 100% B for an additional 5 min. The column was then equilibrated with 100% A for the next 10 min.

The applicability of the method to the analysis of Hyp and Pro content in complex biological systems was evaluated using human gingival fibroblast cultures. For this phase of the study, cells were grown to confluency in 60-mm culture dishes containing Dulbecco's growth medium (DMEM) that was supplemented with ascorbic acid (50 μg/ml) and β-aminopropionitrile (50 μg/ml), but devoid of fetal calf serum and Pro. The medium was then replaced with 5 ml of fresh medium containing 50 μCi of [^{14}C]Pro and the cultures were pulsed for 12 h at 37°C in air–carbon dioxide (95:5).

Immediately upon cooling the cultures to ice-cold conditions, the medium was discarded and the cells were washed three times with 10-ml portions of ice-cold phosphate-buffered saline. Then they were suspended in 0.5 *M* acetic acid, homogenized by sonication and centrifuged for 10 min at 13 000 *g*. Step-wise, the resultant supernatant was lyophilized, hydrolyzed in 6 *M* hydrochloric acid, and again lyophilized.

For the OPA derivatization step, the lyophilized sample was reconstituted in 100 μl water, mixed with 100 μl of OPA reagent, filtered, and 130 μl of the filtrate were injected into the chromatograph. Fractions of 0 to 3 min were collected and lyophilized.

Then sequentially the residue was derivatized with 200 μl of PITC reagent, evaporated to complete dryness, reconstituted in 200 μl of sample buffer, and 130 μl were injected into the chromatograph.

RESULTS

Hydroxyproline and proline recovery after removal of primary amino acids

The amino acid standard mixture containing 250 nmoles of each of the 13 amino acids and [^{3}H]Hyp and [^{14}C]Pro was derivatized with OPA, then injected onto the column. Fractions (1 min) were collected over a period of 0–13 min and individually analyzed for radioactivity by liquid scintillation counting (LSC). It was found that 98% of [^{3}H]Hyp and 95% of [^{14}C]Pro radioactivity was recovered in the 0–3 min fractions. The percentage of recovery was dependent on the time interval between the addition of the OPA reagent and HPLC analysis (Fig. 1). Since the recovery of Hyp gradually decreased after 5 min, it is recommended that the OPA derivatives should be analyzed within 5 min post-OPA mixing. In contrast the recovery of Pro was maximal and constant up to 100 min post-derivatization.

The change in the amounts of removed primary amino acids (fractions 4–13) in Fig. 1 was calculated from the HPLC peak areas. Although their OPA derivatives were found to be stable for at least 4 h following addition of OPA, it is recommended that analyses of primary amino acids be performed within 2 h after the reaction.

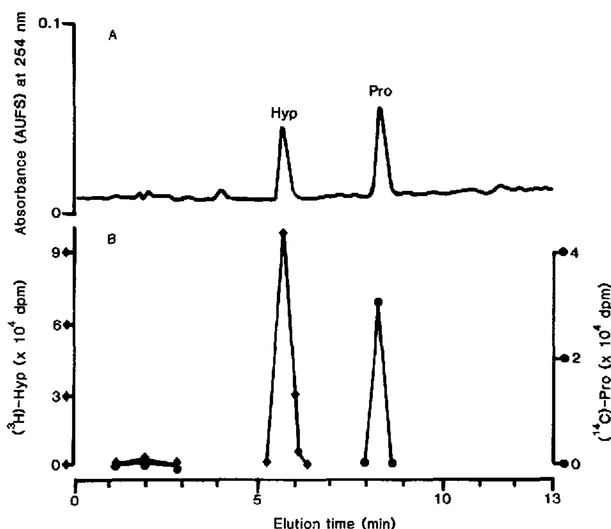

Fig. 2. (A) Chromatogram of OPA and PITC reacted amino acid standard mixture containing Hyp and Pro. (B) The radioactive peaks corresponding to the retention times for Hyp and Pro in A. The [^{3}H]Hyp and [^{14}C]Pro peaks were constructed from LSC counts obtained on fractions that were collected at 15-s intervals.

