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Journal of Chromatography B, 691 (1997) 297–304

JOURNAL OF
CHROMATOGRAPHY B

Rapid method for the isolation of hydroxylysylpyridinoline and lysylpyridinoline from concentrated bone hydrolysates by liquid chromatographic techniques

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Received 22 April 1996; revised 17 September 1996; accepted 24 September 1996

Abstract

A rapid method for the isolation of hydroxylysylpyridinoline and lysylyridinoline from bone by liquid chromatographic methods is described. Decalcified bone is hydrolysed in 7 *M* hydrochloric acid. After evaporation of the acid, the high molecular mass and dark coloured degradation products are removed by adsorption on non-polar adsorbents. The pyridinolines are separated from the majority of the amino acids by adsorption on cellulose. Separation of HP and LP is performed either by cation-exchange chromatography or by reversed-phase ion-pair chromatography. The pyridinoline containing fractions are desalted by size-exclusion chromatography. The progress of the hydrolytic cleavage of collagen and the optimal parameters for purification and separation were examined. As a result the existing method allows the isolation of high amounts of pyridinolines with low amounts of adsorbents and chemicals within a short time.

Keywords: Bone hydrolysate; Hydroxylysylpyridinoline; Lysylpyridinoline; Amino acids

1. Introduction

Hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) are derivatives of hydroxylysine and occur in mature collagen [1,2] as stable and non-reducible trifunctional cross-linking compounds. Their structure differs in one hydroxyl group in the lysyl side-chain (Fig. 1). HP is found in bone and soft tissue, whereas LP occurs nearly exclusively in bone. They are released during degradation of collagen fibrils and excreted in urine in free form (about 40%) and peptide-bound form. The accelerated breakdown of skeletal tissue is correlated with increased levels of the two compounds in urine [3–5] and they are more specific than other chemical markers (e.g., urinary hydroxyproline). LP in particular can be seen as a significant biological marker for measurement of bone turnover. The measurement of the concentration of both compounds in urine is usually carried out by liquid chromatography [4–9] or immunosorbent-assay [4,5,10,11]. For the preparation of standards, HP and LP are isolated from bone [12], tendon [13] or urine [14,15]. After hydrolysis, purification is performed preferably by chromatographic methods such as size-exclusion chromatography [3,6,16], adsorption-chromatography [7,9,12] or ion-exchange chromatography [1,7,11,13,14,17,18]. Usually a combination of different chromatographic methods is applied and only a few procedures apply the same method several times. Due to the complex matrix a great disadvantage of all procedures is that in the first stages of purification,

PII S0378-4347(96)00456-2

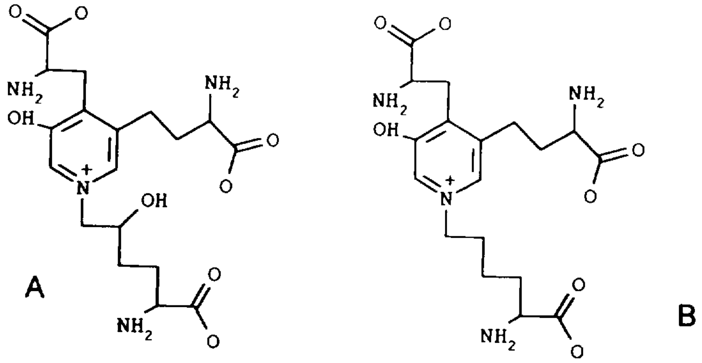

Fig. 1. Structure of (A) hydroxylysylpyridinoline and (B) lysylpyridinoline.

diluted solutions and high amounts of chemicals are involved during sample processing and separation.

Therefore the aim of this work was the development of a simple method which allows the isolation of high amounts of pyridinolines from concentrated bone hydrolysates within a short time. Special attention was paid to the elaboration of suitable purification procedures for the first stages which resulted in a new method for the precleaning of hydrolysates of amino acids obtained by acid hydrolysis. A further aspect was the application of low amounts of regenerable adsorbents and chemicals.

