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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DESMOSINE AND ISODESMOSINE IN TISSUES AND ITS APPLICATION TO STUDIES OF ALTERATION OF ELASTIN INDUCED BY ATHEROSCLEROSIS

YU YAMAGUCHI*, JUN HAGINAKA, MASARU KUNITOMO, HIROYUKI YASUDA and YOSHIO BANDÔ

Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 4-16 Edagawa-cho, Nishinomiya 663 (Japan)

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SUMMARY

A rapid, sensitive high-performance liquid chromatographic method has been developed for the determination of desmosine (DES) and isodesmosine (IDE), the specific cross-linking amino acids of elastin, in the tissue hydrolysates of rats. DES and IDE in the hydrolysate samples were separated on a C_{18} column using 0.1 *M* phosphate buffer–acetonitrile (2.8:1) containing 20 m*M* sodium dodecyl sulphate (final pH 4.5) followed by detection at 270 nm. The recoveries of the added standards of DES and IDE from the aorta hydrolysate samples were $99.6 \pm 2.7\%$ and $98.4 \pm 1.8\%$, respectively ($n = 10$). At DES and IDE concentrations of 2 μg/ml, within- and between-run precisions were 1.11–1.85% and 0.55–1.24%, respectively. The detection limits of DES and IDE were 0.1 μg/ml with a 50-μl injection at a signal-to-noise ratio of 3. The method was successfully applied to a study of the alteration of DES and IDE contents (i.e. elstin contents) in the tissues of rats treated with β-aminopropionitrile and an atherogenic diet. A negative correlation between the contents of these amino acids and of cholesterol was noted in the atherosclerotic aorta.

INTRODUCTION

Elastin is a fibrous protein present in connective tissues, including arterial walls, ligaments, lungs and skin, with elastic recoil properties. The degradation of elastic fibres, progressing during aging, is accelerated in several aging diseases such as atherosclerosis, emphysema, diabetes and several skin diseases [1–3]; on the other hand, they accumulate in the liver with cirrhosis [4]. Since desmosine (DES) and isodesmosine (IDE) are the specific cross-linking amino acids found only in elastin [5], their contents in tissues could reflect that of elastin. Thus, the determination of DES and IDE would be very useful for studying the role of the elastic fibre in the pathology of such diseases.

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The assay method so far employed for the determination of DES and IDE include electrophoresis [6,7], thin-layer chromatography [8], liquid chromatography [9–15], radioimmunoassay [16, 17] and enzyme immunoassay [18]. Above all, a high-performance liquid chromatographic (HPLC) method is the most suitable for the simultaneous determination of DES and IDE in biological samples. The HPLC methods so far developed (including the amino acid analyser procedure) showed poor or no separation of DES and IDE, and required a long run-time (above 40 min). Therefore, they are unsuitable for the determination of DES and IDE in a large number of biological samples (biological fluids or tissue hydrolysates). It is of vital importance to develop a rapid, sensitive HPLC assay method for DES and IDE for the biological study of the synthesis and degradation of elastin.

This paper describes an HPLC method for the determination of DES and IDE in the tissue hydrolysates of rats. DES and IDE were well separated by using sodium dodecyl sulphate (SDS) as an eluent on a C_{18} column and sensitively detected with native UV detection at 270 nm. The application of this method to studies of the alteration of elastin induced by atherosclerosis is also described.

EXPERIMENTAL

Reagents and materials

DES and IDE used as standards were purchased from Elastin Products (St. Louis, MO, U.S.A.). Acetonitrile, SDS and other chemicals of analytical-reagent grade were purchased from Nakarai Chemicals (Kyoto, Japan). Deionized, glass-distilled water was used for the preparation of buffer solutions and HPLC eluents. Phosphate buffer (0.1 *M*) solutions were prepared by using 0.1 *M* sodium dihydrogenphosphate dihydrate, 0.1 *M* disodium hydrogenphosphate dodecahydrate and 0.1 *M* phosphoric acid, adjusted to various pH.

