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Development and validation of a high-performance liquid chromatographic method for the determination of desmosines in tissues

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SUMMARY

The development and the validation of a general strategy for the simple and accurate analysis of desmosines (isodesmosine and desmosine) in tissues coupled with the determination of collagen (as hydroxyproline) is described. The method is based on simplified sample (*i.e.*, lung) pretreatment which involves, in a PTFE screw-capped Pyrex tube, homogenization, collagen estraction with hot 5% trichloroacetic acid and hydrolysis of the elastin-containing residue with 6 *M* hydrochloric acid, followed by cellulose minicolumn purification of desmosines from the hydrolysates, dansyl chloride pre-column derivatization of the purified desmosines and reversed-phase high-performance liquid chromatographic (HPLC) analysis of the dansyl derivatives using a Spherisorb ODS-2 column, an on-column enrichment sample device and a linear gradient of organic modifier (acetonitrile) in phosphate buffer. The simple sample pretreatment, the optimized chromatographic conditions and the short HPLC analysis time (less than 15 min) allow the accurate and rapid determination of desmosine and isodesmosine, thus permitting the determination of elastin in several kinds of tissues with a minimum of sample manipulation.

INTRODUCTION

Structural and metabolic studies on elastin, a protein present in most types of connective tissues, need a suitable method for the determination of desmosine and isodesmosine (DID), the major cross-linking amino acids of elastin. The accurate determination of these cross-linking markers allows the determination of the elastin content in tissues, obviating laborious extraction procedures.

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On the other hand, several problems regarding DID determination make their analysis difficult, as indicated by the high number of methods developed. Conventional ion-exchange chromatography[1–3], thin-layer chromatography[4,5] and immunoassays[6–9] have been employed to determine DID; more recently, high-performance liquid chromatography (HPLC) with[10,11] and without[12–15] precolumn derivatization has been used for the determination of these cross-linkers in pure elastin[10,11] or in tissue hydrolysates[12–14]. Immuno- and conventional ion-exchange chromatographic assays, and also HPLC without precolumn derivatization, often require a multi-step sample manipulation prior to analysis, or they lack specificity and sensitivity or involve time-consuming chromatographic analysis. More success has been achieved with reversed-phase chromatography by using precolumn derivatization with dansyl chloride (DNS-Cl)[10] or naphthalenedialdehyde–cyanide[11]; however, the method was limited to the determination of desmosines in a pure elastin sample, not in tissues.

In this paper, we report a general strategy for the determination of DID in tissues. The method is based on a DNS-Cl precolumn derivatization of the cross-linkers purified by cellulose minicolumns from tissue hydrolysates and a rapid separation and determination of the derivatives by reversed-phase HPLC with a precolumn sample enrichment device. Owing to the concentration and purification pretreatment of the samples and the precolumn enrichment chromatographic system, the method allows the determination of DID in the picomole range, thus permitting the determination of elastin in tissue samples of low elastin content, such as foetal lungs.

EXPERIMENTAL

Chemicals

Crystalline DNS-Cl and a standard mixture of free amino acids were purchased from Sigma (St. Louis, MO, U.S.A.). Pure desmosine, isodesmosine and elastin from bovine ligamentum nuchae were supplied by Elastin Products (Pacific, MO, U.S.A.). Potassium dihydrogenphosphate, hydrochloric acid, acetic acid, potassium hydroxide, sodium hydrogencarbonate and *n*-butanol were analytical-reagent grade products from Merck (Darmstadt, F.R.G.). Cellulose powder (Watman CF-1) was obtained from W. & R. Balston (Maidstone, U.K.). HPLC-grade acetonitrile, acetone and water were obtained from Inalco (Milan, Italy).

Standards

Stock solutions of desmosine and isodesmosine were prepared in 0.01 *M* hydrochloric acid. The concentration of DID solutions was adjusted to 2 μmol/ml by spectrophotometric determination using $\varepsilon = 4900$ at 268 nm for desmosine and $\varepsilon = 7850$ at 278 nm for isodesmosine[16]. Aliquots (0.1 ml) of cross-linker solutions and of standard mixture of amino acids (2.5 μmol/ml) were diluted to 2 ml with 6 *M* hydrochloric acid and processed according to the cellulose minicolumn method[17]. The aqueous eluate from the cellulose column was lyophilized and the residue dissolved in 2 ml of 0.01 *M* hydrochloric acid. This solution was used as a working standard solution for the chromatographic calibration.