2. Experimental

2.1. Chemicals

Bone splinter and bone gelatine (ossein) were obtained from Deutsche GelatineFabriken Stoess (Eberbach, Germany). Analytical grade hydrochloric acid, methanol, acetic acid, citric acid, di-sodiumhydrogenphosphate, ninhydrin, EDTA and pure 1-butanol were purchased from Merck (Darmstadt, Germany). *n*-Octanesulfonic acid (OSA) was supplied by Sigma (St. Louis, MO, USA).

2.2. Methods and apparatus

2.2.1. Demineralisation of bone

Bone splinters were decalcified in 2 *M* hydrochloric acid for 24 h. After several washings with distilled water they were freeze-dried.

2.2.2. Hydrolysis

A 50-g sample of decalcified bone or bone gelatine was transferred into a 250-ml flask (Schott, Mainz, Germany) and filled up with 200 ml of 7 *M* hydrochloric acid. The bottle was sealed with a screw cap containing a teflon liner at the inner side and heated on a boiling water bath for 40 h. After cooling the solution was filtered through a paper filter and evaporated at 50°C in a rotary evaporator at reduced pressure generated by a water pump. Evaporation of volatile compounds of the viscous and dark residue was prolonged at low speed of rotation till no bubbles were formed.

2.2.3. Separation of the high molecular mass degradation products

The dark coloured viscous liquid (about 50 g) consisting of liberated amino acids and high molecular weight sample components was dissolved in 20 ml water and adsorbed on either 15 g of LiChroprep octadecylsilyl silica gel (ODS) of particle size 40–63 μm obtained from Merck or 20 g of XAD-2 polymer-based resin of particle size 50–100 μm purchased from Machery-Nagel (Düren, Germany). In practice, the adsorbents, which had been prewetted in methanol, were added batchwise to the stirred solution of hydrolysis products. After settling of the adsorbent, the solution was filtered (filter porosity G2) and the adsorbent subsequently washed with two 15 ml portions of water. A transparent yellow filtrate was obtained.

2.2.4. Regeneration of the adsorbent

The adsorbent was regenerated with methanol acidified with hydrochloric acid to pH 1.5–2.0. A volume equivalent to 3–4 fold the bed volume was necessary for the elution of the contaminants from octadecyl-silica. If XAD was used as adsorbent, twice the volume of methanol was used for cleaning. Methanol was recovered by distillation, the residue was dissolved in water and discarded.

2.2.5. *Separation of amino acids*

Cellulose of type CF11 (13 g) obtained from Whatman (Maidstone, UK) was suspended in water and poured into a 25×3.3 cm I.D. glass column. After washing with water, the column was equilibrated with 1-butanol–acetic acid–water (4:1:1, v/v). The volume of the hydrolysate was reduced to 100 ml by evaporation under reduced pressure and divided into three equal volumes. A mixture of 1-butanol–acetic acid–hydrolysate (4:2:1, v/v) was prepared immediately before application onto the column.

Subsequently the column was washed with 220 ml of 1-butanol–acetic acid–water (4:1:1, v/v). HP and LP were eluted with 130 ml water. The butanolic layer was separated and the aqueous solution was evaporated to dryness.

For the separation of diluted hydrolysates a solvent mixture of 1-butanol–acetic-acid–hydrolysate (4:1:1, v/v) was used.

2.2.6. *Separation of HP and LP*

Two alternative methods, cation-exchange chromatography as well as ion-pair chromatography were used.

