Amino Acids (1994) 6: 57–63

Amino
Acids

Rapid determination of desmosine and isodesmosine in tissue hydrolysates by isocratic high performance liquid chromatography and precolumn derivatization

P. Charpiot, R. Calaf, C. Chareyre, P. H. Rolland, and **D. Garçon**

Laboratory of Biological Chemistry (PC, RC, CC, DG) and INSERM (PHR), School of Pharmacy, Marseilles, France

Accepted June 11, 1993

Summary. A rapid and sensitive isocratic high performance liquid chromatographic method has been developed for the single and specific determination of low concentrations of desmosine (Des) and isodesmosine (Ide), the major specific crosslink aminoacids in elastin.

Samples of isolated elastin or whole tissue were hydrolysed in 6N HCl, and the hydrolysates were prefractionated on cellulose CF1. Des, Ide, γ-glutamylglutamic acid as internal standard were dansylated and derivatives were extracted from reaction mixture by ethylacetate. Their separation on a Lichrosphere 100-NH_2 column, using methanol-water as mobile phase containing acetic acid and 0.25 M sodium acetate, final pH 6.5, was followed by fluorescence detection (340–510 nm). The overall reproducibility was 5.9% for Des and 5.0% for Ide. The limits of detection were 2.2 pmol and 2.5 pmol, respectively. The method was successfully applied for the determination of Des and Ide in normal pig aortas.

Keywords: Amino acids – Elastin crosslinks – Desmosine – High-performance liquid chromatography – Isocratic

Introduction

The degradation of elastic fibers is involved in aging, acute inflammatory diseases and also degenerative disorders such as emphysema, atherosclerosis or aortic aneurysm.

Desmosine (Des) and isodesmosine (Ide) are lysine derived crosslink amino acids, specific to mature elastin. Assuming the ratio of Des and Ide to total aminoacids to be constant in elastin (Sandberg et al, 1981), the content of desmosine and isodesmosine has been frequently used as an index of elastin

amount in tissues. In biological fluids, especially in urine, these crosslinks are also useful markers of elastin breakdown.

Therefore, accurate methods for the quantification of very low levels of desmosine and isodesmosine are of major interest to investigate the turn-over of mature elastin as well as elastase activities in physiological and pathological processes. Radioimmunoassay and enzyme immunoassay, are highly sensitive but show some cross-reactivity (Gunja-Smith, 1985; King et al., 1980). Some of the chromatographical methods so far employed, including the use of ion exchange chromatography on an autoanalyzer, or reverse phase HPLC with precolumn derivatization and spectrophotometrical or electrochemical detection, intent to analyse the overall aminoacid content in samples (Zarkadas et al., 1986; Negro et al., 1987; Lunte et al., 1989). These methods are rather time consuming, and do not perform reliable quantification of Des and Ide in the whole tissue hydrolysates, due to the low Des and Ide concentrations, highly unbalanced compared to total aminoacid composition.

The purpose of our method was to assay low concentration of Des and Ide, using HPLC methodology after a prefractionation step. Such technics have already been reported, but their sensitivity remain low due to direct UV detection (275 nm) (Faris et al., 1981; Soskel, 1986; Yamaguchi et al., 1987; Schwartz et al., 1990). We describe here a rapid, sensitive, specific, accurate and easy to perform isocratic HPLC method for the quantification of Des and Ide in hydrolysates of purified elastin as well as of whole tissue or biological fluids. This method is based on optimized dansyl precolumn derivatization, ion-exchange chromatography allowing separation of polycarboxylic acid molecules, and subsequent fluorescence detection.

Materials and methods

Chemicals

5-(dimethylamino)-naphtalene-1-sulfonyl-chloride (Dansyl chloride, DnsCl), ethyl acetate, phosphoric acid were purchased from Merck (Darmstadt, Germany), acetonitrile, methanol, acetic acid, sodium acetate were from Carlo Erba (Milan, Italy). Borax, γ-glutamyl glutamic acid (γ-Glu-Glu), bovine serum albumin (BSA) were from Sigma (St. Louis, Mo.). CF1-cellulose was from Whatman. Desmosine, isodesmosine and bovine neck ligament elastin were from Elastin Products (Owensville, Mo.). Water was deionized. Dansyl chloride solutions were always freshly made.

