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Review

Progress in the methodological strategies for the detection in real samples of desmosine and isodesmosine, two biological markers of elastin degradation

Desmosines are crosslinking amino acids unique to mature elastin in humans. Owing to this unicity, they have been discussed as potentially attractive indicators of connective tissue disorders whose clinical manifestations are mostly the result of elastin degradation. This review covers advances in immunochemical, chromatographic, and electrophoretic procedures applied in the last 25 years to detect and quantitate these crosslinks in a variety of biological samples. Recent applications of CE with LIF detection (CE-LIF) for investigating the content of desmosines in different fluids will also be discussed.

Keywords: Biological fluids / Biomarkers / Desmosine / Isodesmosine

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1 Introduction

Three scientists (J. Thomas, F. Eldsen, and M. Partridge) from the University of Cambridge (UK) have pioneered the research on crosslinkages in elastin. In fact, in 1963, they published the first reports on the isolation of two new amino acids that were named desmosine and isodesmosine [1, 2], obtained as the products of the acid hydrolysis of purified elastin from bovine ligamentum nuchae. On the basis of physical and chemical procedures that included proton magnetic resonance studies, they were also able to provide a structure, although partial, for both compounds [2, 3]. A few years later, Anwar and Oda [4] studied the biosynthesis of these two amino acids by incorporating uniformly labeled lysine-^{14}C in cultures of chicken embryo aortas. The results of their investigations provided evidence that four lysines condense to form desmosine or isodesmosine, strongly supporting the view forwarded by other authors who had previously hypothesized the same mechanism of formation [5, 6]. It is now well established that these compounds, whose structures are shown in Fig. 1, are polyfunctional crosslinking amino acids unique of mature elastin in humans [7, 8]. Owing to this unicity, desmosines have been discussed as potentially attractive indicators of connective tissue disorders whose clinical manifestations are mostly the result of elastin degradation. In fact, given that peptides containing the desmosines derived from the destruction of elastic fibers are quantitatively excreted in the urine, their determination may provide a measurement of body elastin destruction [9–12]. Obviously, the same determination may help in the definitive evaluation of any therapeutic agent, by showing the effectiveness (or not) of agents with potential in reducing the elastin breakdown process [13–15]. The fact that detection of desmosines may represent an indirect measurement of extracellular matrix degradation or of elastase activity in several diseases with high morbidity and mortality provides the rationale for understanding why so many efforts have been devoted to achieve their determination in biological fluids. Chronic obstructive pulmonary disease (COPD), a class of disorders characterized by massive destruction of the elastic fibers of the alveoli [16–19] and pseudoxanthoma elasticum (PXE), a rare heritable disorder of connective tissue, whose clinical manifestations are the result of mineralization, fragmentation, and degradation of elastic fibers [20–23], are two typical examples of the disorders mentioned above. Although characterized by a very different etiology, both diseases share a common trait, *i.e.*, the uncontrolled degradation of elastin with abnormal excretion of elastin-derived fragments. To prove out the value of desmosines as candidate markers of these (and other) disorders, several laboratories in the world have developed different procedures for their

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Abbreviations: AAT, α_1-antitrypsin; **COPD,** chronic obstructive pulmonary disease; **PXE,** pseudoxanthoma elasticum