2.2.7. *Cation-exchange chromatography*

The sample was reconstituted in 15 ml eluent A, which was an aqueous solution of 0.02 *M* citric acid and 1 m*M* EDTA, adjusted to pH 2.3 with hydrochloric acid. Eluent B, which consisted of 0.02 *M* Na_2HPO_4 and 1 m*M* EDTA, was adjusted to pH 7.0. The eluent was delivered by a Model L-6200 low-pressure gradient pump obtained from Merck. Separation of HP and LP was performed at 62% A and 38% B either on a 250×7 mm I.D. or 125×4 mm I.D. stainless steel column, packed with a strong acidic cation-exchanger (Nucleosil SA) of 10 μm particle size obtained from Machery-Nagel. Sample volumes containing up to 10 g demineralized bone mass were injected. Detection was performed by a Model L-4500 diode array detector and a Model F-1050 fluorescence detector purchased from Merck and coupled in series. The excitation wavelength was set to 295 nm and the emission measured at 395 nm. Data were recorded on a personal computer by use of computer programmes obtained from Merck and Nelson analytical (Cupertino, CA, USA). Fractions were collected with a Model Foxy programmable fraction collector from Isco (Lincoln, NE, USA) in 1.0-min intervals. After each run data were analysed and the fractions containing HP and LP pooled and evaporated to dryness.

2.2.8. *Ion-pair chromatography*

After evaporation of the solvent the residue was dissolved in 4 m*M* OSA, 1 m*M* EDTA and 3% (v/v) methanol in water adjusted to pH 4.2 with acetic acid. Separation was performed on a 250×10 mm I.D. stainless steel column, packed with octadecyl-silica of type LiChrospher (Merck) of 5 μm particle size. Flow-rate was set to 6.0 ml/min. The column was thermostatted at 35°C by a Model T-6300 column thermostat from Merck. Sample volumes of 1.0 ml were injected. Detection and fraction collection were performed as described above.

2.2.9. *Desalting*

Biogel P2 (Bio-Rad, Richmond, CA, USA) was suspended in the mobile phase of 10% (v/v) acetic acid and filled into a 1.0 m×1.6 cm I.D. glass column. The sample was dissolved in the mobile phase and applied onto the column. Fractions were collected in intervals of 15 min corresponding to a fraction size of 4.5 ml. Their pyridinoline and salt content was determined by measuring the refractive index and fluorescence response (excitation at 295 nm, emission at 395 nm). The fractions containing pure HP or LP were pooled and freeze-dried.

2.2.10. *Estimation of HP and LP in the fractions*

Content of amino acids. The desalted fractions were separated by TLC on a 100×200 mm Merck TLC cellulose plate using 2-propanol–formic acid–water (20:1:5, v/v) as solvent. After drying the plate was immersed in a 0.3% (w/v) solution of ninhydrin in 1-propanol–acetic acid–water (94:5:1, v/v) and subsequently heated at 90°C for 2 min. Scanning was performed by a Model TLC-Scanner II from Camag (Berlin, Germany) setting the wavelength to 570 nm.

2.3. Determination of HP and LP in the fractions

HP and LP were separated on a 125×4.6 mm I.D. LiChroCART column packed with octadecyl-silica of type LiChrosphér (Merck) of 5 μm particle size and thermostatted at 35°C. The eluent consisted of 4 m*M* octanesulfonic acid, 0.08 *M* citric acid, 1 m*M* EDTA and 2.6% (v/v) methanol adjusted to pH 3.6. The flow-rate was set to 1.0 ml/min. Fluorescence detection was performed as described above.

3. Results and discussion

3.1. Hydrolysis

HP and LP were released by acid hydrolysis in a boiling water bath. The time course of the hydrolytic cleavage in 4–7 *M* hydrochloric acid is shown in Fig. 2. The highest yields of HP and LP were found at 7 *M* hydrochloric acid. More than 70% of the final yield of HP and LP was achieved after 30 h of exposure to the hydrolysis conditions and from this time the concentration increases slowly at about 1%/h and remains nearly constant after 40 h.

