Journal of Chromatography, 229 (1982) 200—204
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1183

Note

Rapid method for the purification of the elastin cross-links, desmosine and isodesmosine

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(First received October 6th, 1981; revised manuscript received November 18th, 1981)

Elastin is the protein component which confers unique recoil properties upon certain connective tissues. The importance of the disorganisation and degradation of the elastin architecture which occurs in atherosclerosis and pulmonary emphysema is increasingly appreciated [1]. There is also strong evidence to suggest that the corticosteroids used for the prevention of neonatal respiratory distress cause changes not only in pulmonary surfactant but also in the elastin content of the neonatal lung [2].

The identification of desmosine and isodesmosine as the major cross-links in elastin [3] has led to their acceptance as analytical markers for this connective tissue protein [4]. Since neither desmosine nor isodesmosine is absorbed from the intestinal tract [5, 6] their concentration in urine is a reliable index of elastin degradation in vivo. Thus the assay of elastin cross-links has already achieved some recognition as a potential diagnostic tool, particularly in inherited diseases [7] and in degenerative lung diseases [8].

The analytical methods for elastin employing gravimetric techniques [9] are time consuming and sometimes inappropriate [10] while the more recent methods involving amino acid analysis for desmosine and isodesmosine [4] often require preliminary purification [11, 12]. Recent radioimmunoassay techniques [8, 13, 14] have increased the speed of analysis but may also require a preliminary purification step [14]. For these reasons a rapid mini column method for isolating desmosine and isodesmosine from acid hydrolysates was developed. The method is based on the adsorption of desmosine and isodesmosine onto cellulose in organic acid—alcohol mixtures [11, 15].

0378-4347/82/0000—0000/$02.75

EXPERIMENTAL

Materials

n-Butanol, ethanol, acetone, pyridine, acetic acid and hydrochloric acid were of analytical grade and water was redistilled. Cellulose powder (Whatman CF_1) was obtained from W. & R. Balston (Kent, Great Britain). Silica gel GF_{254} 0.25 mm layers were from Merck (Darmstadt, G.F.R.). Isodesmosine and desmosine were purified from bovine ligamentum nuchae as described previously [15].

Mini columns were made from Samco No. 202 one-piece disposable plastic Pasteur pipettes (Saint-Amand Manufacturing Co., San Fernando, CA, U.S.A.) by cutting off the top hemisphere of the bulb (which then becomes a 4-ml solvent reservoir) and plugging the tip with a single glass bead of 3—4 mm diameter. The barrel of the pipette is conveniently clipped in a frame and 30 may be adequately managed at one time.

Methods

A slurry is prepared by mixing CF_1 cellulose (10 g) with the mobile phase, *n*-butanol—acetic acid—water (4:1:1) 200 ml in a 250-ml wide-mouth reagent bottle. The mixture is shaken to approximate homogeneity and then sonicated for 2—4 min to remove air bubbles. The columns (7 mm I.D.) are then packed to 45—50 mm height by pipetting 4—5 ml of the slurry into the bulb reservoir and allowing the cellulose to settle with occasional tapping. The column bed will not dry even if completely drained for several hours. A further 5 ml of mobile phase is added to each column to wash fine particles from the bulb reservoir.

Samples, typically 5—50 mg of sheep fetal lung tissue or 0.25 ml amniotic fluid, are hydrolysed in 6 *M* hydrochloric acid (0.5 ml) under nitrogen in sealed glass ampoules for 56 h. The vials are opened and the 6 *M* hydrochloric acid hydrolysate mixed, in order of addition, with acetic acid (0.5 ml), cellulose slurry (0.5 ml) and *n*-butanol (2 ml). During this process most of the desmosine and isodesmosine are adsorbed on the cellulose (results not shown). The slurry is transferred onto a prepared column and the ampoule washed with 1.5 ml mobile phase which is also transferred to the column. This additional slurry increases the column length by about 5 mm. The columns are then eluted with a further 15 ml mobile phase which removes hydrochloric acid and amino acids. The desmosine and isodesmosine are eluted from the column with water (5 ml) into plastic tubes. The majority of the residual butanol is displaced from the column by the first 1 ml of water and is either discarded or pipetted from the top of the aqueous fraction containing the desmosine and isodesmosine. The aqueous fraction is usually lyophilized before analysis to remove residual butanol and acetic acid and to concentrate the sample.

