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Quantification of hydroxyproline in small amounts of skin tissue using isocratic high performance liquid chromatography with NBD-F as fluorogenic reagent

Miwa Kakinuma [a,*], Yasuo Watanabe [b], Yusuke Hori [c], Tsunao Oh-i [a], Ryoji Tsuboi [a]

[a] *Department of Dermatology, Tokyo Medical University, 6-7-1 Nishi-Shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan*
[b] *Department of Pharmacology and Pharmacotherapy, Nihon Pharmaceutical University, Saitama 362-0806, Japan*
[c] *Lab of Pharmaceutical Sciences, Warabi Central Hospital, Saitama 333-0851, Japan*

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Abstract

In order to determine the collagen content of small amounts of skin tissue, we developed a new, simple and highly sensitive method of measuring the quantity of hydroxyproline (Hyp) using isocratic high performance liquid chromatography (HPLC) with a fluorogenic agent, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F). The recovery rate of Hyp and reproducibility of the assay were high, and the test was sensitive enough to detect Hyp in less than 1 mg of skin tissue. This method is clinically useful for ensuring accurate diagnosis and for monitoring specific skin conditions using small human skin samples collected in biopsies.

Keywords: Hydroxyproline; Collagen; NBD-F; Skin; High performance liquid chromatography (HPLC)

1. Introduction

Collagen is a major constituent of the dermis, and is therefore involved in many facets of skin diseases as well as the recovery process. For instance, increased collagen production due to the activation of fibroblasts is seen during wound healing, systemic scleroderma and keloid formation [1]. On the other hand, collagen production is suppressed in the Ehlers-Danlos syndrome [2]. In both instances, the change in the collagen content of the skin is likely to be one of the important indicators of the severity of the disease. Therefore, an accurate measurement of collagen content is useful for correct diagnosis as well as for the development of specific drugs, cosmetics and supplements.

Most methods for determining collagen content are based on measurements of the quantities of proline (Pro) and/or hydroxyproline (Hyp), the principal amino acid components of collagen. High performance liquid chromatography (HPLC) is a popular method for measuring Hyp levels quickly and accurately. However, previously reported methods incorporating HPLC require the spectrophotometric detection and the gradient elution systems, both of which involve the low sensitivities and the complicated procedures such as column back flushing, respectively [3,4].

Recent attempts have been made to address these methodological difficulties in HPLC and to find the optimal conditions for separation with the use of a new derivatizing agent and elution method. A highly sensitive method for measuring Hyp, Pro and other amino acids was developed [5–7] using fluorogenic reagents, such as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). Dunphy et al. reported an isocratic analyzing HPLC method using phenylthiocarbamyl (PTC) or phenylthiohydantoin (PTH) as a derivatizing reagent for amino acids [8]. Moreover, Watanabe et al. have described a method using 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F), which showed greater reactivity

* Corresponding author. Tel.: +81 3 3342 6111x5824; fax: +81 3 3342 2055.
E-mail address: kakimiwa@aol.com (M. Kakinuma).

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to both primary and secondary amines than did NBD-Cl [9].

However, HPLC had yet to be used with a derivatizing agent and the aforementioned elution method to determine Hyp levels in the skin tissues.

The purpose of the present study is to develop a new and simple HPLC method for measuring Hyp with the use of NBD-F that is sensitive enough to quantify collagen content in small skin samples taken from human skin biopsies, and useful for diagnosing various skin diseases and monitoring specific skin conditions.

2. Experimental

2.1. Animals

Male Sprague–Dawley rats (7 week old: 270 ± 10 g) were anesthetized to unconsciousness with pentobarbital-Na (45 mg/kg, i.p., Abbot Laboratories, IL, USA) and sacrificed by exsanguinations. Non-muscle connective tissues (i.e. heart valves) or tissue samples devoid of tendons were collected from the diaphragm, heart, skin and thigh muscles. The samples were dissected to isolate the mid-belly region of the thigh, the mid-fiber region of the diaphragm and a longitudinal section of the left ventricle in the middle of the muscular area. Circular rat dorsal skin samples 8 mm in diameter and the samples from the other organs were stored at −80 °C until analyzed.

2.2. Reagents

NBD-F was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The standards for HPLC were Hyp (Wako Pure Chemical Industries Ltd., Osaka, Japan) and a standard amino acid mixture Type AN-2 (Wako pure chemical industries Ltd., Osaka, Japan). The Wako standard amino acid solution contains an equimolar mixture of Asp, Cys, Glu, Ser, Gly, His, Arg, Thr, Ala, Tyr, Val, Met, Ile, Leu, Phe, Lys, Pro and Hyp. All other chemicals were of analytical reagent grade. Hydrochloric acid (HCl), acetonitrile, potassium dihydrogenphosphate, sodium tetra borate decahydrate and boric acid were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ethylenediamine tetra acetic acid disodium salt dihydrate (EDTA-2Na) was purchased from Pharmacia Biotech AB (Uppsala, Sweden).

