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Short communication

Measurement of desmosine and isodesmosine by capillary zone electrophoresis

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Abstract

Desmosine (DES) and isodesmosine (IDE) were separated and quantitated by a simple and sensitive capillary zone electrophoretic (CZE) method, using hydrostatic injection and direct UV detection at 254 or 185 nm. Two different electrophoretic mobilities for the two isoforms were observed in 90 m*M* phosphoric acid pH 2.2. The presence of a mixture of amino acids in the sample did not affect the separation of DES and IDE. The method was successfully used to quantitate the amounts of DES and IDE in elastin hydrolysates.

1. Introduction

Desmosine (DES) and isodesmosine (IDE) are cross-linking amino acids of elastin, a fibrous protein of vertebrate connective tissue; their determination is used as an indicator of the presence or alteration of elastin, and also to measure elastase activity [1].

A radioimmunoassay method [2] has been used to detect DES and IDE in urine samples, whilst high-performance liquid chromatography has been applied to tissue hydrolysates [3,4], or purified elastin hydrolysates [5]. This latter technique usually requires preliminary extraction of the two amino acids on a cellulose mini-column [3,4] (according to the method described by Skinner [6]), and in some cases an additional precolumn derivatization [4,5,7] in order to increase the sensitivity of the detection.

Current HPLC techniques meet most of the demands of amino acid analysis but the determination of minute amounts, when, for example, sample size is limited, requires the use of capillary zone electrophoretic (CZE) techniques. Femtomole-scale amino acid analysis by CZE generally requires laser-induced fluorescence detection, implying a derivatization reaction. In this work CZE was applied to the separation and quantitation of DES and IDE. As both DES and IDE have a pyridinium ring in their structure, direct UV detection was employed, without any

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prior chemical modification of the molecules. Standard filters of 185 or 254 nm were used.

2. Experimental

2.1. Chemicals

Bovine ligamentum nuchae elastin, 2,4-dinitrofluorobenzene and borax were obtained from Sigma (St. Louis, MO, USA), DES and IDE from ICN Biomedicals (Costa Mesa, CA, USA), phosphoric acid and hydrochloric acid (analytical) were from Prolabo (Paris, France).

2.2. Apparatus

Electrophoresis was carried out on a Quanta 4000 capillary electrophoresis system (Waters, Milford, MA, USA) using a 60 cm (53 cm to the detector) × 75 μm I.D. fused-silica capillary (Supelco, Gland, Switzerland). The system was equipped with a UV detector set at 185 or 254 nm with filters. Separation was performed by applying a positive voltage (10–20 kV) to the capillary. Hydrostatic introduction of the sample was used rather than electromigration, to avoid discrimination between faster and slower ions [8].

In order to obtain high resolution and avoid peak distortion, the injection/capillary volume ratio was 1:100. With an injection time of 30 s, the injection volume was ca. 30 nl (capillary volume: 3 μl).

The capillary was pretreated for 10 min with 0.5 *M* potassium hydroxide and 10 min with deionized water before analysis. The capillary was washed with degassed, filtered (0.45 μm) buffer until equilibrium was reached.

2.3. Sample

Elastin powder was suspended in 6 *M* hydrochloric acid and hydrolysed for 24 h, in sealed tubes, under vacuum, at 105°C. The hydrolysate was dried under vacuum, dissolved in water, filtered on 0.45-μm pore size filters and frozen. Standards of DES and IDE were dissolved in water, filtered and frozen.

2.4. Titration of free–NH_2 groups

The total amino acid content of the hydrolysates was estimated by measuring the free–NH_2 groups using Sanger's reagent, as described by Ghyusen et al. [9].

3. Results and discussion

3.1. Separation of DES and IDE

The structure and size of these amino acids are close to those of an oligopeptide so the choice of the electrophoretic buffer was based on those used for oligopeptide separation. Diluted phosphoric acid used for the separation of peptides by CZE [10], was successfully employed for the separation of DES and IDE. Optimization of the separation was performed by first modifying the molarity of the phosphoric acid solution. A linear relationship was observed between the square root of the molarity and the migration time (slope ± S.D. = -0.147 ± 0.009; intercept ± S.D. = 12.2 ± 0.2; S.D.$y = 0.2$; $n = 12$; $r = 0.97$). Optimal separation of DES and IDE was obtained with 90 m*M* phosphoric acid. Secondly, the influence of pH was estimated by gradual addition of sodium hydroxide to the phosphoric acid solution over a pH range of 1.9 to 2.5. Complete separation was obtained at pH 2.2. As the migration time increased with pH the voltage applied was modified in order to reduce the migration time, without changing the resolution. An optimal value of 15 kV gave complete separation in 13 ± 1 min. A typical electrophoregram obtained with a 1:1 mixture of DES and IDE, is presented in Fig. 1.

All attempts to further separate the two peaks, by addition of agents to complex –NH_2 groups (i.e. trifluoroacetic acid) or by cationic complexation (i.e. Cu, Ca, Cs), were unsuccessful. This presumably reflects the fact that the primary chemical structures of the two amino acids are identical.

