Journal of Chromatography, 382 (1986) 258—263
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3278

Note

Quantification of collagen synthesis by reversed-phase high-performance liquid chromatography utilizing 4-chloro-7-nitrobenzofurazan derivatives

W.C. SESSA, Jr., R.L. RODGERS* and C O. CHICHESTER

Department of Pharmacology and Toxicology, University of Rhode Island, Kingston, RI 02881 (U.S.A.)

(First received April 9th, 1986; revised manuscript received May 30th, 1986)

Hydroxyprolines, *trans*-4-hydroxy-L-proline (t-4-Hyp) and *trans*-3-hydroxy-L-proline (t-3-Hyp), are widely used markers for the quantification of collagen content and synthesis in a variety of tissues. The hydroxylated imino acids are formed via post-translational modifications of peptidyl bound proline residues by specific hydroxylase reactions. Because of its relative infrequent occurrence in other proteins, t-4-Hyp is considered a marker for interstitial collagens, whereas t-3-Hyp is an indicator of basement membrane collagen [1, 2].

Several methods are available to quantify various isomers of hydroxyproline. The most common are amino acid analysis, ion-exchange chromatography and the colorimetric assays [3]. All of these methods are time-consuming, especially when large numbers of samples are involved.

Recently, reversed-phase high-performance liquid chromatography (HPLC) was applied to imino acid analysis with and without precolumn derivatization [4—7]. Prior treatment of the sample with 4-chloro-7-nitrobenzofurazan (NBD-Cl) allows preferential labeling and sensitive fluorometric detection of secondary amines. This approach has been used to assay hydroxyproline in urine, plasma and purified collagen standards [4, 8, 9]. However, it has not been employed to quantify collagen levels or synthesis rates in tissues.

The present report describes the application of reversed-phase HPLC of NBD derivatives to determine tissue collagen levels and synthesis rates in two tissue types: rat aorta and human xenograft tumors. The values for collagen content obtained with this method correlate closely with those using the Juva and Prockop procedure [10]. The method also allows the determination of specific activities of hydroxyproline and proline in each tissue sample.

0378-4347/86/$03.50

EXPERIMENTAL

Reagents

All reagents were of the analytical grade. Materials and their sources are as follows: *trans*-4-hydroxy-L-proline, *cis*-4-hydroxy-L-proline (c-4-Hyp), L-proline, L-glycine and NBD-Cl from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and methanol (HPLC grade) were purchased from J.T. Baker (Medford, MA, U.S.A.). Isotopes, [^{14}C-U]L-proline (specific activity 273 mCi/mmol) and [^{3}H]hydroxy-L-proline (specific activity 5.9 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.).

Sample preparation

Radiolabeled vascular tissue and tumors. Thoracic aortae (approximately 40 mg) from fifteen-week-old male Wistar rats (Charles River Breeding Labs.) were placed into 2 ml of gassed (95% oxygen; 5% carbon dioxide) Krebs-Henseleit (K-H) solution and allowed to incubate for 30 min at 37°C. The ionic composition of the K-H solution was: NaCl 113 m*M*; KCl 4.7 m*M*; $CaCl_2$ 2.5 m*M*; $MgSO_4$ 1.2 m*M*; $NaHCO_3$ 36 m*M*; KH_2PO_4 6.2 m*M*; sodium ascorbate 0.05 m*M*; glucose 11.5 m*M*. At 30 min, the above solution was removed and replaced with fresh K-H solution (2 ml) supplemented with [^{14}C]L-proline (10 μCi/ml) and incubated up to 10 h. At each time point, aortae were washed with phosphate-buffered saline (PBS) pH 7.4, and subsequently homogenized in 1 ml of PBS. Proteins were precipitated with 50% trichloroacetic acid (TCA) (0.1 ml), centrifuged and washed six times with 5% TCA to remove unincorporated label. Residual TCA was extracted via two ether washings (4.0 ml each). Labeled aortae were then hydrolyzed in 2 ml of 6 *M* constant boiling hydrochloric acid for 24 h at 110°C. Hydrolysates were filtered, evaporated to dryness and neutralized with potassium tetraborate, 0.2 *M*, pH 9.5.

Human pancreatic adenosquamous carcinoma xenograft tumors were produced by injecting COLO 357 cells grown in culture into the flanks of six-week-old nude mice as described [11]. When the tumors reached 400—600 mg the tumors were removed from mice, washed with PBS, minced and placed into 2 ml of K-H buffer supplemented with [^{14}C]L-proline (10 μCi/ml) and incubated at 37°C for up to 4 h. At each time point, samples were treated as described above.