To ensure that primary amino acids are removed from the test samples, OPA and PITC derivatizations were performed on a standard amino acid mixture of 13 amino acids (1 μmole each) that lacked Hyp and Pro. After removal of primary amino acids from the sample the Pico-Tag system (Waters Assoc.) was used to detect the presence, if any, of unremoved primary acids. No peaks were observed even though a large excess of these amino acids was used for the study.

Fig. 2A depicts a chromatogram of a standard amino acid mixture containing Hyp and Pro that was processed by the described method and analyzed photometrically at 254 nm. It is evident from this absorbance profile that the procedure yielded a good separation of Hyp from Pro with no evidence of interference from primary amino acids. In order to confirm the identity of the Hyp and Pro peaks, [^{3}H]Hyp and [^{14}C]Pro were added to the amino acid standard. The mixture was then subjected to the OPA and PITC procedures and chromatographed (Fig. 2B). The comparison of Fig. 2A and B shows that radioactive peaks are located in the fractions corresponding to the retention times for Hyp and Pro. No amino acid peaks were discerned in the samples devoid of Pro and Hyp and unknown peaks were not close to or overlapped with the peak areas occupied by Hyp and Pro. The radioactivity profile confirmed the retention times for Hyp and Pro. The retention times for Hyp and Pro were 5.5 min ($\pm$0.1) and 8.1 min ($\pm$0.1), respectively.

In this study, 95% and 98% of [^{3}H]Hyp and [^{14}C]Pro radioactivity in PITC-derivatized systems was associated with Hyp and Pro peaks, respectively. Thus with the HPLC system, column, and derivatization procedures operating at optimum conditions, the results were reliable and reproducible.

Fig. 3 shows a representative chromatogram of 3.3 μl of human blood serum that was processed by the described OPA and PITC procedures. Although the employed injection volume was small in compoarison with volumes used in other studies[3,5], the procedure yielded a good separated small Hyp and a very prominent Pro peak. In addition to these two peaks and an injection response, three other large peaks were observed on these chromatograms. The 2.0- and 3.9-min components

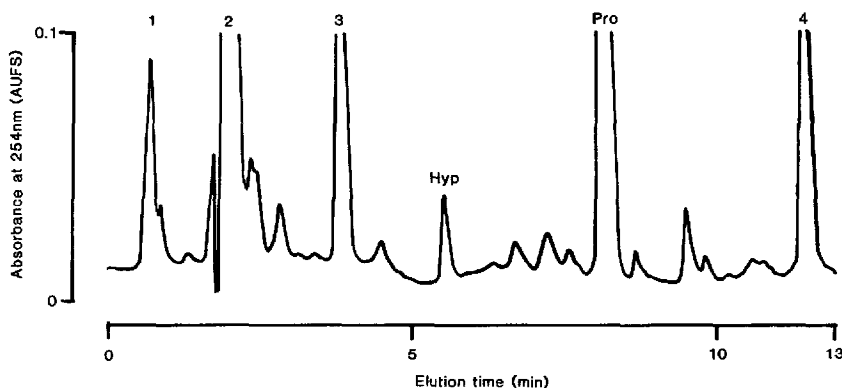

Fig. 3. Separation of PITC-derivatized human blood serum components recovered in the 0–3 min eluate of OPA-derivatized sample. The chromatogram shows a good resolution of Hyp and Pro. The four remaining peaks are: (1) injection response, (2, 3) by-products of OPA derivatization, and (4) by-product of PITC reaction.

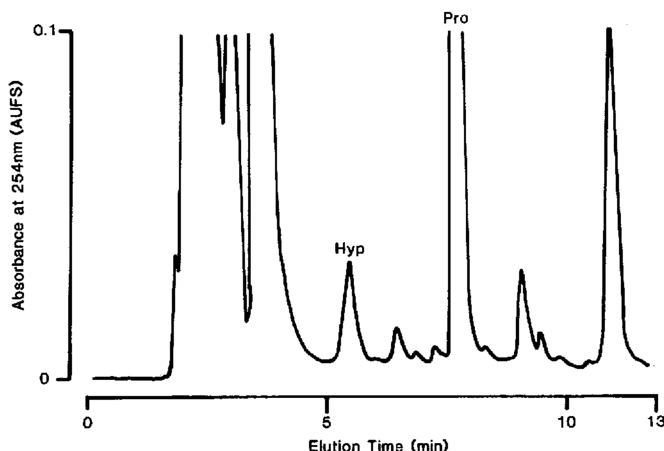

Fig. 4. Separation and quantitation of Hyp and Pro from fibroblast cell cultures. The PITC reaction was carried out on the 0–3 min eluate of OPA-derivatized sample. The peaks represent 1.20 nmoles of Hyp and 12.93 nmoles of Pro, respectively.