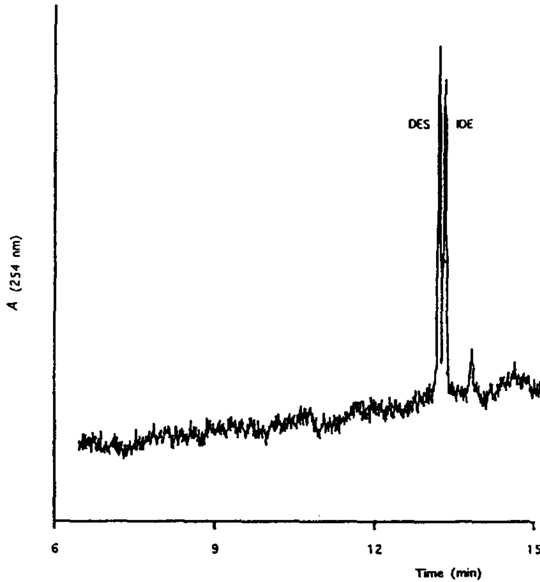

Fig. 1. Electrophoregram of a standard mixture of DES and IDE (1:1) with detection at 254 nm.

3.2. Quantitation of DES and IDE

A linear relationship between the DES and IDE peak areas and the amounts injected was obtained over the range 1–500 pmoles with detection at 185 or 254 nm (Table 1). These two wavelengths were used because they correspond to the standard filters of the Quanta 4000 apparatus. They are not specific for DES, the absorbtion of which shows maxima at 234 and 268 nm in 0.1 M hydrochloric acid [11]. Under the conditions used the minimal amount detected was 2 pmole at 254 nm, with a signal-to-noise ratio of 5, and 0.2 pmoles at 185 nm, with a signal-to-noise ratio of 4. These limits can be compared with that of 100 pmoles obtained with direct UV detection at 275 nm, using the HPLC technique reported by Lunte et al. [5].

Table 1
Linearity of the calibration curves of DES and IDE at 254 and 185 nm

	Slope ± S.D.	Intercept ± S.D.	S.D.y	n	r
DES 254 nm	38 ± 3	222 ± 114	177	8	0.96
IDE 254 nm	22 ± 2	96 ± 63	161	8	0.96
DES 185 nm	430 ± 19	1243 ± 680	1076	9	0.99
IDE 185 nm	654 ± 26	2102 ± 940	1489	9	0.99

3.3. Analysis of elastin

Elastin was hydrolysed and the resulting solution was injected into the capillary without any preliminary extraction or chemical modification. Using detection at 254 nm only two peaks of DES and IDE appear on the electrophoregram (Fig. 2). DES and IDE were identified by adding excess standards. Unidentified peaks are present in the electrophoregram using detection at 185 nm, however they do not interfere with the resolution of DES and IDE (Fig. 3).

Amounts of DES and IDE in elastin were 17 μg/mg and 3 μg/mg respectively. These values are in accordance with values obtained by Lunte et al. using HPLC [5]. The number of DES + IDE residues compared to the total of amino acid residues (estimated by Sanger's method) was 3 DES + IDE for 1000 amino acids of

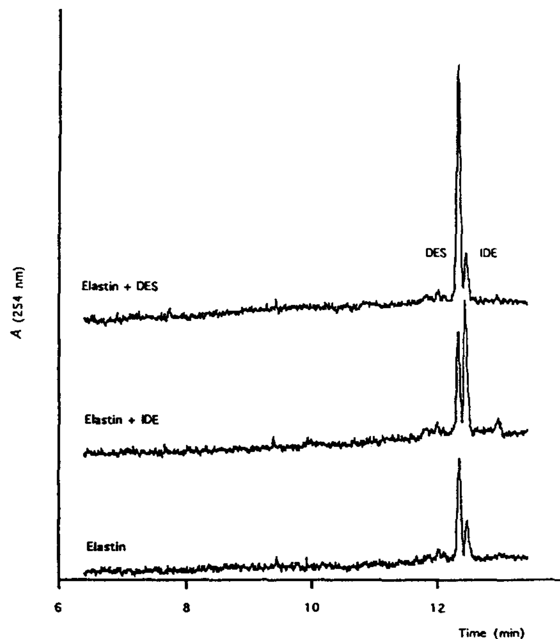

Fig. 2. Electrophoregram of elastin hydrolysate with detection at 254 nm. Identification of DES and IDE by addition of excess standards.

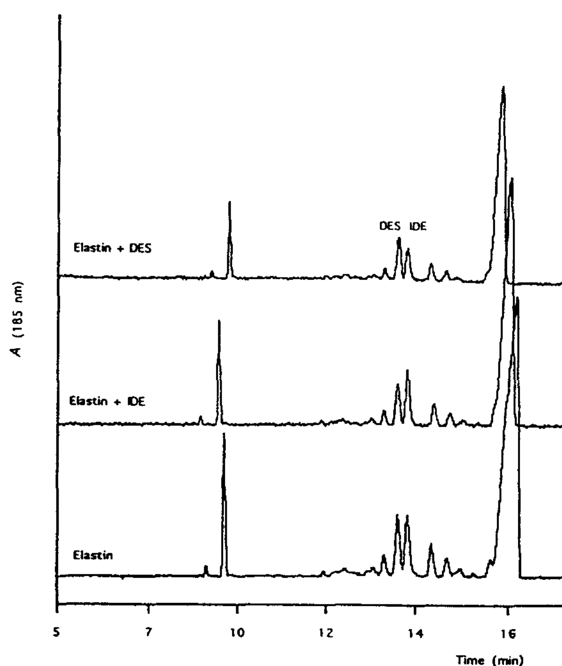

Fig. 3. Electrophoregram of elastin hydrolysate with detection at 185 nm. Identification of DES and IDE by addition of excess standards.

elastin. This is in agreement with published data [12].

4. Conclusion

A sensitive method for the separation and quantitation of DES and IDE by CZE was developed. This method allows the detection of DES and IDE without preliminary purification or chemical modification of the amino acids. We are now investigating the application of this method to the analysis of tissue hydrolysates.

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