Chromatography

A reversed-phase column (150×4.5 mm I.D.) packed with Nucleosil 5C-18 (5 μm) (Macherey-Nagel, Düren, F.R.G.) was used, together with a guard column (30×4.6 mm I.D.) packed with the same material. The HPLC system consisted of an 880-PU pump (Japan Spectroscopic, Tokyo, Japan), a Rheodyne 7125 loop injector (Cotati, CA, U.S.A.) and a UVIDEC 100-IV variable-wavelength detector (Japan Spectroscopic). The eluent was 0.1 *M* phosphate buffer–acetonitrile (2.8:1, v/v) containing 20 m*M* SDS (final pH 4.5), which had been degassed prior to use in an ultrasonic water-bath. The flow-rate was 0.8 ml/min. The optical density was monitored at 270 nm. All chromatographic operations were carried out at ambient temperature.

Animal experiments

Three-week-old (weaned) male Sprague–Dawley rats (Shizuoka Laboratory Animals, Shizuoka, Japan) were housed in an air-conditioned room (23±1°C; humidity 60±10%) and maintained on a purified basal diet or an atherogenic diet supplemented with or without 0.3% β-aminopropionitrile fumarate (BAPN).

The basal diet contained 20% casein, 63.2% sucrose, 10% corn oil, 2% agar, 0.8% vitamin mixture and 4% salt mixture. The atherogenic diet consisted of the basal diet with 1.5% cholesterol and 0.5% cholic acid in place of an equal amount of sucrose. The animals were given their respective diets by pair-feeding and allowed tap water ad libitum for a period of six weeks. Thirty-two animals were divided into four groups. Group I (control group) was fed the basal diet. Group II was fed the basal diet supplemented with 0.15% BAPN. Group III was fed the atherogenic diet. Group IV was fed the atherogenic diet supplemented with 0.15% BAPN.

Tissue samples

At the end of the feeding, the rats were killed under diethyl ether anaesthesia, and aortas, lungs and livers were excised. After lyophilization, the tissues were cut into small segments, and lipids were subsequently extracted with chloroform–methanol (2:1, v/v). Total cholesterol was enzymatically determined as described previously [19]. The delipidated tissues were transferred to hydrolysis tubes, mixed with 6 *M* hydrochloric acid, flushed with nitrogen, sealed and hydrolysed for 72 h at 105 °C. The hydrolysate samples were treated by the cellulose "mini-column" method reported by Skinner [20]. The solvent of the eluate was removed by evaporation, and the residue was dissolved in distilled water. After brief centrifugation to remove insoluble substances, a 50-μl portion of the solutions was loaded onto the column. Before centrifugation, the solution of the liver hydrolysate sample was further treated with a mixture of anion-exchange resin and charcoal to decolourize it. The loss of DES and IDE due to adsorption was less than 4%.

RESULTS AND DISCUSSION

Chromatographic conditions

Since DES and IDE have amino and carboxyl groups in their molecules, ion-exchange [12] and ion-pair reversed-phase [13,14] HPLC systems have been used to separate them. First, we tried to use a cation-exchange HPLC system: it took ca. 20 min to separate DES and IDE completely. Next, the use of a cationic ion-pair agent, such as tetrabutylammonium bromide, on a C_{18} column resulted in the elution of DES and IDE in the void volume; on the other hand, DES and IDE were well retained on the C_{18} column when an anionic ion-pair agent, such as an alkyl sulphonate (C_5–C_8) or alkyl sulphate (SDS) was used. The use of SDS gave the most satisfactory results for the separation of DES and IDE, and the various factors affecting this (concentrations of SDS and phosphate buffer, eluent pH and content of acetonitrile) were examined as described below.

