Comparison of ELISA and HPLC for the Determination of Desmosine or Isodesmosine in Aortic Tissue Elastin

Toru Osakabe,1 Yoshiyuki Seyama,2 and Saburo Yamashita2
1Drug Discovery Laboratory, Pharma Research & Development Division, Hoechst Japan Limited, Kawagoe, Saitama, Japan, 2Clinical Chemistry, Hoshi College of Pharmacy, Shinagawa, Tokyo, Japan

We developed a rapid and simple method for estimating tissue elastin content by measuring desmosine (D) in tissue hydrolysates by competitive ELISA. We compared the ELISA previously reported HPLC methods. When D or isodesmosine (ID) in hydrolysate of the same elastin preparation were measured by the two different methods, a good linear relationship was obtained (r = 0.854 for human aorta or r = 0.938 for rabbit aorta, respectively). The ELISA method can detect as little as 6 pmol/ml and it may be useful in monitoring elastin metabolism in patients with various connective tissue diseases.

Key words: elastin peptides, diagnosis, aortic tissue

INTRODUCTION

Determination of desmosine (D) or isodesmosine (ID) in tissue hydrolysates has been used to monitor elastin metabolism because D or ID is a specific cross-linked component in elastin. Therefore, various methods of D or ID detection by RIA (1), ELISA (2), and HPLC (3) have been reported. Previously we developed an HPLC method that was a modification of Covault's procedure (4). This paper describes a method to determine desmosine content in tissue hydrolysates by ELISA, modified from the Gunja-Smith procedure (2). We examined the correlation of tissue desmosine content between ELISA and HPLC from various aortic tissue elastin samples. Moreover, we studied the relation between desmosine and elastin peptides with the ELISA method.

MATERIALS AND METHODS

Materials

Standard desmosine and isodesmosine, albumin-conjugated desmosine, and rabbit antidesmosine-keyhole limpet hemocyanine (KLH) antibody were purchased from Elastin Products Co. (Pacific, MO). HRP-labeled swine antirabbit immunoglobulins were purchased from DAKO Immunoglobulins a/s, Denmark. Polystyrene microtiter plates (Immuino Module Maxisorp U8) were obtained from Nunc (USA). Porcine pancreatic elastase was obtained from Worthington (USA). Other chemicals were of analytical grade or reagent grade and were obtained from Wako Pure Chemical Co. (Tokyo, Japan). Thirty-two human aortas, which were the coronary artery, were obtained from autopsy cases from Tokyo Metropolitan Hospital. The study subjects were 8 patients (mean age 25 ± 8 (mean ± SD) years) with acute cardiac failure, 12 patients (mean age 43 ± 12 years) with chronic alcoholism, and 12 patients (mean age of 65 ± 11 years) with myocardial infarction. Rabbits were purchased from Sankyo Labo-service (Tokyo, Japan). Female rabbits, aged 3 months (n = 11) and 6 months (n = 11), had a 1% cholesterol diet for 12 weeks at our facilities.

Production of Desmosine

For the rabbit aorta, rabbits were sacrificed; the aorta was then immediately freed of blood and adipose tissue by washing with cold saline. These aorta were defatted and dried with a mixture of 2:1 chloroform–methylene and acetone. Aortic elastin fractions were prepared according to a previously reported method (5). The elastin fractions were weighed and hydrolyzed in 6 N HCl for 48 hr at 110°C. The hydrolysate was evaporated and dried over P2O5 and solid NaOH in a dessicator. After neutralization with a 0.05 N NaOH solution, an aliquot of hydrolysate was used for the determination of desmosine. Procedures of human aorta were identical to those described above for rabbit aorta.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; D, desmosine; ID, isodesmosine; RIA, radioimmunoassay; BSA, bovine serum albumin; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; HRP, horseradish peroxidase.

Received November 23, 1994; accepted January 26, 1995.

Address reprint requests to Dr. Toru Osakabe, Drug Discovery Laboratory, Pharma Research & Development Division, Hoechst Japan Limited, 1-3-2 Minamidai, Kawagoe, Saitama 350-11, Japan.

© 1995 Wiley-Liss, Inc.
Production of Elastin Peptides

Insoluble elastin was prepared from human aorta using methods described in a previous report (5). The human insoluble elastin was weighed and solubilized using porcine pancreatic elastase, at an enzyme/protein ratio of 1:100 for 24 hr in 0.05 M borate buffer (pH 8.0).

Measurement of Desmosine by Competitive ELISA

Determination of desmosine concentration was by the competitive ELISA procedure used for collagen, fibronectin, and laminin, using methods described by Rennard et al. (6). Polystyrene microtiter plates were coated with 50 µl of albumin-conjugated desmosine (1 µg/ml) in PBS to each well. Following incubation for 2 hr at 25°C or overnight at 4°C, the plates were washed three times with POD Washing Solution (Behringwerke AG, Marburg, Germany). In another set of test tubes, desmosine standard or test samples diluted in PBS were mixed with 400-fold diluted rabbit antidesmosine–KLH in 0.5% casein 0.5% BSA–TBS. Aliquots of solution (50 µl) obtained from these test tubes were added to each well; plates were reincubated at 25°C for 90 min. After washing three times with POD Washing Solution, 50 µl of 1,000-fold-diluted HRP-labeled swine antirabbit immunoglobulins in 0.5% casein–TBS were added to each well. Plates were reincubated at 25°C for 90 min, washed three times with POD Washing Solution, and then incubated for another 30 min, after the addition of 50 µl of chromogen–tetramethylbenzidine (TMB; Behringwerke AG, Marburg, Germany). The reaction with TMB was stopped by the addition of 50 µl of 0.5 N H2SO4. Absorbance was measured at 450 nm, using an ELISA processor BEP-II (Behringwerke AG, Marburg, Germany).