HPLC

All HPLC equipment was from Merck (Darmstadt, Germany) and consisted of a L6200A ternary low pressure gradient system, with a 50 μl injection loop. Analysis were performed using a Lichrosphere 100 NH_2 5-μm column (250 $\times$ 4.6 mm I.D.) from Merck. Mobile phase consisted of a methanol:water mixture (85:15), containing acetic acid and sodium acetate at various molarities as described below, the flow rate remaining at 1 ml/min. As a routine, the mobile phase was 0.25 M acetate and final pH 6.53. Solvents were filtered on 0.45 μm, degassed prior to use, and gently stirred during analysis. Effluent was monitored using a F1050 fluorescence spectrophotometer, with a 12 μl flow cell. Excitation and emission wavelengths were 340 nm and 510 nm, respectively. Data were recorded using a D2500 integrator. All chromatographic analysis were run at room temperature.

Sample preparation

Pig aortas were washed in 0.15 M NaCl and stripped of adhering tissue, dried and ground in liquid nitrogen. Aorta elastin was isolated by an alkaline extraction procedure (Lansing et al., 1972), as follows: the tissue was homogenized in 0.125 NaCl phosphate buffer, pH 7.4, and centrifugated. The pellet was delipidated in chloroform:methanol (2:1), washed, and then treated by 0.1 N sodium hydroxyde at 95°C for 45 min. The insoluble fraction, representing elastin, was washed three times with distilled water, and dried. Commercially available bovin neck ligament elastin was also used. Purified elastin, other proteins, or whole tissues were then hydrolysed for 18 hours in 6N HCl at 105°C, and hydrolysates were dried under vacuum.

CF1 column

Des and Ide were extracted from hydrolysates using CF1-cellulose procedure, essentially as described by Skinner (Skinner, 1982). The CF1-cellulose was washed four times with water prior to use, then suspended (5 g/100 ml) in an organic mobile phase consisting of n-butanol-acetic acid-water (4:1:1). Dried proteinic hydrolysates were dissolved in 0.5 ml of water, to which 0.5 ml of acetic acid, 5 ml of CF1 slurry and 2 ml of butanol were added, and mixed for 30 min. The mixture was then transfered onto the top of a 10 cm × 1 cm I.D. sintered-glass mini-column (Biorad). Non-cross-linking amino acids first were eluted three times with 5 ml of the mobile phase, then cross-linking amino acids were eluted with 6 ml of distilled water and samples freeze-dried.

Dansylation

Des and Ide standard solutions (0.01–10 nmoles) and purified hydrolysates were derivatized according to Gray's method (Gray, 1972; Tapuhi et al., 1980; Tapuhi et al., 1981), modified as follow. 500 μl of 0.05 M borax-HCl buffer (Bayer et al., 1976), pH 8.6, containing 250 μl of 10 mM DnsCl in acetonitrile (10 mg/4 ml), and 50 μl of γ-Glu-Glu as internal standard were added to dried samples. Concentration of internal standard solution was 5 μM or 50 μM, according to the expected concentration of Des and Ide. Reaction was carried out in the dark in a screw-top tube at 45°C during at least 15 min, and stopped by the addition of 50 μl of 4% ethylamine furthered by a 5 min incubation at 45°C (Tapuhi et al., 1981).

Extraction

Des and Ide derivatives were purified by extraction from reaction mixture. Dansylation mixture was adjusted to pH 4.2 with 0.1 M H_3PO_4 (250 μl), then briefly mixed with 800 μl ethylacetate, and centrifuged. 750 μl of the upper layer was dried at 45°C under N_2. The dried residue was stored at 4°C or diluted with mobile phase, the volume depending on the desired concentration ratio, then an aliquot of 50 μl was injected onto the column.

