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DETERMINATION OF RADIOLABELLED PROLINE AND HYDROXYPROLINE IN COLLAGEN HYDROLYSATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE RADIOMETRIC DETECTION

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SUMMARY

Radiolabelled proline and hydroxyproline were separated on a C_8 column (10 cm × 4.6 mm I.D.) with 10.4 m*M* sodium dodecyl sulphate in water–*n*-propanol (88·12, v/v) (pH 2.6) as the mobile phase at a flow-rate of 0.6 ml/min. The retention times of hydroxyproline and proline were 5 and 8 min, respectively. On-line radiometric detection was performed either in a homogeneous mode (liquid scintillator was added to the column effluent in the ratio 3.33:1) or in a heterogeneous mode (the detection cell was packed with a solid scintillator and 0.1 *M* ammonia was mixed with the column effluent in the ratio 1:6 in order to prevent adsorption of amino acids on the cell packing). Detection limits were in the range 100–900 dpm for individual isotopes and detection modes and the reproducibilities were better than 10%. The application of the method to a collagen synthesis study is reported.

INTRODUCTION

Collagen is one of the oldest proteins and it forms about one third of all animal proteins. Collagen has many functions, e.g., effects on cell differentiation, migration, adhesion and proliferation, platelet aggregation, mechanical properties of the tissues and immunogenic properties, etc. Under certain pathological circumstances collagen metabolism may be affected on different levels: (1) during the synthesis on ribosomes and (2) during post-translational modifications – the hydroxylation of proline is in this respect one of the most important, because of stabilization of the collagen molecule through hydrogen bonds formed by hydroxyproline.

Collagen metabolism may be studied in cell cultures derived from normal or pathological tissues. Radiolabelled proline, which is added to the culture, is incorporated into procollagen and partially hydroxylated to 4-hydroxyproline (or to 3-hydroxyproline in special cases) by a post-translational enzymatic reaction.

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The amount of newly synthesized collagen is directly proportional to the amount of radioactivity incorporated.

For many years the amount of newly formed radioactive hydroxyproline was taken as a measure of the amount of synthesized collagen. Classical methods for the radiochemical assay of hydroxyproline [1–3] were tedious and encountered serious losses of radioactivity. A high-performance liquid chromatographic (HPLC) method for the determination of hydroxyproline was developed in our laboratory [4]. For radiometric measurements the peak of hydroxyproline was collected and measured in a scintillation vial. However, the activity of hydroxyproline is proportional to the amount of newly synthesized collagen only under the assumption of constant hydroxylation ratio (ratio of hydroxyproline to proline). If this condition is not met, e.g., with vitamin C deficiency and under the influence of some other exo- or endogenous compounds, then radiolabelled proline incorporation has also to be measured. A method for the separation of radiolabelled proline, hydroxyproline, lysine and hydroxylysine by low-pressure liquid chromatography [5] was recently published, but the analysis time was 5.5 h.

The aim of this study was to develop an automatic and rapid method for the determination of radiolabelled proline and hydroxyproline by HPLC with on-line radiometric detection.

EXPERIMENTAL

Chemicals

Trichloroacetic acid was obtained from International Enzymes (Windsor, U.K.), sodium dodecylsulphate (SDS) from Serva (Heidelberg, F.R.G.) and DL-hydroxy[2-^{14}C]proline, L-[U-^{14}C]proline and L-[5-^{3}H]proline from Amersham (Little Chalfont, U.K.). Supersolve X liquid scintillator was obtained from Zinsser Analytic (Frankfurt, F.R.G.) and YG 30 solid scintillator from the Institute for Research, Production and Application of Radioisotopes (Prague, Czechoslovakia). Minimal essential medium (MEM), penicillin and streptomycin were from ÚSOL (Prague, Czechoslovakia), bacterial collagenase (EC 3.4.24.3) from Sigma (St. Louis, MO, U.S.A.), foetal calf serum (FCS) from the Veterinary School (Brno, Czechoslovakia) and vitamin C from Biotika (Slovenská Lupča, Czechoslovakia). All other chemicals were of analytical-reagent grade and were purchased from Lachema (Brno, Czechoslovakia).