3.2. Separation of the high molecular mass degradation products

The hydrolytic degradation products which cause the dark colour of the solution are probably attributable to aldehydes and amines. They are of high molecular mass (estimated by size-exclusion chromatography) and formed in hot and strong acidic solutions by polymerisation of the hydrolytic products. In chromatography they tend to blockade the active sites of adsorbents and ion exchangers. As a consequence high amounts of adsorbents and washing solutions and the application of diluted hydrolysates are required. A rapid and efficient purification by chromatographic methods is only possible, when these compounds have previously been removed. Although being very hydrophilic, it was found that they can be adsorbed on non-polar adsorbents such as ODS matrices or resins of the type XAD. Under the experimental conditions the pyridinolines leave the column unretained.

The best results were achieved when the residue after evaporation of the acid was dissolved in water, and the adsorbent-wetted with methanol-was batchwise added to the solution. Although the pH of the solution was kept between 1.0 and 1.8, no loss of the capacity of the ODS matrix, due to dissociation of octadecylsilyl substituents, was observed.

As an alternative analytical tool, polymer-based resins of the type XAD, which are stable under strong acidic conditions, are also suitable. Good results were obtained with XAD 2 of small particle size (50–100 μm). The efficiency is drastically reduced when XAD of greater particle size is used. This may be explained by the fact that the high molecular mass degradation products could not penetrate into the pores of the adsorbent. The complete removal of the dark coloured compounds from the adsorbent is only possible if two requirements are met. On the one hand, the pH of the reconstituted hydrolysate in water should not be changed, otherwise only incomplete cleaning of the adsorbent is achieved. The colour of the adsorbent will then turn to grey and the capacity is drastically reduced. On the other hand, cleaning is only possible with an acidified organic solvent such as methanol acidified with hydrochloric acid, which yields excellent results. Furthermore, regeneration of the ODS matrix is more successfully achieved compared with the XAD-2 resin.

The same cleaning procedure was applied with great success to other protein hydrolysates in either column or batch mode and proved to be an excellent method for sample preparation.

3.3. Separation of the fractions containing the amino acids

3.3.1. Adsorption chromatography on cellulose.

HP and LP are separated in an eluent preponderantly consisting of 1-butanol from most of the released amino acids by adsorption on cellulose. The method was originally introduced by Skinner [19] for the detection of elastin crosslinks and has now been adapted for the purification of concentrated solutions of amino acids. The high concentration of the hydrolysate in the eluent leads to an accelerated