Unlabeled vascular and tumor tissues. Thoracic aortae (approximately 40 mg) from fifteen-week-old male Wistar rats and aliquots of lyophilized tumor samples were hydrolyzed for 24 h at 110°C. After filtration, samples were neutralized with potassium hydroxide and analyzed by HPLC and by the method of Juva and Prockop [10].

Amino acid derivatization

Neutralized hydrolysate (0.1 ml) was placed into a test tube containing 0.4 ml of 0.2 *M* potassium tetraborate (pH 9.5). c-4-Hyp (5 m*M*, 0.020 ml) was added to each sample as an internal standard due to its convenient elution pattern between t-4-Hyp and proline. Derivatizing agent, NBD-Cl (0.5 ml of 16 m*M* in methanol), was added to the above mixture and incubated at 60°C for 3 min in the dark [8, 9]. The reaction was halted by immediate immersion

into an ice bath and quenched by the addition of 0.05 ml of 3 *M* hydrochloric acid. A 0.1-ml portion of the reaction mixture was injected onto the column for analysis. Total hydroxyproline was determined by comparing the ratio of peak heights of t-4-Hyp/c-4-Hyp of hydrolysates to those generated from standards. Collagen synthesis was quantified as the amount of radioactive hydroxyproline collected under the corresponding peak. Derivatization was expressed as a percentage of the total radioactivity recovered under the hydroxyproline and proline peaks. The mean derivatization of radiolabeled proline and hydroxyproline in tissue samples was 90 ± 5%. All samples were corrected to 100% for calculations of collagen content and synthesis.

Instrumentation and chromatographic conditions

The equipment used consisted of a Waters M-45 constant-flow pump and Gilson fluorimeter (excitation filter 330—380 nm, emission filter 460—600 nm) connected to a Linear chart recorder. Radioactive eluents (0.8 ml) were collected into 5-ml liquid scintillation vials using an Alpha 200 fraction collector. Radioactivity was quantified by liquid scintillation spectroscopy at 85% efficiency for C_{14}. Analytical separation was performed on a 30 cm × 3.9 mm, 10-μm silica μBondapak C_{18} column (ASI) preceded by a direct connect precolumn (Alltech) with pellicular C_{18} packing. The mobile phase consisted of 21.75% acetonitrile and 0.015% phosphate (pH 2.85) with a flow-rate of 1.2 ml/min at room temperature. The mobile phase was filtered and degassed by vacuum suction and sonication for 30 min.

RESULTS AND DISCUSSION

Characterization of the procedure

Two parameters were taken into consideration for optimizing the conditions of derivatization: (1) NBD-Cl concentration; (2) the time of incubation. We sought to provide maximal derivatization of hydroxyproline without sacrificing the selectivity of NBD-Cl for secondary amines.

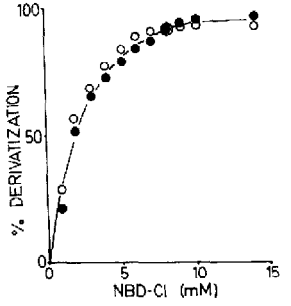

Fig. 1. Effect of NBD-Cl concentration on percentage derivatization of [^{3}H]t-4-Hyp (○) and [^{14}C]proline (●). Each reaction mixture contained 0.1 ml of t-4-Hyp (5 m*M*), 0.02 ml of c-4-Hyp (5 m*M*), 0.02 ml of L-glycine (10 m*M*) and 0.1 ml of L-proline (5 m*M*) in 0.2 *M* potassium tetraborate buffer, pH 9.5 Each sample was spiked with 0.01 μCi of [^{3}H]hydroxyproline and [^{14}C]proline and derivatized with 0.5 ml NBD-Cl for 3 min at 60° C. Of each sample, 0.1 ml was injected for quantitation of percentage derivatization. Recovery of radioactivity was 97%.

TABLE I

EFFECT OF INCUBATION TIME ON PEAK-HEIGHT RATIOS OF *trans*-4-HYDROXY-L-PROLINE AND GLYCINE, AND PERCENTAGE DERIVATIZATION OF [^{3}H]HYDROXYPROLINE AND [^{14}C]PROLINE

Each reaction mixture contained 0.1 ml of t-4-Hyp, 0.03 ml of c-4-Hyp, 0.1 ml of L-proline (5 m*M*) and 0.02 ml of L-glycine (10 m*M*) in 0.2 *M* potassium tetraborate buffer, pH 9.5. Each sample was spiked with 0.01 μCi of [^{3}H]hydroxyproline and [^{14}C]proline and derivatized with 0.5 ml of 16 m*M* NBD-Cl (8 m*M* final concentration) at 60°C.

