Amino Acids (1993) 4: 45–51

Amino
Acids


# Separation of amino acids using ion-paired reversed-phase high-performance liquid chromatography with special reference to collagen hydrolysate

**J. Frey, A. Chamson,** and **N. Raby**

Laboratoire de Biochimie, Faculté de Médecine, Saint-Etienne, France

Accepted March 12, 1992

**Summary.** The collagen study includes the analysis of its characteristic amino acids: proline, hydroxyproline, lysine, hydroxylysine. HPLC offers an interesting device if associated with on-line radiometric detection for the determination of radiolabelled amino acids in the case of metabolism studies. To avoid pre or post-column derivatization which may be poorly quantitative in the case of the hydrolysate of unpurified samples, we developed an ion-paired reversed-phase chromatography using a C8 column (econosphere C8 5 $\mu$m, length: 250 mm, ID: 4.6 mm from Alltech Ass.) and an elution carried out with an acetonitrile gradient in heptane-sulfonate solution. A direct detection at 210 nm was used. Nineteen amino acids were separated within 40 min. Lag time was 7.3 min between hydroxyproline and proline, and 6.9 min between hydroxylysine and lysine. In the case of radiolabelled amino acid, there was a linear correlation ($r = 0.92$) between HPLC and ion-exchange chromatography.

**Keywords:** Amino acids – High-performance liquid chromatography – Ion-pairing – Collagen amino acids – Hydroxyproline – Hydroxylysine

High-performance liquid chromatography (HPLC) has many applications in the field of amino acid analysis. Its high sensitivity and its speed offer great advantages in rapidly analysing small quantities of a sample. Method performance mainly depends on the use of the reverse phase procedure on a hydrophobic stationary phase. But in the case of amino acid, their polar functions must be derivatized to increase their hydrophobicity. Moreover, derivatization achieves a sensitive detection by converting the amino acids to derivatives that either fluoresce or absorb in the UV or visible. Nevertheless, in the case of hydrolysate from unpurified biological samples, the reagent used for the derivatization process may interact with biological components other than the amino acids so that no, or very little, pretreatment of the solutes is reproducible or quantitative.

To avoid these problems, we chose not to use derivatization and to rely only on an ion-pairing agent to increase hydrophobicity (Walker and Pietrzyk, 1985 and 1987) with a conventional reversed-phase column and direct ultraviolet detection (Molnar, 1977). Moreover, in the case of radiolabelled amino acids, this procedure allowed direct connection of the HPLC apparatus to the flow radioactivity detector.

The improvements in the method were focused on the separation of collagen amino acid in metabolic studies. The characteristic amino acid of collagen are hydroxyproline (Hyp) and hydroxylysine (Hyl) which derive from proline (Pro) and lysine (Lys) respectively during the metabolic process (Frey and Raby, 1991). The aim of this work therefore was to separate these four amino acid with a good yield and a good reproducibility.

## Material and methods

### *Apparatus*

A Kontron high-pressure liquid chromatograph with two T-414 LC pumps and a 432 variable-wavelength UV detector was used. The pumps were monitored by 457 HPLC controller software from Kontron. The sample was introduced by a Rheodyne Model 7125 sample injection valve using a 20 $\mu$l loop. The HPLC instrument was connected in line (Fig. 1) with a radioactive flow detector, Flo-One $\beta$ detector (Radiomatic Ins. Tampa, Fl.).

### *Column*

The column was an Alltech Econosphere C8 cartridge, 250 mm × 4.6 mm I.D., packed with 5 $\mu$m spherically shaped silica particles loaded with 5 % C8.