Apparatus

An SP-8100 liquid chromatograph (Spectra-Physics, San Jose, CA, U.S.A.) was connected to an LB 506 D radioactivity monitor (Laboratorium Prof. Dr. Berthold, Wildbad, F.R.G.) equipped with either a 150-μl cell packed with YG 30 solid scintillator (yttrium glass), particle size 32–54 μm, or an empty 500-μl Z 500-4 cell used for liquid scintillation counting.

The liquid scintillator was delivered by a BESTA E 100 pump (Bernhard Stadler, Heidelberg, F.R.G.) and mixed with the column effluent in a T-piece. The

same pump was used for adding ammonia solution for measurements with solid scintillator.

A switching valve with an electric actuator (Valco, Houston, TX, U.S.A.) was added after the column to enable the detection cell to be by-passed when desired.

Radiochromatography

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 (88:12, v/v). SDS (3 g/l) was added as an ion-pairing reagent and the pH was adjusted to 2.6 with 1 *M* trichloroacetic acid. The flow-rate of the mobile phase was 0.6 ml/min and the pressure at ambient temperature (23–28°C) was 8.5 MPa. The pressure generated in solid scintillator cell was 2 MPa.

The liquid scintillator was mixed with column effluent in the ratio 3.33:1 (scintillator flow-rate 2 ml/min).

For measurements with the solid scintillator cell, 0.1 *M* ammonia solution was mixed with the column effluent in the ratio 1:6 (flow-rate 0.1 ml/min). The column effluent was directed to the cell via a switching valve only between 3.5 and 12 min after injection.

The discriminator settings were as follows: for the ^{3}H channel the upper limit was 700 and for the ^{14}C channel the lower limit was 25 and the upper limit was 700. These settings were the same for both detection techniques used.

Incubation experiment

Human skin fibroblasts were grown to confluence in MEM with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin in an incubator at 37°C in atmosphere of carbon dioxide (5%). Incubation with radioactive precursor ([^{14}C]proline, 37 000 Bq/ml) in the presence of vitamin C (40 μg/ml) lasted 24 h.

Sample preparation

The culture medium was collected and processed by a modified procedure of Bellon et al. [6] as follows. Briefly, medium was dialysed 3×24 h against 0.5 *M* acetic acid, lyophilized and 10 ml of collagenase buffer (0.1 *M* ammonium formate, pH 7.8, containing 0.001 *M* calcium acetate and 20 μg/ml of bacterial collagenase) were added in order to release collagen peptides. The mixture was incubated at 37°C for 18 h. The incubation was stopped by the addition of 10 ml of cold 12% trichloroacetic acid solution and the samples were kept at 4°C overnight. They were centrifuged at 47 000 *g* for 30 min and the supernatant was hydrolysed in 6 *M* hydrochloric acid for 16 h at 110°C, evaporated to dryness and the residue dissolved in 1 ml of water.

RESULTS AND DISCUSSION

Chromatography

The radiochromatogram of a mixture of [^{14}C]hydroxyproline and [^{14}C]proline is shown in Fig. 1.