Incubation time (min)	Peak-height ratio		Derivatization (%)	
	t-4-Hyp	Glycine	[^{3}H]Hydroxyproline	[^{14}C]Proline
1	1.38	0.16	48.5	65.0
3	1.78	0.65	90.1	90.0
5	1.56	1.02	94.0	95.9
7	1.38	1.15	94.1	95.4
10	1.34	1.21	94.0	96.0
20	1.45	1.21	92.8	96.0

Increasing concentrations of NBD-Cl increased the percentage derivatization of [^{14}C]proline and [^{3}H]hydroxyproline in the presence of four amino acids (Fig. 1). As NBD-Cl concentration approached 8 m*M*, the derivatization plateaued at approximately 90%. The magnitude of t-4-Hyp peak height was proportional to the concentration of NBD-Cl added. The ratio of peak heights and linearity of response remained consistent throughout a range of concentrations of standards, confirming previous studies [9, 12]. Based on the above observations, 8 m*M* NBD-Cl was used throughout the remainder of the study.

Increasing the time of incubation at 60°C beyond 3 min did not significantly increase the extent of derivatization of [^{14}C]proline and [^{3}H]hydroxyproline (Table I). However, the selectivity of NBD-Cl for t-4-Hyp diminished as time of incubation increased beyond 5 min. These data support previous studies, suggesting that shorter reaction times favor the selective derivatization of

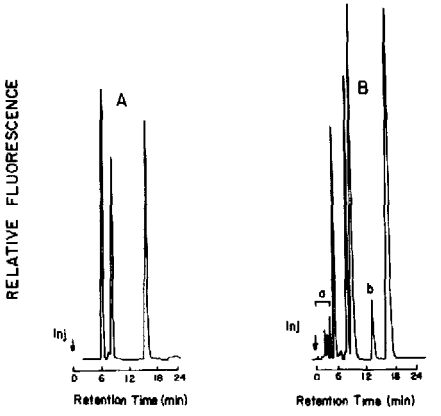

Fig. 2. Typical chromatograms of standards (A) and 0.1 ml aortic hydrolysates (B). The elution profile (from left to right) is as follows: t-4-Hyp (5.7 min), c-4-Hyp (7.8 min), L-glycine (8.1 min) and L-proline (17.5 min). Peaks a and b in chromatogram B are unknown.

secondary amines [4, 8, 9]. Therefore, all subsequent incubations were carried out for 3 min at 60°C.

Standard curves for t-4-Hyp and proline were linear (r = 0.996 and 0.997, respectively) up to approximately 5 μg (38 and 43 nmol, respectively, per 0.1-ml injection; data not shown). The lower limit for reliable detection was approximately 0.5 μg per 0.1-ml injection (4 nmol) at the fluorimeter setting used (10 × 0.5). Higher (picomolar) sensitivities could be obtained; however, this was not necessary for tissue analysis.

The separation of amino acid peaks from aortic hydrolysates was comparable to that of the corresponding standards (Fig. 2). Therefore, prior treatment of the tissue samples with Dowex columns [13] or with *o*-phthalaldehyde [9, 14] was not necessary.

Quantification of collagen content and collagen synthesis from tissue hydrolysates

Collagen content. Aorta and tumor hydroxyproline values obtained with reversed-phase HPLC were well correlated with those from the standard colorimetric method of Juva and Prockop [10] (Fig. 3). The data from aortic samples also show that the method is reproducible when samples are assayed on different days. The mean value for all samples (9.31 μg hydroxyproline per mg wet weight) is similar to previously reported values for thoracic aorta [15—17].

Collagen synthesis. The rates of incorporation of [^{14}C]proline into aortic and tumor collagen in vitro are shown in Fig. 4. The rates were linear for at least 10 and 4 h for aortae and tumors, respectively. The slopes of the lines represent the rate of net de novo collagen biosynthesis, assuming: (1) a constant pool size of intracellular proline; and (2) relatively low rates of degradation of newly formed collagen. Specific activities of hydroxyproline can be determined at each time point.