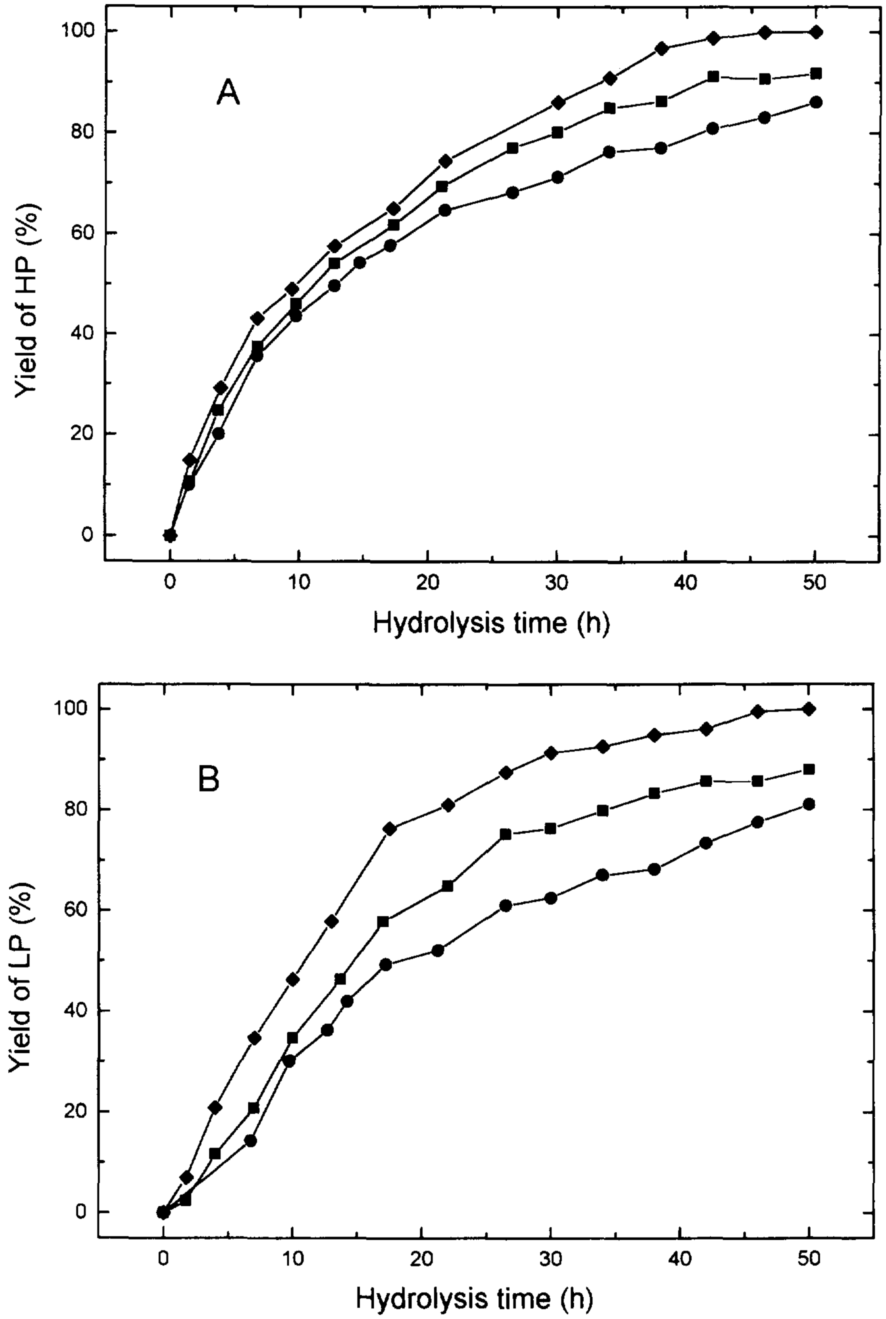

Fig. 2. Release of (A) HP and (B) LP by acid hydrolysis in a boiling water bath. Conditions: concentration of hydrolysate, 0.2 g ossein/ml; concentration of hydrochloric acid, (●) 5 *M*, (■) 6 *M*, (◆) 7 *M*.

phase separation and the formation of gas bubbles in the column. Therefore samples of the mixture were prepared immediately before application onto the column. The loadability of a CF11-column under the conditions described above is shown in Fig. 3. In the batch mode the recovery of HP and LP was found to