2.3. Hydrolysis of proteins

Tissue samples (mean ± S.E.: 51.7 ± 1.4 mg, $n = 84$) were hydrolyzed for 22 h at 108 °C in evacuated flame-sealed Pyrex tubes with 400 μl of 6N HCl, to which 1% phenol was added. The hydrolysates were dried in vacuo. For the HPLC analysis, the dry residues of the hydrolysates were re-suspended in 500 μl of 50 mM borate buffer containing 20 mM EDTA-2Na (pH 8.0). The re-suspended samples were diluted 20–2000 times with 50 mM borate buffer containing 20 mM EDTA-2Na and 7.5 μM L-homoserine (pH 8.0).

2.4. HPLC conditions

An EP-300 (EICOM Corp., Kyoto, Japan) pump equipped with an L-7200 auto sampler (HITACHI, Tokyo, Japan) was employed. A guard column (20 mm × 3.9 mm) and a main column of SC-5ODS (200 mm × 4 mm, 10 μm, EICOM Corp., Kyoto, Japan) were used. All solvents were filtered and degassed prior to use. The flow-rate was 0.8 ml/min and the column temperature was kept at 30 °C using a column oven. A HITACHI L-7485 spectrofluorometer equipped with a 12 μl flow cell was used with an excitation wavelength of 470 nm and emission at 540 nm. The phosphate buffers (pH 5.0) containing 2–16% acetonitrile was used in the mobile phases.

2.5. Derivative procedure

A 30 μl of either hydrolyzed tissue samples or a mixed amino acid standard solution diluted 1/100 times with 50 mM borate buffer containing 20 mM EDTA-2Na (pH 8.0) was poured into a 500 μl conical tube. A 10 μl of NBD-F (10 mM in acetonitrile, freshly prepared) was added to this solution and the tube was capped and covered with an aluminum foil. The vessel was then heated to 60 °C for 1 min. After cooling in ice-water, 40 μl of 0.05 M HCl was added to the reaction mixture. A 10 μl volume of the solution was injected into the column.

3. Results

3.1. Separation of standard amino acids

Hyp and Pro were analyzed using HPLC in conjunction with the isocratic method. As a standard concentration of the mobile phase in the NBD-F gradient elution method, a 16% acetonitrile has been initially used [10]. In Fig. 1A, the retention time of Hyp and Pro is shown to be about 2 and 9 min, respectively. However, in the analyses of the standard amino acid mixture, the peak readings for Hyp and Pro overlapped with those of Asp, Glu, Ser, His, Gly, Arg, Thr and Ala (Fig. 1B). In order to define the most clearly separated peaks, various concentrations (i.e. 2, 4, 8, 12% and so on) of acetonitrile were then examined. Consequently, with 8% acetonitrile, Hyp and Pro were eluted at 7 and 30 min, respectively (Fig. 2A), and clearly separated from the standard amino acid mixture (Fig. 2B).

3.2. Analysis of linearity and accuracy

The standard curve with five different concentrations of Hyp up to 150 pmol showed a straight line and a linear regression of 1.0 ($y = 10.628 \times -0.498$). Thus, the newly developed