Results

Derivatisation procedure optimization

Whatever the temperature (45°C or 60°C), the dansylation yield of desmosine, isodesmosine, and γ-Glu-Glu rose to a maximum at 15 min, and remained constant even if the reaction time exceed one hour. Fig. 1 shows the effect of buffer pH on reaction yield. The optimum pH, around 8.6, proved to be slightly lower than the optimum range 9–10 described for other aminoacids (Gray, 1972). As shown in Fig. 2, the extraction yield was nearly constant when the pH

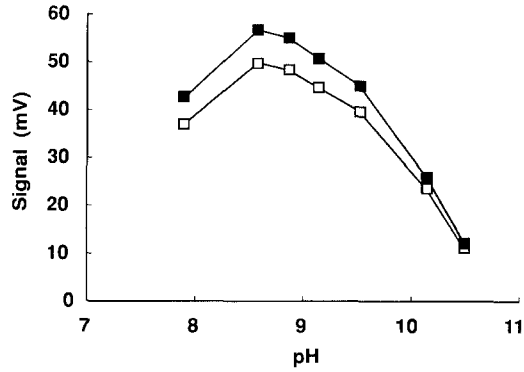

Fig. 1. Effect of pH on the efficiency of dansylation of Des (■) and Ide (□). Standard solutions of Des and Ide (2.5 nmol) were derivatized using 0.05 M borax-HCl buffer at different pH. Other conditions are described in Materials and methods

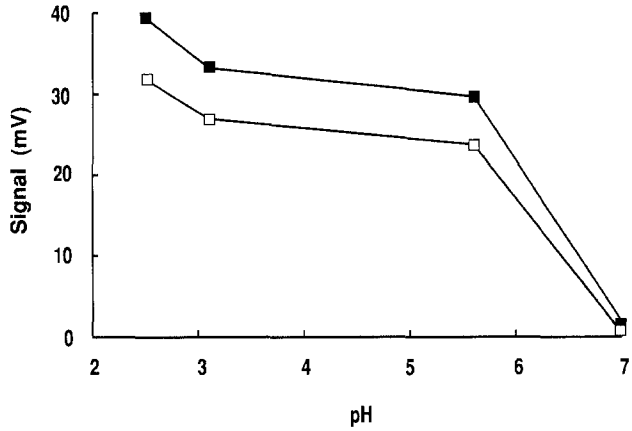

Fig. 2. Effect of pH on the efficiency of extraction of Des (■) and Ide (□) derivatives by ethylacetate. PH of reaction mixture was adjusted at different values by 0.1 M H_3PO_4 before extraction. Other conditions are described in Materials and methods

of dansylation mixture was in the range 3–5.5, but decreased down to 0 at neutrality.

In order to improve Des and Ide analysis in proteinic hydrolysates, dansylation was also carried out with various amounts of bovine serum albumin (BSA) hydrolysate added to standards of Des and Ide (10 nmol). When the amount of BSA varied up to 100 μg, i.e. about 2 mg of protein in the sample before CF1 fractionation, the reaction yield remained unchanged.

Chromatographic separation

Capacity factors and resolution of Des and Ide decreased when buffer molarity was increased from 0.2 M to 0.3 M. At 0.3 M no separation occured at any pH in the range 6.1–6.7. At 0.2 M and 0.25 M, the observed resolution was very poor at pH 6.1, especially with a coelution of γ-Glu-Glu and Des, but increased with pH. The use of 0.25 M acetate, pH 6.53 proved to be a correct compromise.