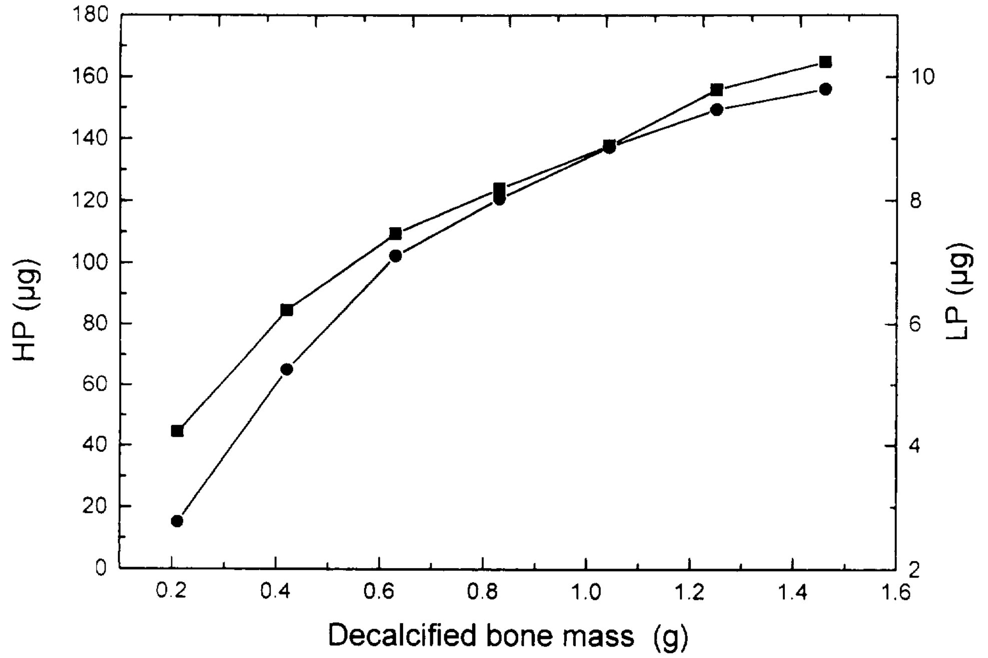

Fig. 3. Recovery of (■) HP and (●) LP after adsorption chromatography on CF11 dependent on column loading. Conditions: column, 60×8 mm I.D., adsorbent 160 mg CF11; sample, 1-butanol–acetic acid–hydrolysate (4:2:1, v/v), concentration of hydrolysate in the sample=70 mg decalcified bone/ml.

be generally lower. Although the dark coloured compounds of high molecular weight formed during acid hydrolysis comprise only a few mass percent of the whole liberated compounds, the application of the simple precleaning step leads to an improvement of the capacity of the adsorbent and a reduction of the applied volume of organic eluent of one order of magnitude.

The mixture of the sample with 1-butanol and acetic acid undergoes a slow phase separation leading to the formation of an organic and an acidic aqueous layer. Because HP and LP are enriched in the aqueous layer, it represents a simple alternative method. The enrichment is better for HP (99%) than for LP (87%), but the separation from other amino acids is poor.

3.3.2. Cation-exchange chromatography

In order to avoid the use of non-recoverable organic solvents, alternative methods were examined. The best results were obtained by cation-exchange chromatography. Silica-based cation exchangers were found to be more suitable for that problem than polymeric ones (e.g., Dowex 50WX8, IR120). The separation and the recovery of HP and LP on silica-based strong acid cation exchangers (e.g., Nucleosil SA) was found to be superior by a factor of three. Optimal conditions for enrichment are diluted solutions (<10 mg decalcified bone/ml) and a pH of 2.3. In either batch or column operation the same recovery of the two compounds was obtained. However, although the conditions of operation were carefully examined, in comparison to adsorption-chromatography on cellulose the capacity and the separation of HP and LP from other amino acids is poorer.

3.4. Separation of HP and LP

The separation of HP and LP was performed either by ion-exchange chromatography on a strong acid cation exchanger (Fig. 4) or by ion-pair chromatography using *n*-octanesulfonic acid as ion-pairing reagent (Fig. 5). The separation efficiency of both methods is comparable, but ion-pair chromatography suffers from a long equilibration time, and the

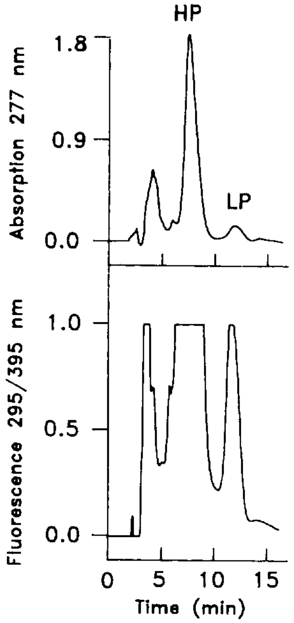

Fig. 4. Separation of HP and LP by cation-exchange chromatography. Conditions: eluent, A=0.02 *M* citric acid, 1 m*M* EDTA, adjusted to pH 2.3 with hydrochloric acid, B=0.02 *M* Na$_2$HPO$_4$, 1 m*M* EDTA, pH 7.0, A+B=62+38% (v/v); column, 125×4 mm I.D. packed with Nucleosil SA, 10 μm; flow-rate, 1.0 ml/min; sample, 3.5 g decalcified bone/ml corresponding approximately to 3 mg prepurified extract; injection volume, 2.0 ml; temperature, 35°C.