Tissue samples

Aorta and liver samples from a 120-g rat were removed, rinsed in 0.9% sodium chloride solution, stripped of adhering tissue, weighed, cut into small pieces, poured into a PTFE screw-capped Pyrex tube, frozen, lyophilized and then dried at 40°C under vacuum and weighed again. Rabbit foetuses of 30-days gestational age were used as a source of foetal lungs; the organs were removed from the thorax and treated as the aorta sample.

Dried tissue samples (usually 50–100 mg) were homogenized in water (1:5, w/v) with a Polytron PT 10-35 homogenizer using a PT 10/TS probe (Brinkman Instruments, Westbury, NY, U.S.A.). The homogenate was mixed with an equal volume of 10% (w/v) cold trichloroacetic acid (TCA) solution and centrifuged at 9000 *g* at 4°C for 10 min. The supernatant was discarded and the residue was treated with 5 ml of 5% TCA at 90°C for 30 min in order to extract collagen[18]. After centrifugation, the TCA extract can be saved and used for the determination of collagen (as hydroxyproline) according to the procedure described previously[19]. The TCA-extracted residue was washed with acetone by centrifugation and dried at 60°C under vacuum. The dried material was suspended in 2 ml of 6 *M* hydrochloric acid; after flushing with nitrogen, the tube was sealed and the sample was hydrolysed for 48 h at 120°C. Isodesmosine and desmosine were isolated in almost pure form from the hydrolysate sample using a cellulose minicolumn[16]. Cellulose eluate was lyophilized and the residue dissolved in 0.01 *M* hydrochloric acid. Precolumn derivatization of desmosines was carried out according to a slight modification of Gray's method[20]. Typically, 100 μl of 0.5 *M* sodium hydrogencarbonate in HPLC-grade water and 100 μl of 20 m*M* DNS-Cl in acetone were added to 100 μl of the sample or of the working standard solution in a screw-capped glass tube. After 40 min at 65°C in the dark, the derivatization mixture was diluted to 1 ml with the mobile phase. Sodium hydrogencarbonate and DNS-Cl solutions were always freshly prepared. Volumes of 25–250 μl of derivatization mixture were injected into the chromatograph.

Elastine sample

A 2-mg amount of elastin was dissolved in 2 ml of 6 *M* hydrochloric acid in a Pyrex tube. After flushing with nitrogen, the tube was flame-sealed and the sample hydrolysed at 120°C for 48 h. The hydrolysed sample was processed and derivatized as described above for the tissue samples.

High-performance liquid chromatography

Chromatographic separations were performed using an HPLC apparatus including two Model 2150 pumps and a Model 2152 controller (LKB, Bromma, Sweden), a Model 440 absorbance detector equipped with a 254-nm interference filter (Waters Assoc., Milford, MA, U.S.A.), a Model 910 injection valve (Negretti & Zambra, Southampton, U.K.), the loop being replaced with a Guard-Pak C_{18} module (Waters Assoc.), used as a pre-column and as an on-column enrichment sample device. A Spherisorb ODS-2, 5-μm particle size, column (150 × 4 mm I.D.) was used. Chromatographic profiles and peak areas were determined by using a Model 3390 A integrator (Hewlett-Packard Italiana, Rome, Italy).

Chromatographic analysis was performed with binary gradient elution as described below. Mobile phase A was a mixture of 25 m*M* potassium dihydrogen-

phosphate–25 m*M* acetic acid and acetonitrile (85:15, v/v); mobile phase B was the same mixture but in the proportions 40:60 (v/v); the mobile phases were degassed under reduced pressure and the pH adjusted to 7.2 with 2 *M* potassium hydroxide solution. The column was first equilibrated with mobile phase A at room temperature and a flow-rate of 1.2 ml/min for 8 min. At the beginning of the analysis, with the injection valve in the load position, 25–250 μl of the sample were injected into the Guard-Pak C_{18} cartridge through the needle port by using a Model 750 500-μl Hamilton syringe; then the C_{18} cartridge was washed with 1 ml of mobile phase A by means of a syringe and finally the valve handle was rotated to the inject position to bring mobile phase A from the pump to the opposite end of the Guard-Pak module and to determine the sample to be flushed into analytical column. Elution was carried out with a linear gradient from 25% to 45% B in 20 min. At the end of the chromatography, the column was washed with mobile phase B for 5 min and then conditioned with mobile phase A for 8 min.