have been occasionally detected in the standard samples after OPA derivatization. Similarly the peak at 11.5 min has sporadically appeared following PITC derivatization. Thus these three unidentified components originate as by-products of the derivatization reactions. They, along with a number of smaller peaks, did not interfere with the analyses as they were well removed from the areas on the chromatograms occupied by Hyp and Pro.

The developed method also appears highly suitable for analysis of Hyp and Pro in complex biological systems as cell cultures (Fig. 4). The illustrated spectrometrically determined retention times for Hyp and Pro were confirmed by LSC analysis which showed that all the ^{14}C-activity in the samples was associated with the depicted peaks for Hyp and Pro.

DISCUSSION

The objective of the present study was to develop a practical and reliable method for determining Hyp and Pro concentrations in biological samples. The method is based on removal of primary amino acids by OPA derivatization and subsequent derivatization with PITC of the 0–3 min eluate fraction recovered from the OPA derivatized sample.

Numerous derivatization and elution systems have been developed for quantitation of amino acids. The application of these methods for determination of Hyp presents a number of difficulties[3]. Roth[3] drew attention to low concentrations of Hyp that occur in the biological samples. In order to improve the separation of Hyp, Nakazawa *et al.*[5] developed a standard addition method. While Roth[3] and Ahnoff[1] used NBD-Cl, which has much higher fluorescence intensities for Hyp and Pro, Cassini *et al.*[4] used dabsyl chloride and Wiedmeier[2] dansyl chloride. However, these methods had difficulty in determining Hyp from biological samples due to their low sensitivity, low concentration of Hyp, derivatization problems, poor separation, and use of complicated post-column derivatization systems.

In the present study, primary amino acids, which are generally present in much higher concentrations in comparison to Hyp in biological samples, were removed on a reversed-phase column with OPA derivatization. Then the Hyp and Pro were derivatized again with PITC and again applied to a reversed-phase column. The separation of Hyp and Pro by the present method was very simple as the analysis, including equilibration, took only 20 min to complete. Furthermore, the Hyp and Pro containing fraction has a small volume which is convenient for lyophilization, and OPA derivatization is a simple operation. Also in this system, the same mobile phase and column were used for purification and detection of Hyp and Pro. Therefore, Hyp and Pro detection using PITC can be followed immediately after Hyp and Pro purification without any treatment to the column.

The procedure for PITC derivatization[7] is simple compared with NBD-Cl, dansyl chloride and dabsyl chloride which need special conditions. For example, NBD-Cl has to be protected from exposure to light during incubation. Furthermore, the PITC method was improved by the use of volatile reagents and solvents which produced a derivative that is stable for weeks[7]. Also this method has a high sensitivity so that complicated systems based on post-column derivatization are not required.

The recovery of Hyp by the present method was 93%, approximately the same as that reported by Casini *et al.*[4]. Its sensitivity is at least 10 pmoles higher than the methods employed by Casini *et al.*[4] and Roth[3]. In the present method, Hyp in human serum was separated and detected from an injection volume of 3.3 μl of serum. In the standard addition method[5] which was one of the most sensitive methods previously used to detect Hyp from biological samples, more than 50 μl of serum were needed to determine Hyp. Therefore, in comparison to other methods the present method has the advantage of having a higher sensitivity, no interference, no complicated system such as post-column derivatization, a high stability of the PITC derivatives, and a higher recovery.

The method appears particularly useful for the analysis of biological materials which contain very low concentrations of Hyp and Pro in the presence of disproportionately large amounts of primary amino acids. The results of the analysis of Hyp and Pro in fibroblast cell proteins suggest that it merits consideration as a preferred HPLC method for determining collagen content of connective tissues.

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