retention time of either HP or LP varies more or less from injection to injection. Ion-exchange chromatography was therefore applied preferably. HP and LP can be completely separated from each other in one run at low to medium column load (corresponding approximately to 15 mg prepurified hydrolysate). However at higher sample load pure fractions of LP are only obtained if the fractions containing more or less marked amounts of impurities of HP are individually subjected to a second chromatographic run.

3.5. Identity and purity

Identity of the pyridinolines was verified by plasma-desorption mass spectrometry. HP showed a molecular ion at *m/z* 413, LP at *m/z* 429. Optimal signal responses of HP/LP were obtained at 295 nm in UV absorption measurements or at 295 nm (excitation) and 395 nm (emission) by fluorimetric detection in acidic solutions. These data are in accordance with the results reported by others [14,17,18]. From 50 g of decalcified bone 7.0 mg HP and 0.4 mg LP could be isolated.

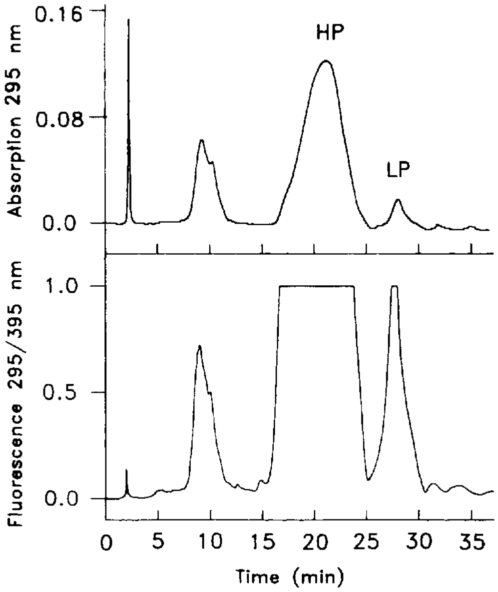

Fig. 5. Preparative separation of HP and LP by ion-pair chromatography. Conditions: eluent, 4 m*M* *n*-octanesulfonic acid, 3.0% (v/v) methanol, 1 m*M* EDTA adjusted to pH 4.2 with acetic acid; column 250×10 mm I.D. packed with LiChrospher 100 RP 18, 5 μm; flow-rate, 6.0 ml/min; sample, 5 g decalcified bone/ml corresponding approximately to 4 mg prepurified extract; injection volume, 1.0 ml; temperature, 35°C.

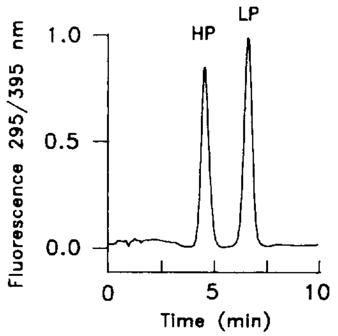

Fig. 6. Separation of a synthetic mixture of HP and LP by ion-pair chromatography with octanesulfonic acid. Conditions: eluent 4 m*M* octanesulfonic acid, 0.08 *M* citric acid, 1 m*M* EDTA, 2.6% (v/v) methanol adjusted to pH 3.6 with acetic acid; column, LiChroCART LiChrospher RP 18, 5 μm, 125×4 mm I.D.; flow-rate 1.0 ml/min; temperature, 35°C.

Purity with respect to the content of other amino acids was determined by thin-layer chromatography. Filtration with octadecyl-silica showed almost no influence on purity. Adsorption chromatography on cellulose increased purity to 43% and after ion-exchange chromatography and desalting, purity was better than 98%.

The amount of HP and LP in the fractions was estimated by ion-pair chromatography with OSA (Fig. 6).

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