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Short Communication

High-performance liquid chromatographic determination of desmosine and isodesmosine after phenylisothiocyanate derivatization

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ABSTRACT

A new sensitive and selective high-performance liquid chromatographic method for the analysis of desmosine and isodesmosine in human and rat tissues is described. This method requires a purification step with column chromatography, followed by precolumn derivatization with phenylisothiocyanate. The reaction products are then separated by isocratic chromatography on a C_{18} column and quantitated by ultraviolet detection at 254 nm. The recovery of standards of both compounds added to tissue samples and analysed by this method is usually greater than 90%, and the absolute detection limit is 0.5 ng for both compounds. The method is sensitive enough to measure both substances in tissue fragments of 30 mg of wet mass, which means that it can be used to study elastin in small human biopsies.

INTRODUCTION

The study of elastin, a fibrous protein of the connective tissue, should be very useful in helping to understand the biochemical basis of several pathological conditions, including emphysema, aneurysms and atherosclerosis, in which the mechano-elastic properties of the tissues are impaired. Since desmosine (DES) and isodesmosine (IDE) are characteristic cross-linking amino acids of elastin, and are not found in any other protein in living organisms [1], their levels in tissues can be used as a measure of the elastin content.

Several methods are available to measure DES and IDE in tissues, including thin-layer chromatography [2], radioimmunoassay [3] and high-performance liquid chromatography (HPLC) [4]. HPLC is the most suitable for simultaneous determination of DES and IDE in a biological sample, but the only HPLC method available up to now [4] lacks the sensitivity to measure DES and IDE in human biopsies. We therefore decided to develop a new HPLC method with the necessary sensitivity and specificity to quantitate DES and IDE in small fragments of human and animal tissue.

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The method described is based on precolumn derivatization with phenylisothiocyanate (PITC) of the tissue hydrolysates after their purification by column chromatography [5]. The PITC derivatives of DES and IDE are subsequently measured by HPLC using a C_{18} column and UV detector at 254 nm, employing tetrabutylammonium (TBA) as ion-pairing reagent. Application of the method to the analysis of DES and IDE in human and animal tissues is described.

EXPERIMENTAL

Chemicals

Bovine ligamentum nuchae elastin was obtained from Sigma (St. Louis, MO, USA), DES and IDE standards were from EPC (Pacific, MO, USA), HPLC-grade ethanol, acetonitrile and methanol were from Farmitalia Carlo Erba (Milan, Italy), TBA phosphate was from Aldrich (Steinheim, Germany), PITC and triethanolamine, sequenal grade (used after redistillation over ninhydrin), were from Pierce (Rockford, IL, USA).

Chromatography

Analyses were done on a System Gold liquid chromatograph (Beckman, Fullerton, CA, USA) equipped with a variable-wavelength detector, and peaks were recorded and calculated by an integrator (Shimadzu, Kyoto, Japan). The column was a Nucleosil 120-5 C_{18}, 5 μm, 250 mm × 4 mm I.D. (Macherey-Nagel, Duren, Germany), held at 32°C, and used in combination with a LiChrocart guard column (4 mm × 4 mm I.D., Merck, Darmstadt, Germany).

The eluent (flow-rate 1 ml/min) was 10 m*M* TBA in 80 m*M* phosphate buffer (pH 6.5)–methanol–acetonitrile (50:16:34). The TBA phosphate solution was used after filtration through a 0.45-μm cellulose filter (Millipore, Bedford, MA, USA) and the eluent was degassed in an ultrasonic water-bath before use and subsequently kept under helium throughout the analysis. DES and IDE were detected at 254 nm.

Sample preparation

Male CD-Cobs rats (Charles River, Calco, Italy), 300 ± 50 g body mass, were decapitated under ether anaesthesia. The lungs and the abdominal aorta were immediately removed, minced, and lyophilized, and 20-mg portions were hydrolysed in 6 *M* hydrochloric acid for 36 h in sealed glass vials. The hydrolysates were subsequently fractioned on a cellulose mini-column, as described by Skinner [5], and the eluted fractions, after evaporation of the solvent under vacuum, were redissolved in 100 μl of triethanolamine–water–ethanol (1:2:2) and dried at 40°C by a mechanical vacuum pump (15 mmHg). This last step was repeated twice to neutralize the excess hydrochloric acid present after elution through the cellulose columns. The residues were dissolved in 20 μl of triethanolamine–water–ethanol (1:1:8) with 5μl of PITC added, before incubation of the solutions for 30 min at

40°C in sealed glass tubes. The derivatized samples were then dried at 40°C by a mechanical vacuum pump (15 mmHg) and dissolved in 500 μl of the eluent before injection into the HPLC column (20-μl aliquots).