Quantification was performed by external calibration. The concentration of the analyte was determined by comparison of the peak area with that of a known standard. A calibration graph was obtained by applying aliquots of working standard solutions containing scalar amounts of DID from 30 to 1500 pmol to the column. Recovery experiments were carried out by adding known amounts of DID to dried aliquots of foetal rabbit lung and processing the samples through the acid hydrolysis and cellulose minicolumn steps. To evaluate the precision of the method, within-run and between-run coefficients of variation (C.V.) were calculated according to Godel *et al.*[21].

RESULTS AND DISCUSSION

Tissue sample treatment

Elastin, together with collagen, is the main component of connective tissue, which ensures the functional integrity of many anatomical systems. The indirect determination of this protein may be performed by the determination of specific amino acids, which are markers for elastin. Thus the determination of desmosines in tissue hydrolysates provides a measure of the elastin content. However, owing to the low concentration of desmosines in tissue samples, a reliable determination of these markers cannot be performed directly in acid hydrolysates of tissue samples by HPLC. Cellulose minicolumn treatment can be used for a simple purification and concentration of DID from acid hydrolysates of samples containing elastin. The purified DID sample can be analysed by reversed-phase HPLC with precolumn derivatization, without serious interferences.

HPLC with pre-column derivatization

Several reagents have been proposed for the precolumn derivatization of amino acid molecules, e.g. *o*-phthaldialdehyde (OPA)[22], DNS-Cl[23], dimethylaminoazobenzenesulphonyl chloride (DABS-Cl)[24], phenyl isothiocyanate (PITC)[25] and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl)[26]. Each of these reagents can be considered as a good candidate for the precolumn derivatization of the desmosines, owing to the presence of four amino groups in their molecules. Nevertheless, only DNS-Cl holds promise of being a suitable reagent for DID derivatization; OPA and PITC, as reported previously[10], DABS-Cl and NBD-Cl, as checked by us (unpublished results), were found to be inadequate.

The HPLC method here presented is dedicated to the determination of DID, purified from tissue hydrolysates, following a simplified gradient programme. Fig. 1 shows the appearance of the DNS derivative of standard DID on the HPLC trace. The profile relates to the injection of 90 pmol of derivatized DID in 100 μl of the reaction mixture. Isodesmosine and desmosine are not separated following this HPLC procedure; as the molar extinction of the derivatives of desmosine and isodesmosine are equivalent[10], the lack of separation of DID is not a drawback with respect to the total determination of the linkers for elastin determination. As can be seen from Fig. 1, by using the column-switching device, the peak at the beginning of the chromatogram, which corresponds to excess of reagent and to the main side-products of the reaction, is small, considering the high sensitivity of the detector and the large volume of sample injected. In addition to the peak of the analyte of interest, other small peaks are detectable in the chromatographic profile, with lower retention times; the major small peak is DNS-NH_2 but the nature of the remaining components is unknown. Analysis is complete in less than 15 min with the solvent gradient chosen. Attempts to perform HPLC under isocratic conditions gave poor results, particularly for biological samples.

Calibration graphs for 30–1500 pmol of DNS-DID in the column showed good linearity of peak-area response *vs.* amount of analyte injected, with a correlation coefficient of 0.998. The limit of detection of DID was 3 pmol, which gave a signal-to-noise ratio of 3 at attenuation $\times 1$.

Fig. 2 shows typical chromatograms for DID in (a) elastin hydrolysate, (b) rat aorta, (c) foetal rabbit lung and (d) from adult rat liver. The DID peak shows almost complete separation from interfering peaks present in purified samples. Chromatogram (a) corresponds to the analysis of 10 μg of pure elastin and the other chromatograms to 1–2 mg of the processed dried sample.