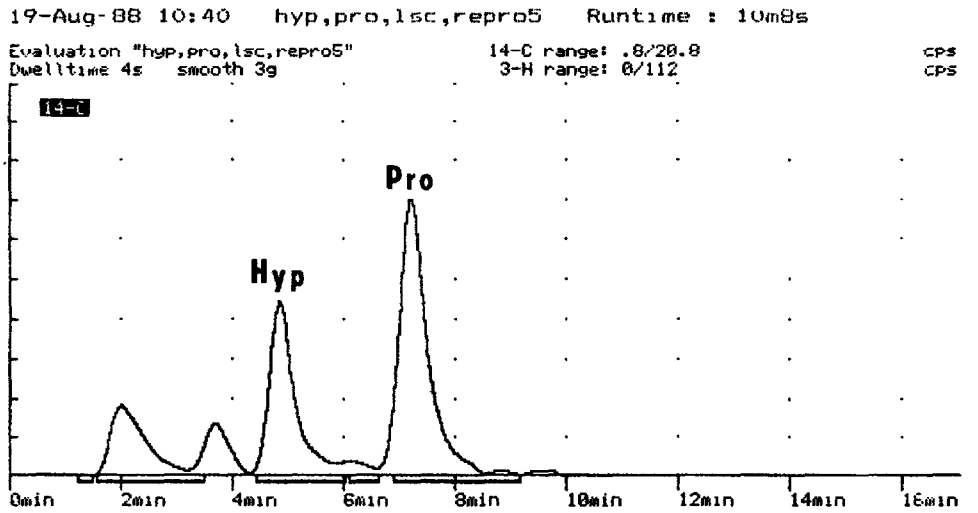

Fig. 1. Radiochromatogram of a mixture of [^{14}C]hydroxyproline and [^{14}C]proline. Detection was performed in the homogeneous mode.

The separation of hydroxyproline from proline is not difficult to achieve [7]. Hydroxyproline is a very polar amino acid and is eluted first from protein hydrolysate amino acids. The difference in the retention behaviours of proline and hydroxyproline is, however, not so great and therefore isocratic elution can be used instead of gradient elution as employed in amino acid analysis.

For radiometric detection it is important to maximize the residence time of a radioactive substance in the detection cell and therefore the flow-rate must be minimized. We increased the *n*-propanol content in the mobile phase from 2.6% used in our previous method [4] for the determination of hydroxyproline to 12.8% in order to decrease the capacity factor (k'). Thus, we were able to decrease the flow-rate to 0.6 ml/min. Acceptable retention times were obtained: 5 min for hydroxyproline and 8 min for proline with a resolution higher than 3.

Detection

Principally two detection modes were studied, using either a solid scintillator (heterogeneous mode) or a liquid scintillator (homogeneous mode). The latter has high efficiency of detection (up to 50% for ^{3}H and 90% for ^{14}C) with almost no increase in pressure in the measuring cell, but involves the consumption of expensive liquid scintillator which must be mixed with column effluent in a ratio of up to 10:1. Large volumes of waste are therefore produced, the disposal of which may produce a problem. Therefore, the optimization of the mixing ratio of scintillator to column effluent is of crucial importance. Fig. 2 shows the relationship between detection efficiency and scintillator flow-rate for ^{14}C- and ^{3}H-labelled compounds. As expected, the higher the volume ratio of the scintillator, the lower is quenching and the efficiency increases. A difference can be observed for ^{14}C- and ^{3}H-labelled compounds, the latter, being weaker in energy, requiring more scintillator to reach acceptable efficiency.

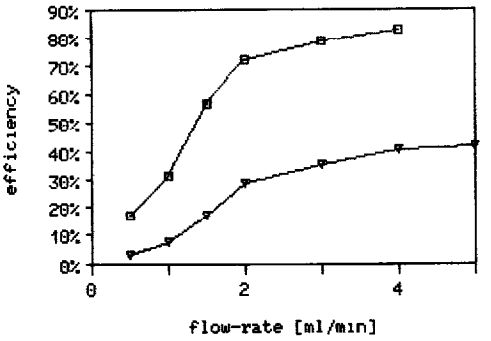

Fig. 2. Relationship between detection efficiency and scintillator flow-rate. □, ^{14}C; △, ^{3}H.