Human veins (long saphenus) surgically removed from patients with varices were immediately frozen and subsequently processed as described for rat tissues.

RESULTS AND DISCUSSION

After derivatization with PITC, DES and IDE still contain four free carboxylic groups (Fig. 1) which, by interacting with the TBA molecules, seem essential for resolution by the HPLC system employed. The molarity of the phosphate buffer is also important for the complete resolution of DES from IDE, and a failure to separate them, which sometimes happens with columns of different batches, can be overcome by slight modifications of this parameter. Complete analysis of DES and IDE in tissues under the conditions described takes 10 min.

A linear correlation was obtained between the peak area and the injected concentrations of DES and IDE in the range 5–500 ng (coefficient of correlation $r = 0.99$ for both compounds), the absolute detection limit being 0.5 ng (12.5 ng/g wet tissue). The means (± S.D.) of ten determinations of standard samples containing 1 μg of DES and 1 μg of IDE were 0.98 ± 0.04 and 1.02 ± 0.06 μg, respectively.

Fig. 2 shows the elution profiles of a standard solution of elastin (1 μg/ml) and of a sample of rat aorta processed as above. Chromatograms obtained from samples of rat lung and human veins were similar to those from rat aorta and are therefore not shown. The recoveries of DES and IDE standards, added to tissue samples (rat lung) and analysed as described, were 93.4 ± 1.9 and 92.1 ± 1.3% (mean ± S.D. of five samples), respectively.

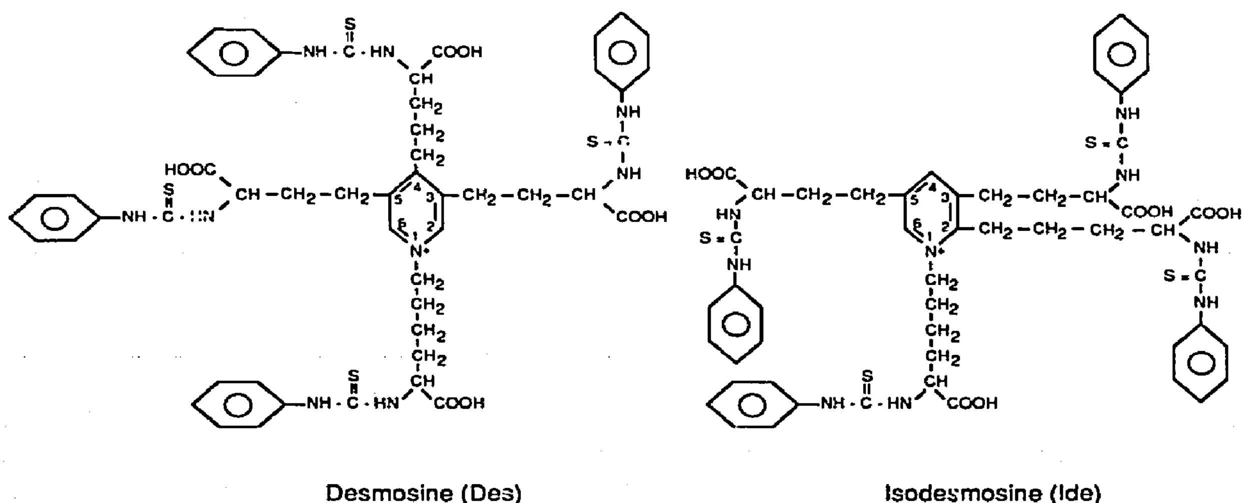

Fig. 1. Structures of DES and IDE after derivatization with PITC.