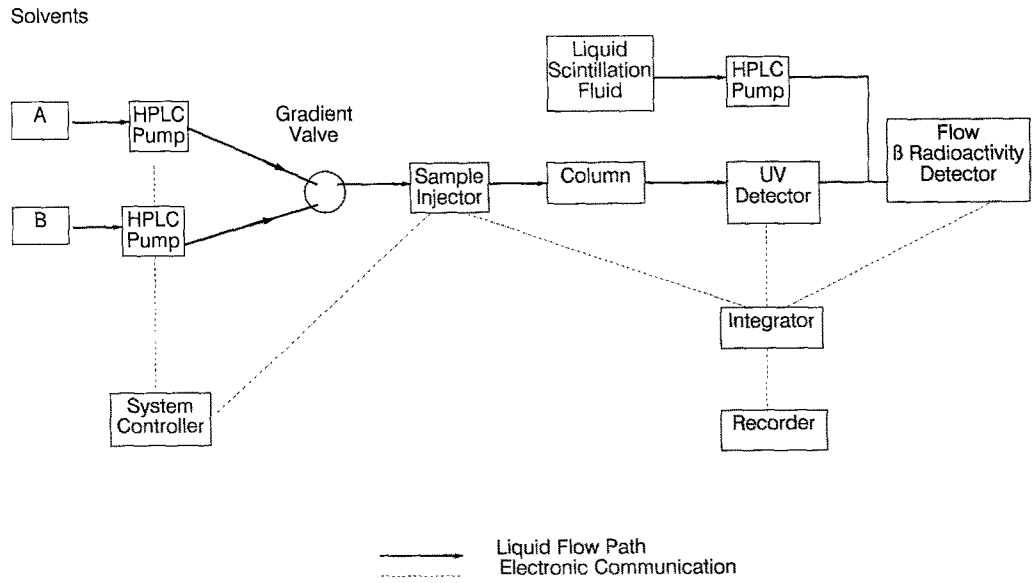


**Fig. 1.** The manifold of the connection between HPLC and radioactive flow detector

### *Chemicals*

1-Heptanesulfonic acid (sodium salt) was supplied by Sigma. Acetonitrile was HPLC—far UV grade (Carlo Erba). Distilled water was free of pyrogenic products. All the other chemicals were reagent grade. The scintillator reagent was Luma-Flow III from Lumac (Olen, Belgium).

### *Operating conditions*

The ion-interaction reagent, sodium heptane sulfonate was dissolved in a concentration of 19.8 mmolar. The pH of the mobile phase had to be adjusted to 2.3 with sulfuric acid. Linear gradients were obtained by mixing solution A which contained sodium heptane sulfonate alone and solution B in which 50 % acetonitrile was added. The two solutions were filtered through a 0.2 $\mu$m filter and degassed under vacuum before use. The shapes of the gradient employed are shown in Fig. 4. The flow rate was 0.4 ml/min at injection for 12 min, then increased to 0.8 ml/min.

In the case of labelled amino acid, the eluate was mixed with the scintillator fluid pulsed at a flow rate of 4 ml/min.

### *Collagen labelling*

Collagen was labelled as previously reported (Chamson et al, 1987). In short, fibroblast cell cultures were incubated with L-[U-$^{14}$C] proline and [4.5-$^{3}$H] lysine (Amersham) for 24 h. The labelled collagen secreted in the culture medium was purified following pepsin digestion and saline precipitation with 4.2 mol/l NaCl (final concentration) for 4 h at 4°C, then after solubilization in 0.5 mol/l acetic acid, precipitation by dialysis against 20 mmol/l disodium phosphate. The purified collagen was hydrolysed with 6 mol/l HCl for 6 h at 100°C (optimal conditions for total amino acid release). The hydrolysate was dried under vacuum and solubilized in water before chromatography.