RESULTS

Initially all 6 *M* hydrochloric acid hydrolysates were evaporated and reconstituted in water before column separation. Later this was found to be un-

necessary as there was no difference between elution volume or recovery of isodesmosine whether the hydrochloric acid was evaporated or not. Similarly there was no effect upon either of these parameters with or without preliminary filtering of the hydrolysate.

In an experiment to monitor column performance and isodesmosine recovery, bovine serum albumin (10 mg) and gelatin (10 mg) were hydrolysed with and without the addition of isodesmosine (274 nmol) and processed as described above but with the collection of fractions (4 × 5 ml mobile phase

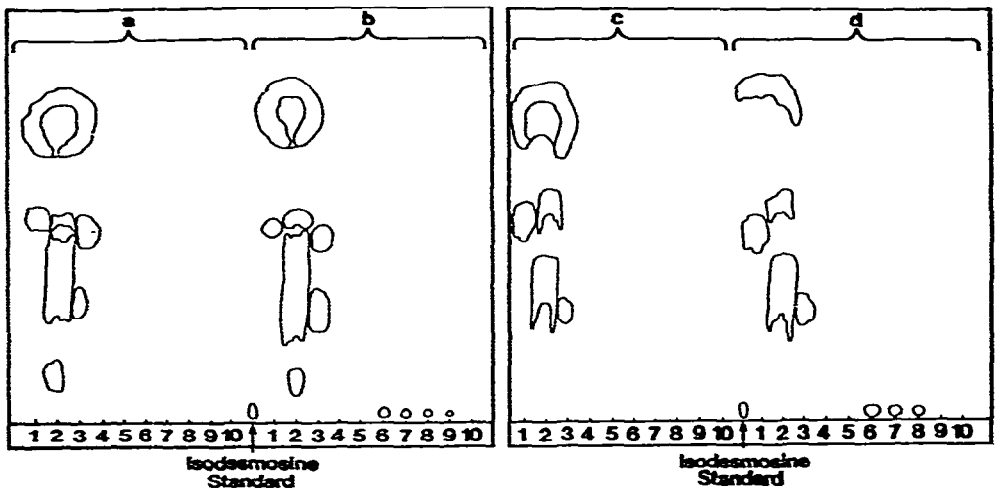

Fig. 1. The location of isodesmosine eluted from CF_1 columns by thin-layer chromatography. 1—4 are 5-ml mobile phase fractions and 5—10 are 1-ml water fractions from the CF_1 columns. Bovine serum albumin (10 mg) hydrolysates with (b) and without (a) isodesmosine (274 nmol) and gelatine (10 mg) hydrolysates with (d) and without (c) isodesmosine (274 nmol) were chromatographed as described in the text. One tenth of each CF_1 column fraction was chromatographed on silica gel GF_{254} layers by ascending development with ethanol—0.5 *M* acetic acid (1:1) to the full plate height. The photographs of the ninhydrin treated plates were pen-contrasted for clarity.