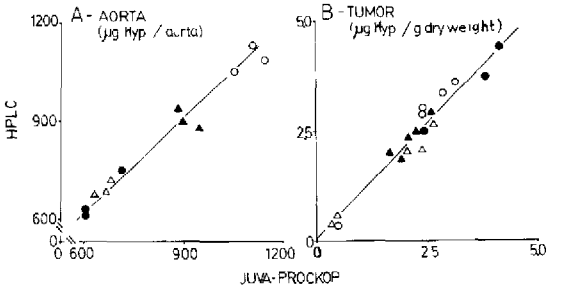

Fig. 3. Quantitation of aortic (A) and tumor (B) hydroxyproline by reversed-phase HPLC and the Juva and Prockop [10] colorimetric assay. The four symbols represent four aortae (A) or four treatment groups (B). The individual points represent single determinations within groups on three different days (A) or on the same day (B). Hydrolyzed aortae were resuspended in 0.4 ml potassium tetraborate (0.2 *M*, pH 9.5) and an aliquot was taken for each assay. Lyophilized tumors were hydrolyzed, filtered and neutralized with potassium hydroxide and an aliquot was taken for each assay. [^{14}C]Proline (0 001 μCi) was added to each sample for determination of percentage derivatization. The mean derivatization was 89 ± 4 and 90 ± 2% for tumors and aortae, respectively. The lines were obtained by regression: $y = 0.90x + 96$ (r = 0 978) for A; $y = 1.05x + 0\ 08$ (r = 0.964) for B.

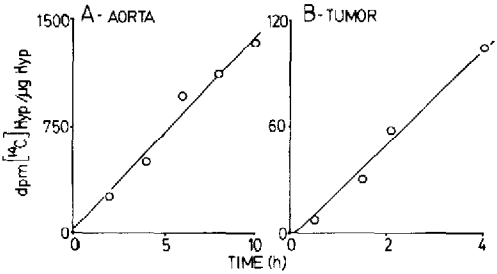

Fig. 4. Time course of [^{14}C]proline incorporation into aortic (A) and tumor (B) [^{14}C]-hydroxyproline. Aortic and tumor samples were metabolically labeled with [^{14}C]proline (10 μCi/ml) for the above specified times at 37°C. Each point represents the mean of duplicate tissue samples. At each time point, the specific activity of t-4-Hyp was determined. Specific activity was calculated by the amount of [^{14}C]hydroxyproline eluted under its corresponding peak, divided by the ratio of peak heights for the sample, compared to those of standards. The lines were obtained by regression: $y = 130x + 33$ ($r = 0.989$) for A; $y = 27.5x - 3.0$ ($r = 0.990$) for B.

This method provides accurate and reproducible quantification of collagen content and synthesis without the necessity for prior sample preparation or gradients.

ACKNOWLEDGEMENTS

This study was supported in part by grants from U.S. P.H.S., No. HL-32120, the American Cancer Society, No. PDT-215B, and the American Heart Association, R.I. Affiliate.

REFERENCES

1 G.J Cardinale and S. Udenfriend, Adv Enzymol., 41 (1974) 245—300.
2 E Adams and L. Frank, Annu Rev. Biochem , 49 (1980) 1005—1061.
3 R. Berg, Methods Enzymol., 82 (1982) 372—398.
4 W.J. Lindblad and R.F. Diegalmann, Anal. Biochem., 138 (1984) 390—394.
5 A. Carisano, J Chromatogr., 318 (1985) 132—138.
6 J.P. Kehrer and S.T. Dydek, Proc. West. Pharmacol. Soc., 27 (1984) 319—372.
7 J. Macek and M. Adam, J. Chromatogr , 374 (1986) 125—128.
8 A.M. Ahnoff, I. Grundevik, A. Arfuidsson, J. Fonsellius and B.A. Persson, Anal. Chem., 53 (1981) 485—489.
9 C.A. Palmerini, C. Fini, A. Floridı, A. Morrelli and A. Vedovelli, J. Chromatogr., 339 (1985) 285—292
10 K. Juva and D.J. Prockop, Anal. Biochem , 15 (1966) 77—83.
11 P.A. Meitner, S.M. Kajiji, N. LaPosta-Frazier, H.A. Bogaars, G A. Jolly, P L. Dexter, P. Calabresi and M D. Turner, Cancer Res , 43 (1983) 5978—5985.
12 A. Bisker, V. Pailler, A. Randax and J.P. Borel, Anal. Biochem., 122 (1982) 52—57
13 S.T. Dydek and J.P Kehrer, Liq Chromatogr., 2 (1983) 536—538
14 G. Bellon, A. Malgras, A Randoux and J.P. Borel, J. Chromatogr., 278 (1983) 167—172.
15 A. Ooshima, G.C. Fuller, G.J. Cardinale, S. Spector and S Udenfriend, Proc. Natl. Acad. Sci. U S.A., 71 (1974) 3069—3073.
16 H.J. Ruskoaho and E R. Savolainen, Cardiovasc. Res., 19 (1985) 355—362.
17 L.A. Ehrhart and C M. Ferrario, Hypertension, 3 (1981) 479—484.