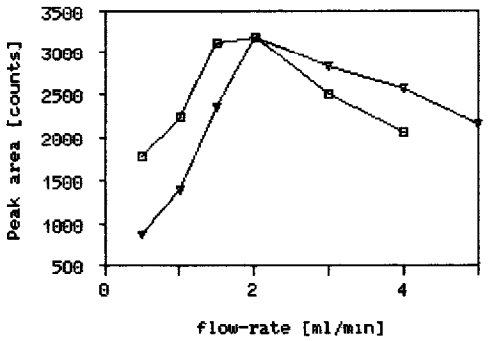

Fig. 3. Relationship between scintillator flow-rate and peak area. □, ^{14}C; △, ^{3}H.

However, as the flow-rate through the detection cell increases, the peak area expressed in counts reaches a maximum at about 2 ml/min and then gradually decreases (Fig. 3), because the residence time in the cell decreases. The best sensitivity is therefore obtained not at maximum efficiency, which requires a high consumption of scintillator, but at a lower mixing ratio.

The variations in scintillator flow-rate also influence the column efficiency (Fig. 4). The large volume of the detection cell (500 μl) causes serious peak broadening, which is most critical at low flow-rates. However, as few as 1000 theoretical plates result in a resolution higher than 3.

The major benefit of the heterogeneous mode is its low cost, as no expensive liquid scintillator is used. The efficiency for ^{14}C-labelled compounds is good (up to 85%), but only 8% can be achieved for ^{3}H-labelled compounds. The chromatogram obtained in the heterogeneous mode is shown in Fig. 5.

A major problem encountered in the heterogeneous mode is adsorption of some components on the surface of the solid scintillator. We eliminated this problem in the following way. The most hydrophilic compounds which are prone to adsorption are eluted from the reversed-phase column with the void volume. Therefore, we directed the column effluent to waste and only after 3.5 min was it directed toward the detection cell via a switching valve. The same arrangement was applied for column washing. However, proline and hydroxyproline themselves

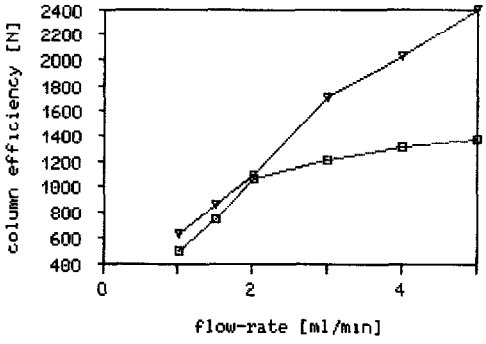

Fig. 4. Relationship between scintillator flow-rate and number of theoretical plates for (▽) proline and (□) hydroxyproline.

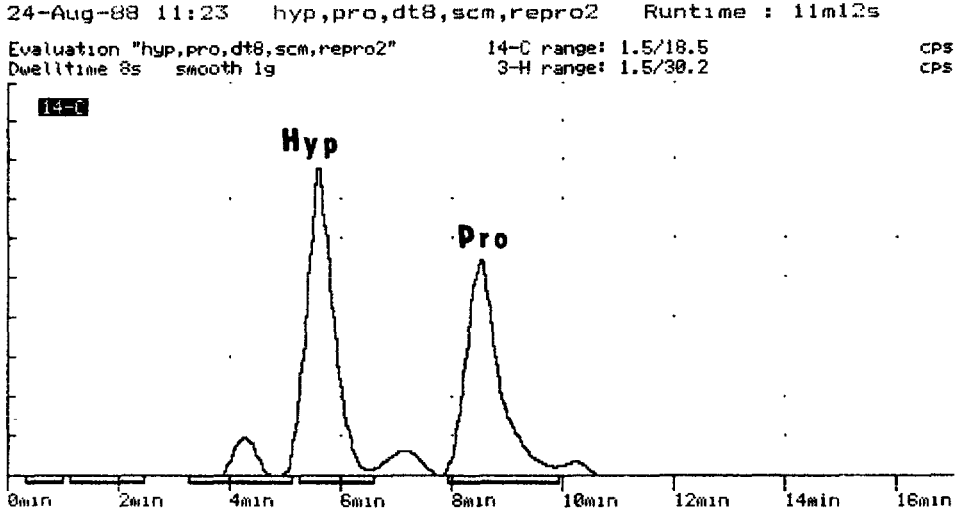

Fig. 5. Radiochromatogram of proline and hydroxyproline obtained in the heterogeneous mode.