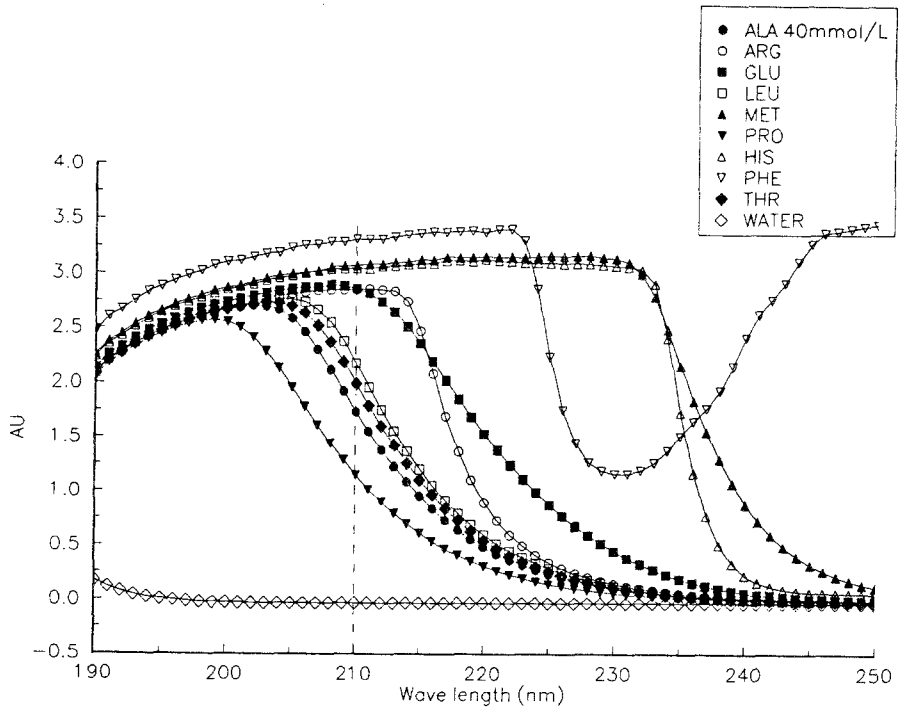


**Fig. 2.** Spectrum of the amino acids

## Results

The optical absorbance was monitored at 210 nm. Fig. 2 shows that at this wavelength the absorbance of all amino acid was sufficient to produce a signal clearly different from the solvent background allowing direct checking of the amino acid elutions.

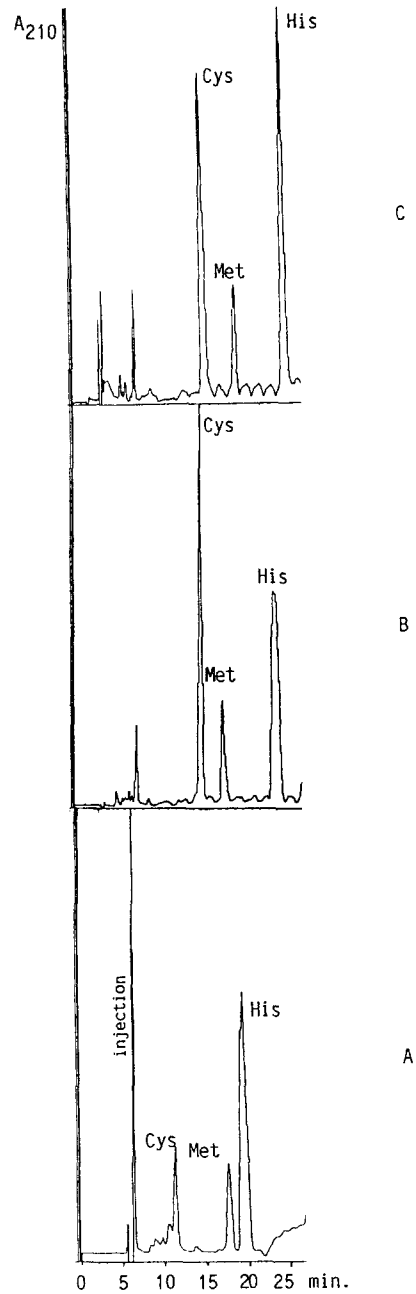


**Fig. 3.** Effect of inorganic salts on the separation of amino acids: **A** No inorganic salt addition; **B** 20 mmol/l $NH_4Cl$ addition; **C** 35 mmol/l $Na_2SO_4$ addition