The concentration of phosphate buffer was changed from 0.1 to 0.02 *M*. The lower buffer concentration resulted in a longer retention time and a tailing peak. Thus, the concentration of phosphate buffer was fixed at 0.1 *M*. Figs. 1 and 2 show the effects of SDS concentration and eluent pH, respectively, on capacity factors (k') of DES and IDE. The capacity factors increased when the concentration of SDS was increased and when the eluent pH was decreased (except for

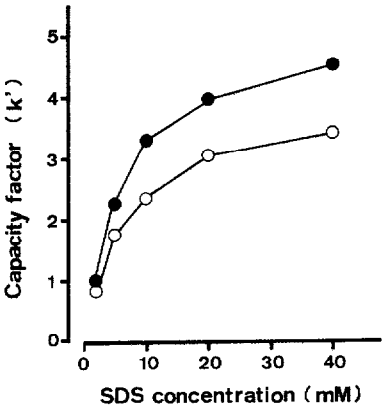

Fig. 1. Effect of concentration of SDS on capacity factors (k') of DES (●) and IDE (○). A 50-μl portion of each sample solution (5.0 μg/ml) was loaded onto the column. The eluent pH was 4.0. Other conditions as in Experimental.

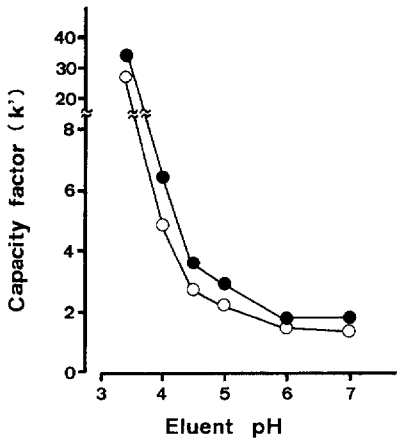

Fig. 2. Effect of eluent pH on capacity factors (k') of DES (●) and IDE (○). A 50-μl portion of each sample solution (5.0 μg/ml) was loaded onto the column. The SDS concentration was 20 m*M*. Other conditions as in Experimental.

eluent pH 7). Taking account of the separation of DES and IDE from the background components of the hydrolysates (especially from the peak between IDE and DES) and the run-time, the conditions described under Experimental were selected for the routine assay of DES and IDE, when the resolution of DES and IDE was 3.4.

Fig. 3 shows chromatograms of DES and IDE from the standard, aorta hydrolysate and lung hydrolysate samples. DES and IDE were separated from the background components of aorta and lung hydrolysates within 10 min. The liver hydrolysates gave a similar chromatogram, except for the elution near the void volume of unknown compounds with a strong UV absorption at 270 nm. The peak between IDE and DES was observed with the standards of DES and IDE as well as the tissue hydrolysates. The structural investigation of this peak component will be discussed elsewhere.

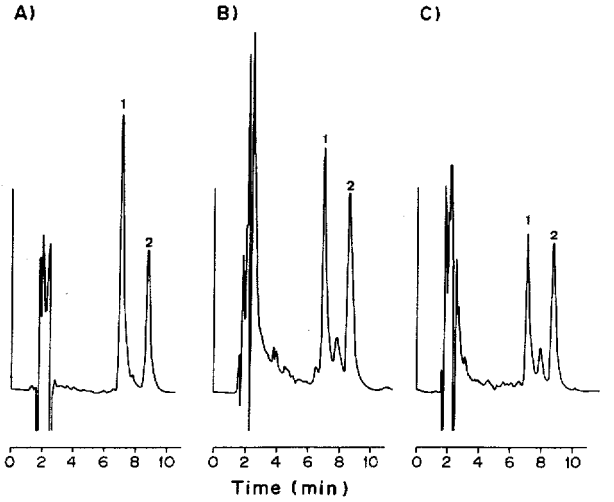

Fig. 3. Chromatograms of DES and IDE in the standard solution (A) and the aorta (B) and lung (C) hydrolysate samples. A 50-μl portion of each sample solution was loaded onto the column. Peaks 1 and 2 are IDE and DES, respectively. In (A), the concentrations of IDE and DES were both 5.0 μg/ml, in (B) 4.9 and 7.0 μg/ml and in (C) 2.8 and 5.3 μg/ml, respectively. Other conditions as in Experimental.

Recovery and precision

The recoveries of the added standards of DES and IDE (final concentration, 10 μg/ml) from the aorta hydrolysates were $99.6 \pm 2.7\%$ and $98.4 \pm 1.8\%$ (mean $\pm$ S.D.), respectively. Table I shows the within- and between-run precisions of DES and IDE for the aorta hydrolysate samples. Similar results were also obtained with the lung and liver hydrolysate samples.