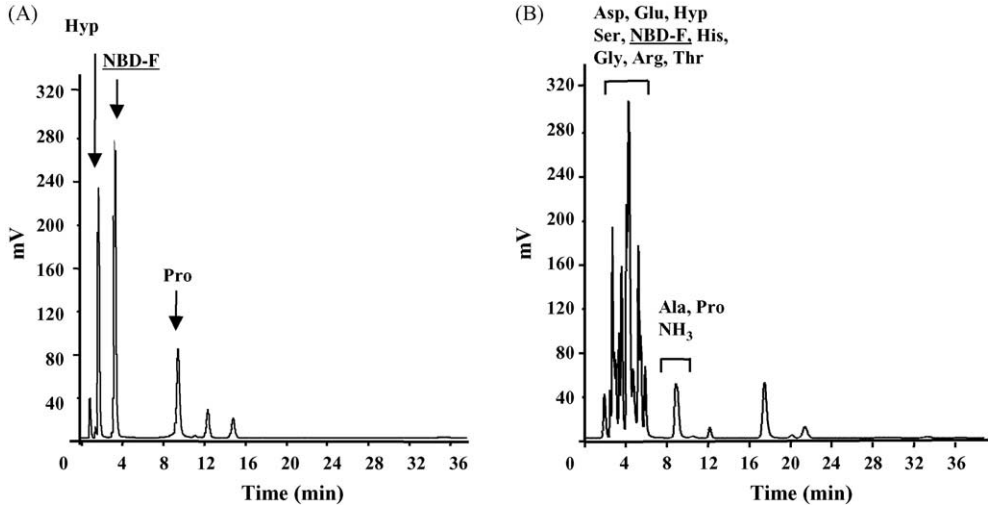

Fig. 1. Chromatogram of (A) derivatized standard amino acid mixture, (B) derivatized Hyp and Pro with 16% acetonitrile in mobile phase. Derivatives were separated by reverse-phase chromatogram as described in the Method. NBD-F is the fluorogenic agent.

assay system appeared to measure at least 400-fold differences in Hyp levels (max: 150 pmol, minimum: 0.375 pmol; as actual measurements). Reproducibility of the assay was recorded as the SEM of the values determined in five successive runs and, the coefficient of variation (CV) was calculated by following the previously reported studies [11]. The average SEM of the retention time of Hyp and Pro was 10 s, while the CV was less than 1.15%.

3.3. Analysis of tissue samples

Hydrolyzed rat skin mixed with or without a known concentration of Hyp was analyzed for the recovery of the Hyp. The recovery rate of Hyp was about 94% (mean ± S.E.: 93.7 ± 0.30%, $n = 6$), and the reproducibility of the Hyp assay was also high. Preliminary experiments with the rat skin samples diluted 20 times with a 50 mM borate buffer containing 20 mM EDTA-2Na with 7.5 μM L-homoserine (pH 8.0) failed to produce accurate measurements (data are not shown). However, as shown in Fig. 3, after the optimal dilution (2000 times) was finally achieved, we were able to use this method to measure the contents of Hyp in skin samples of less than 1 mg.

The levels of Hyp in various rat organs were then measured and compared with those in skin tissue (Fig. 4). Hyp levels (mean ± S.E.) in each organ were as follows: skin 12.44 ± 3.88 μg/mg wet wt; muscle 0.903 ± 0.253 μg/mg wet wt; diaphragm 0.762 ± 0.199 μg/mg wet wt; heart 0.549 ± 0.178 μg /mg wet wt; kidney 0.431 ± 0.128 μg/mg wet wt; and liver 0.294 ± 0.087 μg/mg wet wt. Hyp levels in the skin were about 20 times higher than those of the other organs.

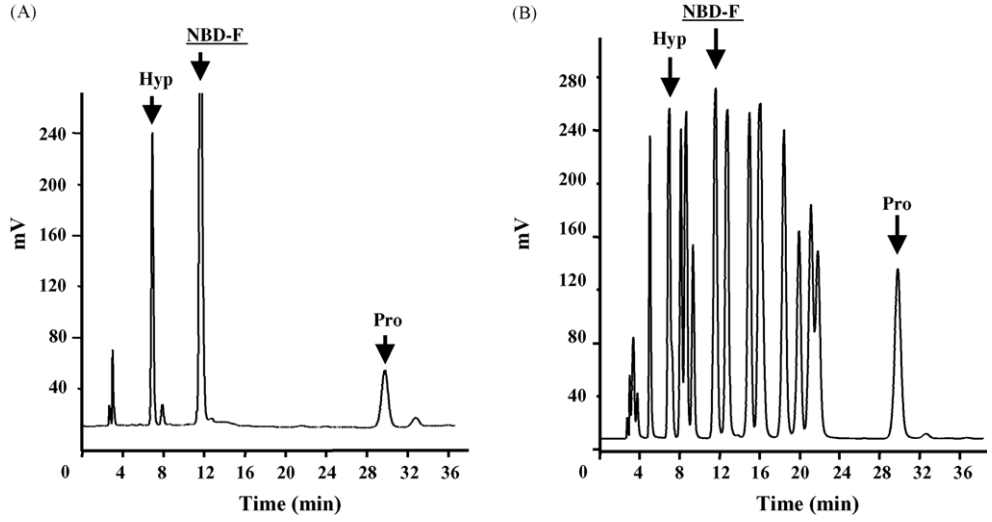

Fig. 2. Chromatogram of (A) derivatized standard amino acid mixture, (B) derivatized Hyp and Pro with 8% acetonitrile in mobile phase. The peak of each amino acid was distinct. The elution time of Hyp and Pro was 7 and 30 min, respectively.