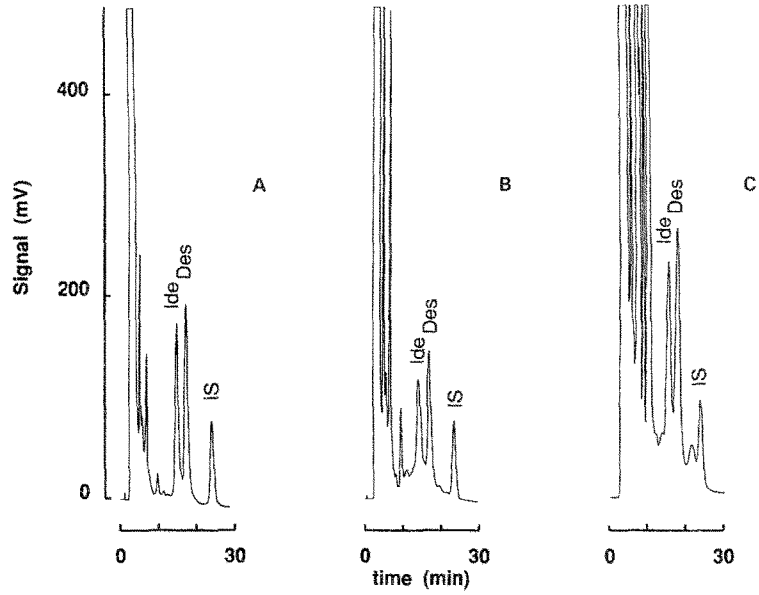

Fig. 3. Chromatograms of Des and Ide in the standard solution (**A**), and elastin (**B**) and aorta (**C**) hydrolysates. The amounts of derivatives loaded onto the column were 315 pmole of Des and Ide in (**A**), 30 μg of elastin in (**B**), 290 μg of dry aorta in (**C**). Internal standard (*IS*) was 300 pmole in each injection

Fig. 3 shows chromatograms of Des and Ide standards, elastin hydrolysate and aorta hydrolysate, obtained under the above conditions. Nearly the same results were obtained using three different columns.

Efficiency of the measurement of Des and Ide

The recoveries of added standards of Des and Ide (2.5 nmol) to elastin hydrolysates were found to be 96.3 $\pm$ 11.0% and 98.8 $\pm$ 6.6% (means $\pm$ S.D.), respectively. The overall reproducibilities of the complete analysis, including CF1 prefractionation, were 5.9% and 5.0% (coefficients of variation). At a signal-to-noise ratio of 3, the detection limits of Des and Ide were 2.2 pmol and 2.5 pmol, respectively.

Discussion

Dns derivatives of acidic aminoacids were separated by a reverse-phase-ion-exchange HPLC, on a 3-aminopropyl column; derivatives of basic and neutral aminoacids were first eluted in the void volume before selective elution of polyacid Dns derivatives. Under the conditions described above, Des, Ide and the internal standard γ-Glu-Glu were well separated. No peak eluting with the same retention times as Des, Ide or γ-Glu-Glu, were found in hydrolysates of BSA and gelatin, free of crosslink aminoacids.

The very low levels of Des and Ide compared to other aminoacids, especially in tissue hydrolysates, justify a prefractionation step to remove most of non-

crosslink aminoacids. The CF1 procedure was found to be appropriate by removing about 95% of non-crosslink aminoacids. In addition, the low amino-acid content in CF1 eluates strongly reduced the DnsCl required for dansylation procedure.

In our conditions, dansylation of Des and Ide proved to be faster than that of most other aminoacids. Hence, the relatively small excess of DnsCl, the short time of reaction, and, as previously reported (Tapuhi, 1981), the use of ethylamine to stop the reaction, minimized the arising of undesirable side-products such as DnsOH and $DnsNH_2$, thereby improving the reproducibility of the protocol. Furthermore, the quenching by ethylamine preserved the amino-groups of the bounded phase from reacting with DnsCl, extending the life time of the column. In this settings, it was also found useful to wash the column every 15 runs with a solution of acetic acid in methanol:water (85:15), final pH 3.5, which avoids a shortening in retention times of the two compounds and an associate decrease in their resolution.

Moreover, extraction with ethylacetate removed DnsOH excess, some Dns-aminoacids such as Aspartate, arginine, lysine, glutamate, and, partially, Dns-NH_2 (Seiler, 1970). In addition with CF1 purification, this extraction step also avoided the overloading of the column and improved the aspect of the chromatogram. The uptake of the residue in a small volume of mobile phase, down to 80 μl for a convinient 50 μl injection, allowed the injection of a large part of the Des and Ide present in the sample, increasing the overall sensibility of the method. Thus, less than 5 pmol of Des or Ide could be detected in the hydrolysate. Stability trials showed that after evaporation of ethylacetate, the dry residue can be stored at 4°C during at least 6 months, without affecting neither chromatographic resolution, nor baseline stability.