Linearity and sensitivity

The calibration graphs, constructed with concentration of DES or IDE versus peak height, were linear with correlation coefficients above 0.999 over the concentration range 0.5–50 μg/ml. The slopes of the regression lines of the standard DES and IDE were much the same as those of the added standard. The detection limits of DES nd IDE were 0.1 μg/ml, respectively, in the hydrolysate samples with a 50-μl injection at a signal-to-noise ratio of 3.

DES and IDE contents in tissues of atherosclerotic rats

Table II shows the contents of DES and IDE in the aorta, lung and liver of rats treated with BAPN and/or an atherogenic diet. The ratio of IDE to DES in all the tissues tested was relatively constant (1.0–1.2). The contents of DES and IDE in the lung and liver were approximately a tenth and a thousandth of those in the aorta, respectively. In group II, the contents of DES and IDE significantly decreased by 23 and 27%, respectively, in the aorta and by 34 and 29% in the liver, compared with group I. In the lung, there was a significant decrease in the IDE content by 13%, but not in the DES content. BAPN, a known cause of lathyrism, inhibits the activity of lysyl oxidase [21] and hence blocks the formation

TABLE I

PRECISION OF THE ASSAY OF DES AND IDE IN AORTA HYDROLYSATE SAMPLES ($n=5$)

Concentration added (μg/ml)	Desmosine		Isodesmosine	
	Concentration found (mean ± S.D.) (μg/ml)	Coefficient of variation (%)	Concentration found (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
Within-run				
2.0	2.02 ± 0.03	1.85	2.03 ± 0.03	1.11
10.0	9.98 ± 0.05	0.46	9.99 ± 0.01	0.36
Between-run				
2.0	2.02 ± 0.04	1.24	2.01 ± 0.03	0.55
10.0	9.98 ± 0.05	0.53	10.00 ± 0.05	0.33

TABLE II

DES AND IDE CONTENTS IN AORTA, LUNG AND LIVER OF RATS GIVEN BAPN AND/OR AN ATHEROGENIC DIET

Each value represents the mean ± S.D.; group composition is detailed in Experimental; $n=8$; N.D. = not determined.

	Contents			
	I (control)	II (BAPN)	III (cholesterol)	IV (cholesterol + BAPN)
Aorta (mg/g dry weight)				
Desmosine	1.42 ± 0.04	1.10 ± 0.09*	1.49 ± 0.07	0.93 ± 0.08*
Isodesmosine	1.56 ± 0.04	1.14 ± 0.09*	1.66 ± 0.08	0.95 ± 0.07*
Lung (mg/g dry weight)				
Desmosine	0.121 ± 0.006	0.113 ± 0.005	0.122 ± 0.005	0.109 ± 0.006
Isodesmosine	0.130 ± 0.006	0.113 ± 0.005**	0.131 ± 0.006	0.111 ± 0.006**
Liver (μg/g dry weight)				
Desmosine	1.72 ± 0.19	1.14 ± 0.03**	N.D.	N.D.
Isodesmosine	1.95 ± 0.21	1.39 ± 0.08**	N.D.	N.D.

*Significant difference from group I, $p<0.001$.
**Significant difference from group I, $p<0.05$.

of cross-links in elastin [22–24]. Our findings are in good agreement with those previous results, although the lung was less subject to damage than the aorta and liver.

Although the contents of DES and IDE in group III were much the same as those in group I, group IV showed lower contents of DES and IDE than group II. We also observed induction of atherosclerosis and a remarkable cholesterol accumulation in the aorta of group IV: the total cholesterol content in group IV was 9.92 ± 1.48 to 5.64 ± 0.25 mg/g dry weight in group I. Additionally, we found a

negative correlation between the contents of DES and IDE and total cholesterol in the aorta of group IV ($n=7$), $r=-0.899$, $p<0.02$ and $r=-0.902$, $p<0.02$, respectively. This suggests that the lipid deposition may be associated with DES and IDE contents in the BAPN-induced atherosclerosis.

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