Recovery experiments gave satisfactory results. With standard additions of DID to dried samples, the mean recovery was 87 $\pm$ 3% (S.D., n = 11). Recovery experiments were also performed to demonstrate the linearity between the amount of DID measured and the amount of tissue analysed. Analysis of a larger number (n = 11) of samples from a pool of dried foetal rabbit lungs demonstrates that the DID level found is linearly proportional (r = 0.997) to the amount of tissue between 50 and 250

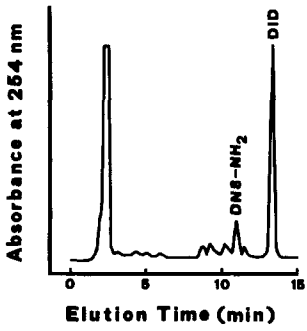

Fig. 1. HPLC of standard DID derivatized according to the procedure described under Experimental. The analyte peak correspond to an injected amount of 90 pmol of DID in 0.1 ml of derivatization mixture. Integrator attenuation $\times 3$; other conditions are described in the text.

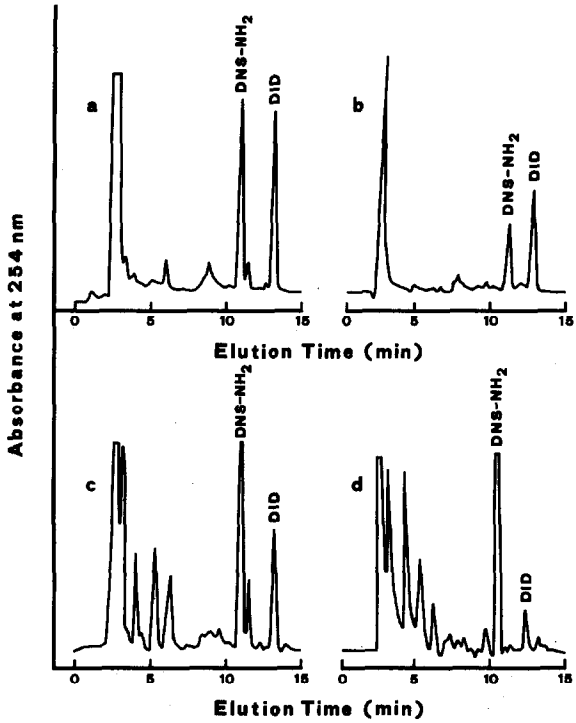

Fig. 2. Representative chromatograms of derivatized samples from (a) elastin, (b) rat aorta, (c) foetal rabbit lung and (d) rat liver. Concentrations of DID: (a) 35 pmol/mg of elastin, (b) 9 nmol/mg of dried sample tissue, (c) 81 pmol/mg of dried sample tissue and (d) 15 pmol/mg of dried sample tissue. Integrator attenuation ×3; other conditions are described in the text.

mg. As little as 50 mg of dried lung sample is sufficient to give routinely reliable results. The within-run reproducibility (C.V.) of the procedure, performed by repeated injections ($n = 11$) of the same sample with a 50 pmol injected amount of DID, was 2.8%. The between-run precision (C.V.), obtained from analyses of aliquots of the same sample carried out on five subsequent days, was 6.2%. In addition, when separate aliquots ($n = 7$) of a pool of dried foetal rabbit lungs were processed and analysed by HPLC, the procedure showed a C.V. of 5.2%.

The procedure described here has been used to assay DID in pure elastin and in foetal rabbit lungs. To measure the DID content in elastin, five separate samples of protein were hydrolysed and processed according to the present procedure. A mean value of 37 ± 1.1 nmol/mg was found, which is comparable to those reported elsewhere[10,11]. Determinations of DID in five samples of foetal lungs (30 days of gestation) gave a mean cross-linker value of 8.5 ± 1.5 nmol per 100 mg of dried sample.

The isolation of desmosines from tissue samples and their determination are technically difficult. Although several efficient HPLC systems are available for the assay of DID in pure elastin, many problems arise in the determination of these cross-linkers in tussue samples. The most important step in the present procedure is the effective purification and concentration of the compound of interest, which permits the HPLC determination of DID to be performed.

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