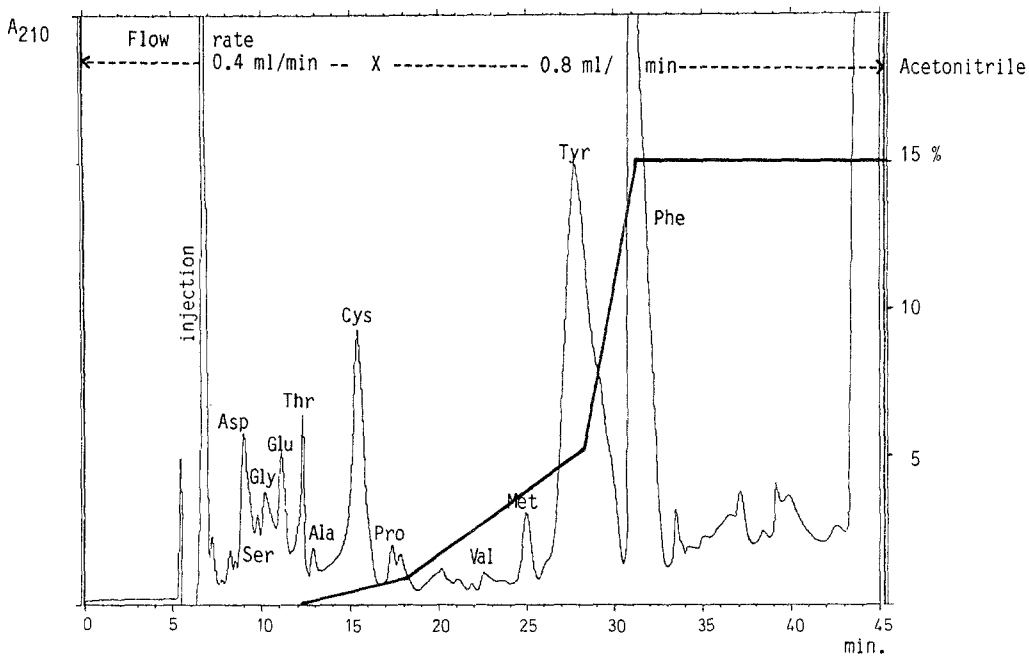


**Fig. 4.** Chromatogram of the protein hydrolysate amino acids. Column: Econosphere C8 (5 $\mu$m, octyl-silica); gradient elution from 19.8 mmol/l sodium heptane sulfonate, pH 2.3, with acetonitrile as the gradient former

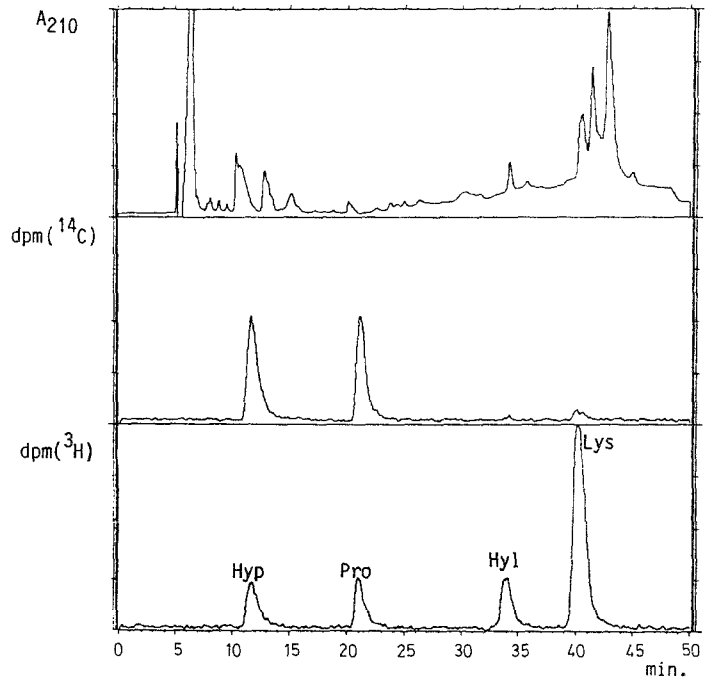


**Fig. 5.** Chromatogram of the amino acids from a labelled collagen hydrolysate. The chromatographic conditions are the same as in Fig. 4. The eluate was mixed with the scintillator reagent (4 ml/min). Absorbance (210 nm) and dpm (channel of $^{14}C$ and $^{3}H$) were monitored respectively

The effect of adding salt to the mobile phase (Martinez et al., 1988) was studied by addition of 35 mmol/l sodium sulfate or 20 mmol/l ammonium chloride respectively. The chromatograms obtained under these conditions (Fig. 3) showed that the addition of salt did not improve resolution of the amino acid separation.

Using an acetonitrile linear gradient with several slopes, then a step gradient, it was possible to separate the amino acid of a protein hydrolysate (Fig. 4). Especially Hyp, Pro, Hyl, Lys were particularly well separated without any contamination (Fig. 5).