Measurement of Elastin Peptides by Competitive ELISA

We have reported previously on the competitive ELISA for elastin peptides (7). Briefly, polystyrene microtiter plates were coated with 50 µl of elastin peptides in PBS (5 µg/ml) to each well. Following incubation for 2 hr at 25°C or overnight at 4°C, the plates were washed 3 times with POD Washing Solution. In another set of tubes, elastin peptides, as the standard or test samples diluted in PBS, were mixed with 100-fold-diluted anti-elastin peptide serum in 0.5% casein–TBS. Aliquots of solution (50 µl) obtained from these test tubes were added individually to each well; the plates were then incubated at 25°C for 90 min. Subsequently, procedures were identical to those described for competitive ELISA of desmosine.

Measurement of Desmosine by HPLC

We have reported previously on a rapid and simple determination of desmosine by HPLC (3). Briefly, the sample was hydrolyzed in 6 N HCl, 110°C, for 16 hr. The sample was evaporated and neutralized, and desmosine and isodesmosine were then separated by the use of an isocratic solvent system of 0.1 M methansulfonic acid (pH 2.0)/acetoneitrile:90/10 in 6 mM heptan sulfonic acid. The HPLC eluates were monitored at 275 nm.

Recovery Test

To test for possible interference with the assay by other tissue constituents, aliquots of tissue samples were assayed both with and without the addition of a known amount of standard desmosine solution. Standard desmosine solution was also assayed in the absence of a tissue sample. Quantitative recovery of desmosine was 109%.

Cross-reactivity Test

To test for possible interference with the assay by isodesmosine, aliquots of isodesmosine standard for HPLC were assayed. The average cross-reactivity of isodesmosine to the competitive ELISA for desmosine was 11.0%.

Calculation

The concentration of desmosine in tissue was calculated from a standard curve for the determination of desmosine by ELISA and was corrected for amounts of elastin fraction.

RESULTS

A typical standard curve was obtained for standard assay conditions (Fig. 1). The curve is approximately linear within the range of concentrations 6–760 pmol/ml. A typical dilution curve is shown in Figure 2. The average recovery of tis-

![Fig. 1. Standard curve of desmosine competitive ELISA.](image-url)
New Methods of Estimating Tissue Elastin Content

Table 2. Interassay and Intra-assay Reproducibility of Desmosine Competitive ELISA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Interassay (n=3)</th>
<th>Intra-assay (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (pmol/ml)</td>
<td>CV (%)</td>
</tr>
<tr>
<td>1</td>
<td>87.1</td>
<td>6.2</td>
</tr>
<tr>
<td>2</td>
<td>170.7</td>
<td>5.3</td>
</tr>
<tr>
<td>3</td>
<td>217.1</td>
<td>12.1</td>
</tr>
</tbody>
</table>

CV, coefficient of variation.

Fig. 2. Dilution curve of desmosine competitive ELISA.

Fig. 3. Comparison of desmosine content in human aorta between HPLC and ELISA.

DISCUSSION

We demonstrated earlier that elastin-specific cross-linking component, isodesmosine, obtained from the extract of aortic samples from Marfan's syndrome, dissecting aneurysm, or aortic aneurysm was greatly decreased in comparison with control (aortic regurgitation) by HPLC assay for isodesmosine (8). Therefore, measurement of D or ID is a useful marker in connective tissue diseases and a useful assay for the clinical laboratory. Several research groups have prepared antibodies against desmosine or isodesmosine as well as developing both RIA (1) and ELISA (2) techniques for quantification. The ELISA procedure for desmosine has proved useful, convenient, and rapid. We have improved the ELISA procedure for desmosine with the use of a commercial antigen (albumin-conjugated desmosine) and antibody (anti-desmosine-KLH) instead of the antigen (gelatin-conjugated desmosine) and its antibody described in a previous report (2). Therefore, ELISA for desmosine has the advantage of convenient and rapid detection without the necessity of conjugation of desmosine to gelatin. Moreover, ELISA for desmosine is more sensitive (>6 pmol/ml) than the HPLC assay for isodesmosine (>95 pmol/ml). However, this system requires a large dilution of the tissue samples. Therefore, the CV value of intra-assay reproducibility was high. We observed that the commercial antibody for desmosine cross-reacted with isodesmosine at a
Fig. 4. Comparison of desmosine content in rabbit aorta between HPLC and ELISA.

Fig. 5. Correlation between elastin and peptides and desmosine in human aorta.

rate of approximately 11%. Therefore, the ELISA values were higher than the HPLC assay results. The higher ELISA results may be due to the cross-reactive properties of the commercial antibody. The ELISA method is useful for determination of desmosine in small tissue specimens, such as human biopsy specimens. Comparative study of estimation of desmosine and elastin peptides has not been reported. Thus, additional studies are necessary to determine whether quantitative elastin levels are also reflected in the desmosine level in the same sample. We measured elastin peptides and desmosine content with the ELISA method in some samples. Among subjects with human aortic elastin, there was correlation between elastin peptides and desmosine levels. Therefore, the amount of desmosine in elastin hydrolysates paralleled to that of elastin peptides obtained from the same elastin aortae. We estimated that both elastin peptides and desmosine may be useful in monitoring metabolic changes with elastin degradation. These ELISA procedures have proved useful for the detection of elastin and its degradation products in biopsies.

REFERENCES