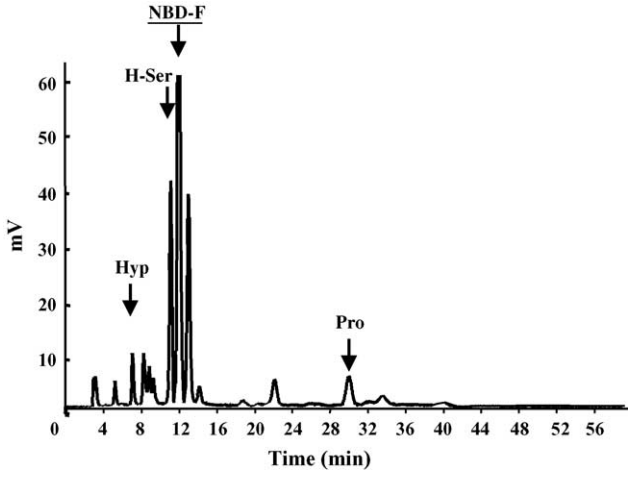

Fig. 3. Chromatogram of derivatized skin sample with 8% acetonitrile in mobile phase. The elution time is same as in Fig. 2. H-Ser is the internal standard amino acid.

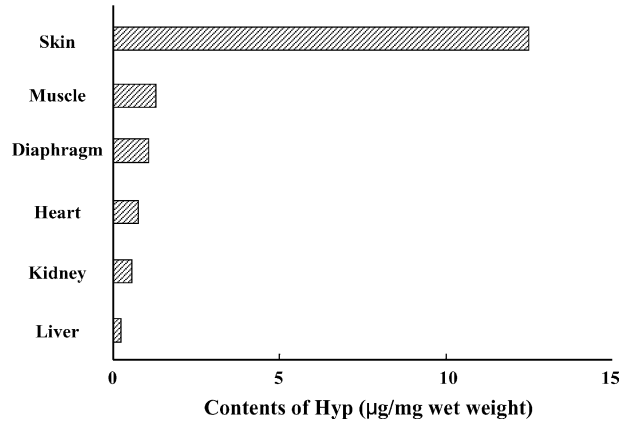

Fig. 4. Collagen content detected in individual rat tissue samples. Each bar shows the mean value of eight experiments.

4. Discussion

The development of methods for amino acid analysis as alternatives to ion-exchanging chromatography has recently relied on the use of reverse-phase HPLC and of various derivatizing reagents. The most widely used techniques employ pre-column derivatization of the amino acids with any one of the following derivatizing agents: *O*-phthalaldehyde (OPA) [12], 5-dimethylaminonaphthalene-1-sulfonylchloride (Dns-Cl) [13], 4-dimethylaminoazobenzene-4-sulfonyl chloride (DABS) [14] and phenylisothiocyanate (PITC) [15], 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride (DMS-Cl) [16], or NBD-F [9]. The detection methods also vary, and include fluorescence (OPA, Dns-Cl, DMS-Cl, NBD-F), ultraviolet absorption (Dns-Cl, PITC), visible absorption (DABS-Cl) and electrochemical detection (OPA). Other studies for determining Hyp levels using a colorimetric method [17] with HPLC [18,19] have also been reported. However, these methods require relatively large sample quantities because of their lower sensitivity.

In the present paper, we developed a simple and highly sensitive method for measuring Hyp in small quantities of skin obtained in routine clinical procedures. Previous studies have shown that the use of DMS in highly sensitive fluorometry often posed methodological difficulties, while the use of OPA and Dns resulted in lower sensitivity than the use of DMS and NBD-Cl [16]. NBD-Cl, one of the most highly sensitive labeling reagents, has been used to measure levels of Hyp and Pro in biological samples. On the other hand, in HPLC using NBD-Cl as a fluorescent labeling reagent [18,19], the derivative of Hyp with NBD-Cl was light-sensitive and required a relatively long chromatographic separation time (50 min) [18]. Determining the optimal conditions for elution allowed us to improve the isocratic method with NBD-F. This modification in turn enabled us to determine Hyp levels in small bio-samples, particularly those of skin, with simple procedure and high accuracy. This method is also useful for measuring Hyp levels in other organs with results corresponding closely to previously reported values [20].

Our method of using a fluorogenic reagent, NBD-F, with the isocratic HPLC enables us to measure Hyp levels in less than 1 mg of tissue or in similarly miniscule samples obtained in human skin biopsies. This method has great clinical application in the diagnosis and assessment of disease states and the formulation of treatments for patients with scleroderma, keloid and Ehlers-Danlos syndrome, where the etiology of the diseases is related to the up-regulation of collagen synthesis in the skin. Moreover, in the field of cosmetic dermatology, our method can be used to measure the amount of Hyp accurately following laser re-surfacing procedures. This simple, sensitive and highly reproducible method has the potential to become a new clinical standard for assessing collagen synthesis in human skin.

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