The accuracy of the labelling measurement depends on the time during which the count increment is observed. The yield of the measure and its reproducibility were calculated at several updates using a known labelled proline sample. Table 1 shows that during a short update the measured counts were low, but the background was also low. On the other hand, using a long update, which may be useful in the case of low radioactivity, the background was at a high level. Moreover, the efficiency and the reproducibility were better at a short update than at a longer one.

The results obtained by HPLC were compared to those obtained by liquid ion-exchange chromatography (Frey and Padieu, 1967) with the same samples of labelled amino acid. Fig. 6 shows the correlation between the two methods in the case of labelled hydroxyproline. A significant correlation was demonstrated by the correlation coefficient ($r = 0.92$) but there was an intercept of 1614 dpm which must be taken into account.

**Table 1.** Radiolabelled proline yield

| Update (sec) | Background (dpm) | Injected (dpm) | Recovery (dpm) | Efficiency (%) | Reproducibility (%) |
|---|---|---|---|---|---|
| 2 | 15 ± 1 | 1009 | 1002 ± 140 | 99 | 4 |
| 15 | 108 ± 4 | 1118 | 1053 ± 126 | 94 | 12 |
| 20 | 142 ± 3 | 1009 | 847 ± 153 | 84 | 18 |

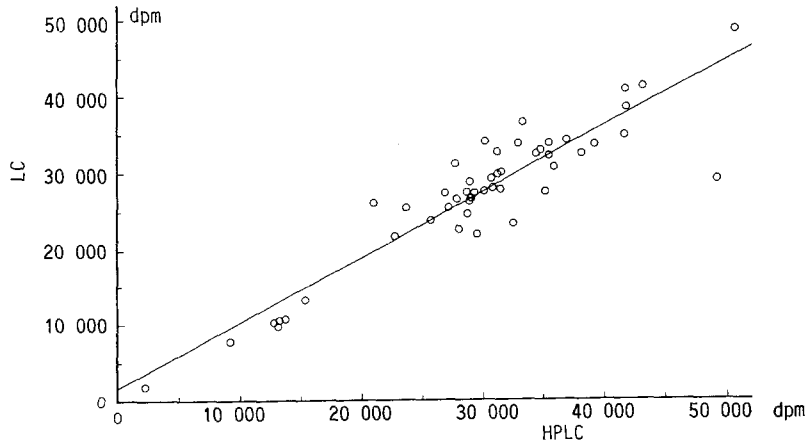


**Fig. 6.** Correlation between HPLC and ion exchange chromatography (*LC*) in the case of radiolabelled hydroxyproline (correlation coefficient $r = 0.92$, slope = 0.87, intercept: 1614)

## Discussion

The separation of amino acid was accomplished by HPLC using a reversed-phase and a ion-pairing reagent to increase the hydrophobicity of the amino acid. With direct detection at 210 nm, derivatization was not necessary.

The effect of adding salt to increase the ionic strength of the mobile phase to favour ion pairing was not demonstrated. It could however modify the stability of the mobile phase and produce background instability. The amino acid protonation was sufficient under acid conditions. To obtain a better separation of the polar amino acid, a low flow rate, 0.4 ml/min, was used.

In the case of radiolabelled amino acid, the chromatographic system can be coupled with a flow radioactivity detector. The separation of characteristic amino acid of collagen could be obtained with a good yield. According to the radiolabelling measurement, a short update must be used to obtain the best recovery and the best reproducibility. The correlation with liquid ion exchange chromatography, however, showed an intercept of 1600 dpm. This difference at a low radioactivity level may be linked to a lower sensititivy of HPLC because of the small amount of the injected sample. It is therefore necessary to use a sample with sufficient specific activity. It seems that an activity greater than 1000 dpm would be required.

## Acknowledgements

This work was supported by "GIP Exercice". We would like to thank S. Peyroche for her excellent technical assistance and C. Moja, O. Rivallier, and S. Oriol for their collaboration.

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**Authors' address:** Prof. J. Frey, Laboratoire de Biochimie, Faculté de Médecine, 15 rue Ambroise Paré, F-42023 Saint-Etienne Cédex 2, France.

Received October 15, 1991