adhere to the solid scintillator at acidic pH; tailing peaks were observed and the background increased gradually. We tested several solutions for cell cleaning and 0.1 *M* ammonia was found to be the most efficient. It would be inconvenient to clean the cell after each injection. Instead, we pumped ammonia solution with a second pump and mixed it with column effluent. A great improvement in the peak shape was observed with no memory effects.

Quantitation

The reproducibility of the method is shown in Table I. The detection limit of the method was 100 dpm for ^{14}C-labelled compounds and 200 dpm for ^{3}H-labelled compounds in the homogeneous mode. In the heterogeneous mode the detection limits were substantially higher, namely 250 dpm for ^{14}C-labelled compounds and 900 dpm for ^{3}H-labelled compounds.

The detection limits and precision were better in the homogeneous system be-

TABLE I

PRECISION OF THE METHOD

System	Label	Counts	Dpm	Relative standard deviation (%)	Efficiency (%)
Homogeneous	^{3}H	709	13167	1.8	28
		3378	62734	2.3	
	^{14}C	91	657	4.5	72
		345	2492	3.4	
Heterogeneous	^{3}H	197	13133	8.5	8
		942	62800	4.9	
	^{14}C	100	627	8.5	85
		410	2572	5.1	

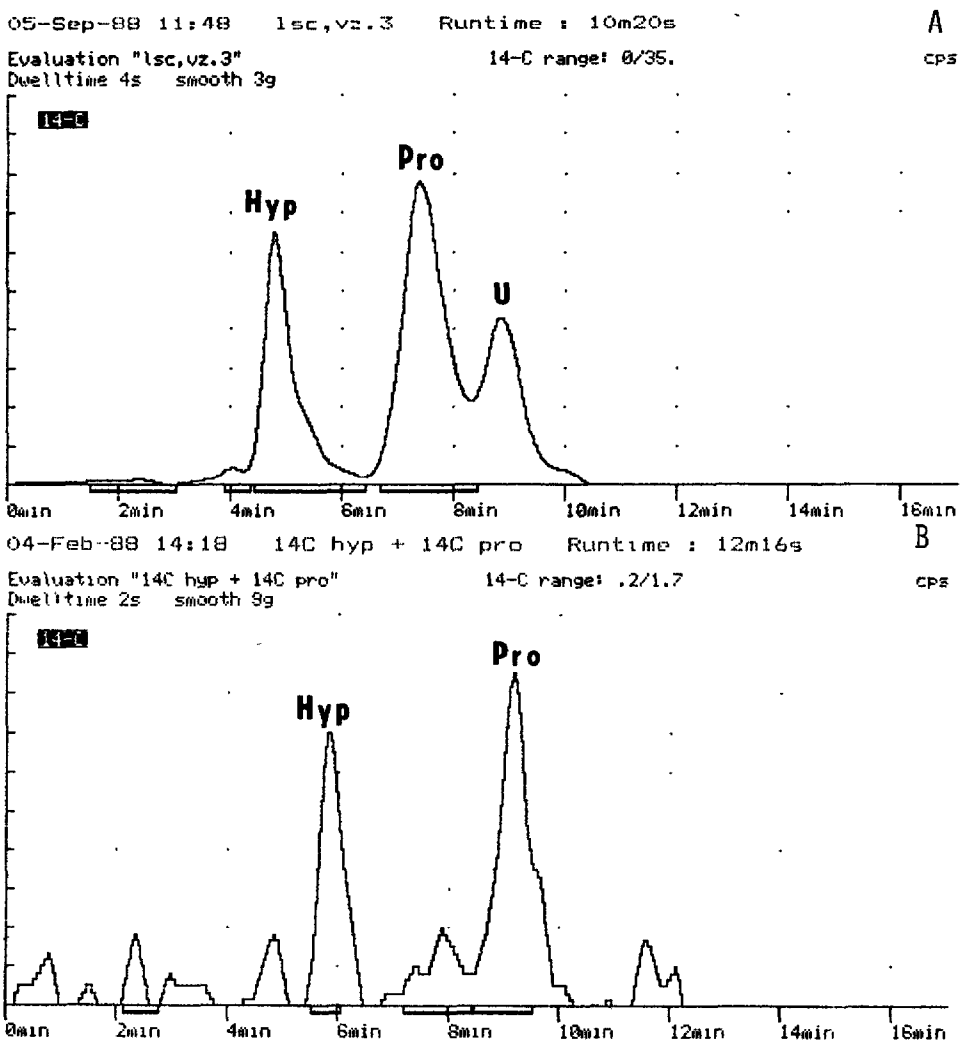

Fig. 6. (A) Radiochromatogram of collagen hydrolysate obtained in the homogeneous mode. Peak areas: [^{14}C]hydroxyproline, 950 counts = 6860 dpm; [^{14}C]proline, 1739 counts = 12 560 dpm. (B) Radiochromatogram of collagen hydrolysate obtained in the heterogeneous mode. The sample was diluted near to the detection limit. Peak areas: [^{14}C]hydroxyproline, 47 counts = 260 dpm; [^{14}C]proline, 87 counts = 483 dpm.

cause the signal-to-background ratio was higher owing to the higher background for ^{14}C-labelled compounds (80 cpm in the heterogeneous mode versus 15 cpm in the homogeneous mode) and the lower efficiency of detection for tritiated compounds.

