Journal of Chromatography, 374 (1986) 125—128
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2836

Note

Method for rapid determination of hydroxyproline by high-performance liquid chromatography and its exploitation for the study of collagen formation

J. MACEK* and M. ADAM

Research Institute for Rheumatic Diseases, Na Slupi 4, CS-128 50 Prague 2 (Czechoslovakia)

(First received June 28th, 1985; revised manuscript received August 18th, 1985)

The accurate determination of hydroxyproline is needed for the estimation of total collagen in tissues. The usual methods are based on spectrophotometric procedures, e.g. the method of Stegeman [1]. This method lacks specificity and therefore it was modified by some authors [2—5], using extraction or ion-exchange chromatography in order to remove interfering substances. With these modifications, however, the method became time-consuming. Since the linear range of the spectrophotometric methods is relatively narrow (1—10 μg of hydroxyproline), concentrated samples have to be diluted and measurements have to be repeated.

For the determination of newly synthesized collagen, the conversion of radioactive proline into hydroxyproline is widely employed. Methods for the radiochemical assay of hydroxyproline [6—8] by silicic acid chromatography following oxidation and extraction into toluene are tedious and encounter serious losses of radioactivity. Dydek and Kehrer [9] have developed a high-performance liquid chromatographic (HPLC) procedure for separating hydroxyproline from proline. These amino acids were detected by colorimetry in the fractions, which is rather cumbersome. Recently, Stimler [10] introduced a method for the separation of hydroxyproline from proline on a high-performance cation-exchange column with detection by absorptimetry. This method, however, has low sensitivity (5 μg) and a long analysis time (40 min). Other HPLC methods [11—18] are more sensitive (generally using pre-column derivatization with fluorescence detection). However, all these methods employ gradient elution and require a minimum of 35 min for separation.

Our new method for hydroxyproline determination is sensitive enough for the desired applications (20 ng per injection or 200 ng/ml) and can be used as

0378-4347/86/$03.50

an alternative method to the spectrophotometric methods. Because it uses column backflushing and isocratic elution, the analysis time is only 10 min. This rapid separation is also attractive for the isolation of radiolabelled hydroxyproline.

EXPERIMENTAL

Chemicals

n-Propanol was purchased from Lachema (Brno, Czechoslovakia), trichloroacetic acid was from International Enzymes (Windsor, U.K.), sodium dodecyl sulphate (SDS) and *trans*-4-hydroxy-L-proline were obtained from Serva (Heidelberg, F.R.G.). Trione ninhydrin reagent was from Pickering Labs. (Mountain View, CA, U.S.A.).

Apparatus

Chromatographic equipment was obtained from Spectra-Physics (San Jose, CA, U.S.A.). The SP-8100 liquid chromatograph was connected to an SP-8440 variable-wavelength UV/VIS detector and an SP-4200 computing integrator. Detection was accomplished by post-column reaction with ninhydrin at 128°C using a CRX 390 post-column reactor (Pickering Labs.). The volume of the injection loop was 100 μl. For column backflushing, a six-port switching valve (Valco Instruments, U.S.A.) was used (Fig. 1). If fraction collection is not desired, the backpressure regulator set at 0.4 MPa can be added to the detector outlet. This prevents the formation of bubbles in the post-column reactor, which allows the reaction to be carried out at 140°C.

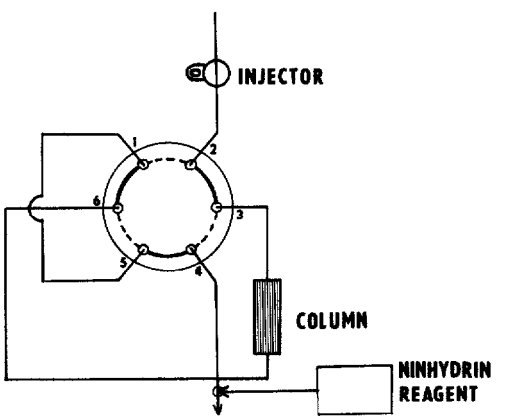

Fig. 1. Flow path of the mobile phase. Dashed lines are used for backflushing.

Chromatography

A silica presaturator column was placed between the pump and the injector. An HPLC cartridge (10 cm × 4.6 mm I.D.) packed with octyl reversed-phase (Spheri-5 RP-8), particle size 5 μm, was obtained from Brownlee Labs. (Santa Clara, CA, U.S.A.). The mobile phase was water—*n*-propanol (97.4:2.6, v/v). SDS (3 g/l) was added as ion-pairing reagent and the pH was adjusted to 2.6 with 1 *M* trichloroacetic acid. The flow-rate of the mobile phase was 0.9 ml/min and

the pressure at ambient temperature (ca. 25°C) was 7 MPa. Ninhydrin reagent flow-rate was 0.2 ml/min. The eluate was monitored at 440 nm.

Sample preparation

Homogenized cells and media from tissue cultures (in our case, from human chondrocytes) are dialysed 2 × 24 h against 0.5 *M* acetic acid and 24 h against deionized water. The dialysate is lyophilized and hydrolysed in 1 ml of 6 *M* hydrochloric acid at 110°C for 16 h. The hydrolysate is evaporated to dryness and dissolved in 0.5 ml of deionized water. A 100-µl aliquot of the mixture is injected on the column.