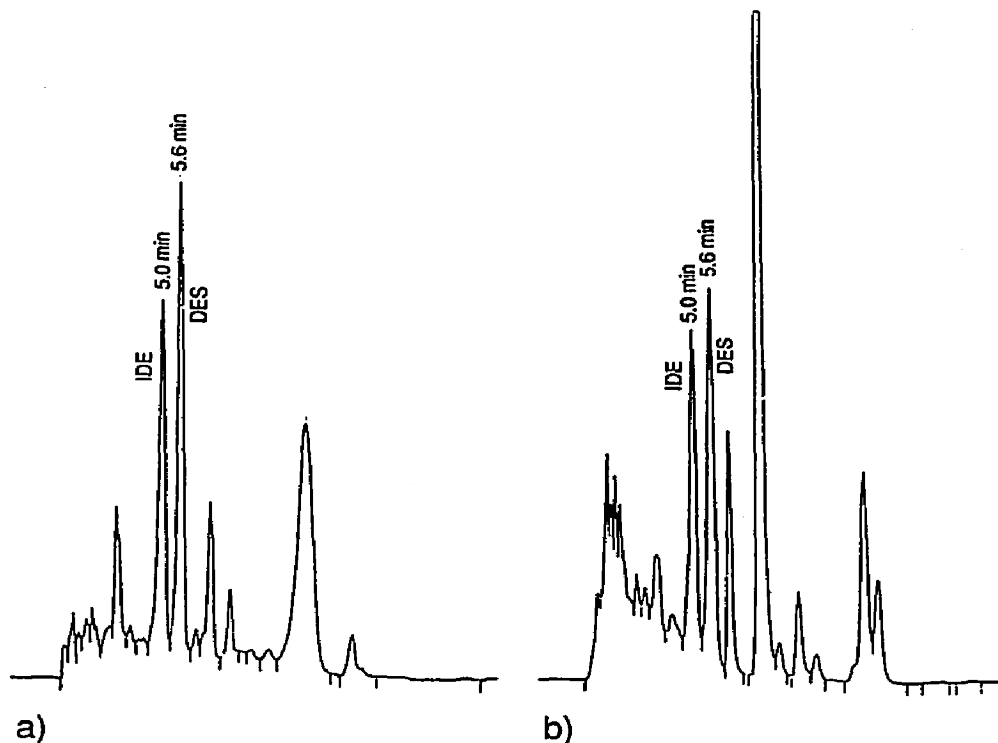

Fig. 2. Chromatograms of (a) an elastin standard (1 μg/ml) and (b) a rat aorta sample.

Table I shows DES and IDE levels in rat lungs and aorta and in human veins. DES and IDE levels in tissues were comparable with those reported in the literature by Yamaguchi *et al.* [4], obtained by another HPLC method. The advantage of our method over that of Yamaguchi *et al.* [4] is that, being ten times more sensitive, it enables us to measure DES and IDE in tissue fragments as small as 30 mg (wet weight); it can therefore be used for human biopsies.

Our limit of detection is comparable with that reported by Harel *et al.* [3] with RIA and by Gunja-Smith [6] with an enzyme-linked immunosorbent assay. However, these authors measured only DES and only in urine. Their method does not measure IDE and they did not report on DES in tissues. Moreover HPLC, espe-

TABLE I

DESMOSINE AND ISODESMOSINE LEVELS IN RAT AND HUMAN TISSUES

Values are mean ± S.D.; n = 5.

Tissue analysed	DES (mg/g dry mass)	IDE (mg/g dry mass)
Rat aorta	0.95 ± 0.07	0.89 ± 0.06
Rat lung	0.08 ± 0.01	0.10 ± 0.02
Human vein	0.71 ± 0.25	0.48 ± 0.10

cially in comparison with RIA, has the advantage of being simple and less expensive and therefore lends itself to better DES and IDE measurements in screening programmes.

In conclusion, the HPLC method described is sensitive enough to measure DES and IDE in human and rat lung, aorta and vein fragments as small as 30 mg, hence in small human biopsies. It can be used to study elastin in these tissues *ex vivo*, and could therefore provide new insights into pathologies such as emphysema, varices and atherosclerosis, involving changes in elastin properties and/or elastin turnover rates.

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