The peak shapes were greatly improved by smoothing of the raw data, especially at low activities.

Calculation of the disintegration per minute is not so important as in static counting. Owing to the chromatographic separation, the constituents of samples causing quenching are separated from proline and hydroxyproline and the efficiency of detection is therefore constant regardless of the sample matrix used.

Application

A chromatogram of collagen hydrolysate is shown in Fig. 6. The $(Hyp \times 100)/(Hyp + Pro)$ ratio was 35.5 ± 0.56 ($n = 3$), which is in accordance with values found by Bellon et al. [6] for human skin fibroblasts.

CONCLUSIONS

This study has demonstrated the usefulness of on-line radioactivity detection for the rapid determination of radiolabelled proline and hydroxyproline and thus for the elucidation of collagen metabolism. It also stresses benefits of optimization of the experimental parameters, e.g., scintillator flow-rate, and minimization of the adsorption of the radioactive compound on the solid scintillator.

For economic reasons, the heterogeneous mode of detection is to be preferred. Only when the sensitivity and precision are critical should the homogeneous system be used. The method is very rapid, reproducible and sensitive. It will be used in further investigations of collagen metabolism, especially in studies of the influence of xenobiotics on collagen synthesis.

REFERENCES

1 D.J. Prockop, S. Undenfried and S. Lindstedt, J. Biol. Chem., 236 (1961) 1395.
2 K. Juva and D.J. Prockop, Anal. Biochem., 15 (1966) 77.
3 N. Blumenkrantz and G. Asboe-Hansen, Anal. Biochem., 66 (1975) 330.
4 J. Macek and M. Adam, J. Chromatogr., 374 (1986) 125.
5 A. Rattner, A. Chamson and J. Frey, Spectra 2000, 126 (1987) 31.
6 G. Bellon, A. Randoux and J.-P. Borel, Collagen Rel. Res., 5 (1985) 423.
7 J. Macek, L. Miterová and M. Adam, J. Chromatogr., 364 (1986) 253.