RESULTS AND DISCUSSION

Chromatography

In our elution system, *trans*-4-hydroxy-L-proline is eluted prior to all other protein hydrolysate amino acids (retention time 4.3 min). Immediately after its elution, the flow through the column is reversed. The amino acids are back-flushed from the column within 10 min. A typical chromatogram is shown in Fig. 2. *trans*-4-Hydroxy-L-proline is completely resolved from aspartic acid, which is eluted after 5.0 min.

Contamination with radioactive proline is not possible, because this amino acid has a retention time of 21 min and is normally eluted with the backflush peak.

More than 100 samples have been analysed up to now, and no interfering peaks were observed. For satisfactory resolution of hydroxyproline from

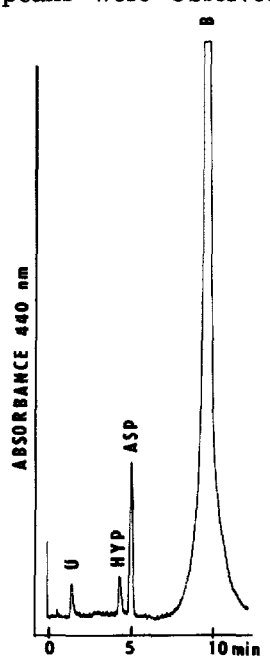

Fig. 2. Chromatogram of hydrolysate of dialysed chondrocyte culture homogenate. Peaks: U = unknown, HYP = *trans*-4-hydroxy-L-proline (160 ng injected or 1.60 µg/ml), ASP = aspartic acid, B = backflush peak.

aspartic acid, the column must be sufficiently equilibrated. The pump was therefore automatically switched on 2 h before the first analysis was run. At the end of day, the column was flushed with methanol to prevent corrosion and packing degradation. Approximately 40—50 samples can be assayed within 8 h. The probe of radioactive hydroxyproline was collected into scintillation vials and, after addition of scintillator, the radioactivity was measured.

Sensitivity, precision, linearity and accuracy

The detection limit was 40 ng per injection (signal-to-noise ratio of 3:1) or 20 ng per injection with backpressure regulator added and post-column reaction at 140°C.

At 2.88 μg/ml, the reproducibility was 3.4% (n = 5). The calibration curve was obtained by weighted linear regression and was linear in the range 0.7—28.8 μg/ml (r = 0.9998, n = 5). Accuracy was 6.1% (n = 5). Recovery of *trans*-4-hydroxy-L-proline after hydrolysis was 95% (this is in accordance with the value found by Bellon et al. [19]). The loss can be explained by epimerization to *cis*-4-hydroxy-D-proline.

CONCLUSIONS

A rapid and selective method for determination and isolation of *trans*-4-hydroxy-L-proline was developed. In our laboratory, the method has proved to be suitable for routine work. The specific radioactivity values are easily obtained in a single run and there are no losses of radioactivity as in previous methods [6—8]. If desired, the method can be easily automated. The back-flushing for rapid hydroxyproline determination can also be employed in amino acid analysers, but longer analysis times are to be expected.

REFERENCES

1 H. Stegeman, Z. Physiol. Chem., 33 (1958) 41.
2 K.I. Kivirikko, O. Laitinen and D.J. Prockop, Anal. Biochem., 19 (1967) 249.
3 H. Stegeman and K. Stadler, Clin. Chim. Acta, 18 (1967) 267.
4 S. Inayama, T. Shibata, J. Ohtsuki and S. Saito, Keio J. Med., 27 (1978) 43.
5 J.F. Woessner, Jr., Arch. Biochem. Biophys., 93 (1961) 440.
6 D.J. Prockop, S. Undenfried and S. Lindstedt, J. Biol. Chem., 236 (1961) 1395.
7 K. Juva and D.J. Prockop, Anal. Biochem., 15 (1966) 77.
8 N. Blumenkrantz and G. Asboe-Hansen, Anal. Biochem., 66 (1975) 330.
9 S.T. Dydek and J.P. Kehrer, Liq. Chromatogr., 2 (1984) 536.
10 N.P. Stimler, Anal. Biochem., 142 (1984) 103.
11 A. Casini, F. Martini, S. Nieri, D. Ramarli, F. Franconi and C. Surrenti, J. Chromatogr., 249 (1982) 187.
12 H. Konomi, T. Hayoshi, I. Takehana and M. Arima, J. Chromatogr., 311 (1984) 375.
13 A. Carisano, J. Chromatogr., 318 (1985) 132.
14 R.L. Heinrikson and S.C. Meredith, Anal. Biochem., 136 (1984) 65.
15 J.D.H. Cooper, M.T. Lewis and D.C. Turnell, J. Chromatogr., 285 (1984) 490.
16 K. Imai, J. Watanabe and T. Toyooka, Chromatographia, 16 (1982) 214.
17 W.J. Lindblad and R.F. Diegelmann, Anal. Biochem., 138 (1984) 390.
18 K. Nakazawa, H. Tanaka and M. Arima, J. Chromatogr., 233 (1982) 313.
19 G. Bellon, R. Berg, F. Chastang, A. Malgras and J.P. Borel, Anal. Biochem., 137 (1984) 151.