γ-Glu-Glu proved to be suitable as an internal standard for dansylation, extraction and chromatography. As it did not elute with Des and Ide during CF1 procedure, it was added only after separation. However, with the optimized conditions described above, the use of the internal standard did not significantly improve the reproducibility, so it may be omitted.

Its accuracy, sensitivity and reproducibility, allow us to consider this procedure as a mean of precise evaluation of the crosslinking aminoacids of elastin in whole tissue. Extension of this method will be relevant to vascular disorders, especially to atherosclerosis. Moreover, we suggest this method could be used, with slight modifications if required, for the determination of collagen crosslink aminoacids, pyridinoline and deoxypyridinoline.

References

Bayer E, Grom E, Kaltenegger B, Uhmann R (1976) Separation of amino acids by high performance liquid chromatography. Anal Chem 48: 1106–1109

Faris B, Ferrera R, Glembourtt P, Mogayzel PJ, Crombie G, Franzblau C (1981) Rapid quantitation of desmosine content in tissue hydrolysates by high-performance liquid chromatography. Anal Biochem 114: 71–74

Gray WR (1972) End group analysis using Dansyl chloride. Methods Enzymol. 25: 121–138

Gunja-Smith Z (1985) An enzyme-linked immunosorbent assay to quantitate the elastin crosslink desmosine in tissue and urine samples. Anal Biochem 147: 258–264

King GS, Mohan VS, Starcher BC (1980) Radioimmunoassay for desmosine. Conn Tissue Res 7: 263–267

Lansing AI, Rosenthal TB, Alex M, Dempsey EW (1952) The structure and chemical characterization of elastic fibers as revealed by elastase and electron microscopy. Anat Rec 144: 555

Lunte SM, Mohabbat T, Wong OS, Kuwana T (1989) Determination of desmosine, isodesmosine, and other amino acids by liquid chromatography with electrochemical detection following precolumn derivatization with naphtalenedialdehyde/cyanide. Anal Biochem 178: 202–207

Negro A, Garbisa S, Gotte L, Spina M (1987) The use of reverse-phase high performance liquid chromatography and precolumn derivatization with Dansyl chlorid for quantitation of specific aminoacids in collagen and elastin. Anal Biochem 160: 39–46

Sandberg LW, Soskel NT, Leslie JG (1981) Elastin structure, biosynthesis, and relation to disease states. N Engl J Med 304: 566–579

Schwartz E, Cruickshank FA, Lebwohl M (1990) Determination of desmosine in elastin-related skin disorders by isocratic high-performance liquid chromatography. Exp Mol Pathol 52: 63–68

Seiler N (1970) Use of the dansyl reaction in biochemical analysis. Methods Biochem Anal 18: 259–337

Skinner SJM (1982) Rapid method for the purification of the elastin crosslinks, desmosine and isodesmosine. J Chromatogr 229: 200–204

Soskel NT (1986) High performance liquid chromatographic quantitation of desmosine plus isodesmosine in elastin and whole tissue hydrolysates. Anal Biochem 160: 98–104

Tapuhi Y, Schmidt DE, Lindner W, Karger BL (1981) Dansylation of amino acids for high-performance liquid chromatography analysis. Anal Biochem 115: 123–129

Tapuhi Y, Miller N, Karger BL (1980) Practical considerations in the chiral separation of Dns-amino acids by reversed-phase liquid chromatography using metal chelate additives. J Chromatogr 205: 325–337

Yamaguchi Y, Haginata J, Kunitomo M, Yasuda H, Bando Y (1987) High-performance liquid chromatographic determination of desmosine and isodesmosine in tissues and its application to studies of alteration of elastin induced by atherosclerosis. J Chromatogr 422: 53–59

Zarkadas CG, Zarkadas GC, Karatzas CN, Khalili AD, Nguyen Q (1986) Rapid method for determinating desmosine, isodesmosine, 5-hydroxylysine, tryptophan, lysinoalanine and the amino sugars in proteins and tissues. J Chromatogr 378: 67–76

Authors' address: Dr. P. Charpiot, Laboratory of Biological Chemistry, School of Pharmacy, 27 Bd. J. Moulin, F-13385 Marseille Cedex 5, France.

Received November 5, 1992