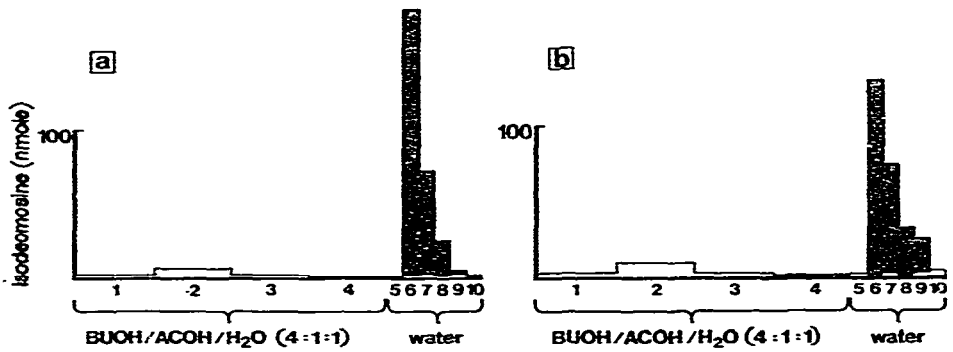

Fig. 2. Recovery of isodesmosine from CF_1 columns. The numbers of the *X* axis refer to the CF_1 column fractions. Isodesmosine was measured in aliquots (1/5000) of column fractions by radioimmunoassay. Shaded columns represent hydrolysates with isodesmosine (274 nmol) added to (a) gelatin and (b) bovine serum albumin. Unshaded areas represent material cross-reacting in the immunoassay which is present in the protein hydrolysates without added isodesmosine. BUOH = *n*-butanol, ACOH = acetic acid.

and 6 × 1 ml water). These fractions were lyophilized, the residues dissolved in water (100 μl) and an aliquot (10 μl) chromatographed on silica gel GF_{254} layers developed with ethanol—0.5 *M* acetic acid (1:1). The isodesmosine was located under ultraviolet light and, as other amino acids, with ninhydrin in acetone—pyridine (50:1). In this chromatographic system all the amino acids have R_F values $\geqslant$ 0.1 whereas the desmosine and isodesmosine remain close to the origin (Fig. 1). The isodesmosine was eluted in the water fractions, principally in fractions 6—9 (Fig. 1b and d), while all other amino acids were eluted with mobile phase, fractions 1—3. There was no appreciable ninhydrin-positive material in the water fractions from the chromatographed hydrolysates without added isodesmosine (Fig. 1a and c). The isodesmosine contents of the fractions were measured by radioimmunoassay [16] of an aliquot of the recovered fractions (Fig. 2). Summation of the quantities of isodesmosine measured by radioimmunoassay in column fractions 6—9 accounts for 99—102% of the 274 nmol isodesmosine added to the hydrolysates (Fig. 2). The small quantity of immunoreactive material in the mobile phase washes (mainly in the second fraction) is all accountable as non-specific cross-reacting material present in bovine serum albumin and gelatin.

DISCUSSION

The method presented appears to be effective in removing contaminating amino acids and leaving desmosine and isodesmosine in relative purity (Fig. 1). The material isolated has been found to be suitable for radioimmunoassay and is completely recovered (Fig. 2). Increments of isodesmosine (0—60 pmol) added to gelatine samples (5 mg) before hydrolysis were also measured, after column purification, with acceptable accuracy (r^2 = 0.992, data not shown) suggesting that the method is sufficiently reliable for routine use.

An equivalent method [11] using paper chromatography requires the 6 *M* hydrochloric acid protein hydrolysate to be filtered, evaporated, reconstituted in water, streaked on Whatman 3M chromatography paper and developed for 48 h, after which the desired material is eluted from the origin with water.

With this method the time from unsealing the 6 *M* hydrochloric acid hydrolysates to the end of the collection of the desmosine/isodesmosine fractions is 2—3 h for 30 or more samples. The only apparent source of error arose from gross inhomogeneity of the column bed due to air bubbles. This problem was eliminated by prior sonication of the cellulose slurry and minimal care in column packing.

The procedure is economical of both cost (*n*-butanol, cellulose and disposable pipette <20 cents/sample) and time. Evaporating water containing traces of acetic acid and *n*-butanol is less demanding on equipment than evaporating 6 *M* hydrochloric acid.

With increasing interest in elastin biochemistry [1], particularly in degenerative lung diseases [8], and atherosclerosis [17], this method may find wide application.

ACKNOWLEDGEMENTS

The author thanks G.C. Liggins, FRS, for his discussion and the Medical Research Council of New Zealand for financial support.

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