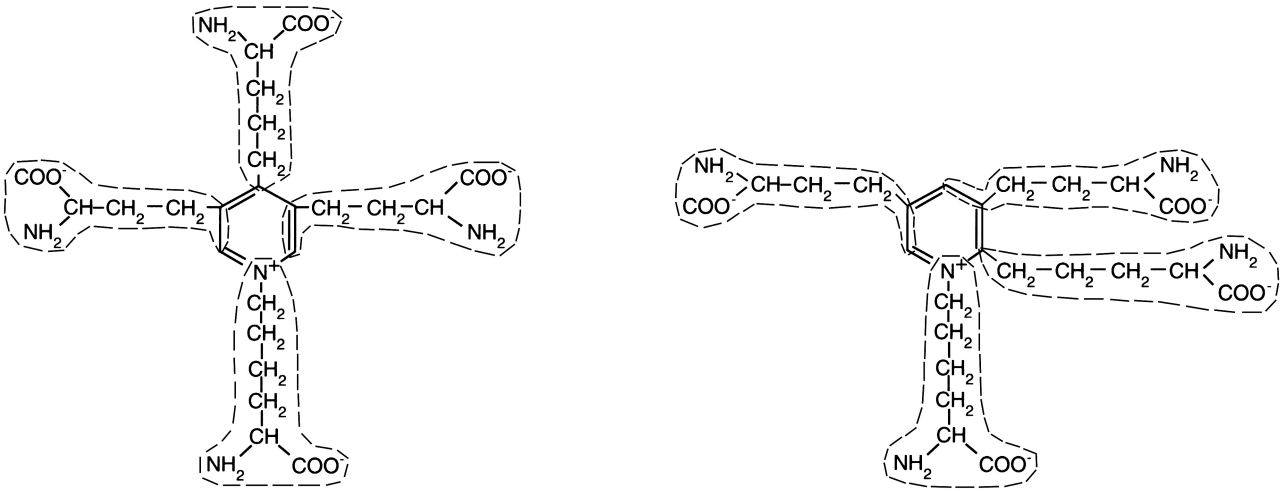

Figure 1. Structure of desmosine and isodesmosine. The dotted lines indicate the lysine residues that participate in the formation of crosslinks.

screening and quantification [24–38]. Although they have been mostly applied on urine, all of them have shown to be promising methods to assess possible correlations between desmosine concentration and the disease responsible for their production. Nevertheless, the advent in recent years of high-resolutive and high-sensitive techniques allowed to yield reliable information also from other matrices including plasma, sputum, and tissues.

The aim of this report is to review the literature dealing with the determination of desmosines as surrogate markers of elastic fiber destruction. The applications performed on real samples will be critically discussed in terms of their analytical and clinical interest, also in the light of our own laboratory experience.

2 Amino acid analysis

Since desmosine and isodesmosine are ninhydrin-positive analytes, it seems plausible that amino acid analysis based on this reaction could be applied for their quantitation. However, the first chromatographic systems developed to resolve these amino acids in real samples required buffers other than those utilized in standard approaches, thus making the method very laborious [6, 39, 40]. To overcome these problems Corbin [41] developed a procedure in which a third ion-exchange column was added to the "classical" equipment based on the use of only two columns. This modification was applied to an elastin hydrolyzate producing excellent resolution and quantification of the two compounds.

Starcher [42] used an amino acid analyzer to determine the elastin content of young animals and human tissues (uterus and skin) by measuring the amount of desmosines. Although the procedure was complex and time-consuming, analyses were accurate and reproducible and allowed to determine the concentration of crosslinkers in the elastin samples isolated from the organ under study. Likewise, Goldstein and Starcher [43], using the same procedure and working on hamsters' urine, showed that elastin turnover may be measured by quantitating the urinary desmosine content.

Desmosines (Des, Ides, and Merodesmosine) from elastin of bovine ligamentum nuchae were measured by Mecham and Foster [44] using amino acid analysis to identify the amino acids around elastin crosslinking sites. Gunja-Smith and Boucek [45] have used amino acid analysis for the detection of desmosines in 24 h human urine samples. They found that patients with Marfan's syndrome excrete consistently smaller amounts of desmosines than do comparable controls during the early developmental period. Also this method was time-consuming, a series of preliminary fractionation and purification steps being necessary for isolation of analytes. A lithium citrate buffer gradient was used by Lonky *et al.* [46] to determine desmosine and isodesmosine from acid hydrolysates of hamster and human lungs. As suggested by the authors, this method could also be used for the quantification of elastin in biopsy specimens. Both an amino acid analyzer and HPLC (see Section 4) were utilized by Covault *et al.* [47] to measure desmosines, in 13 canine and 11 human aortic samples, with the purpose of estimating tissue elastin. Des and Ides were almost completely separated using a synthetic resin and applying a step-elution with a series ($n = 3$) of citrate buffers from pH 3.45 to 5.35. Amounts of analytes around 0.1 nmol *per* sample could be detected.

Amino acid analyzer was used by Velebny *et al*. [48] to detect desmosines in rat liver with carbon tetrachloride-induced cirrhosis. Their results (a four-fold increase of desmosines in comparison with those of rat liver from controls) allowed to conclude that insoluble elastin may accumulate in cirrhosis. Bruce *et al*. [49] have estimated lung elastin degradation by quantitating desmosines excreted in a 24-h period urine by 16 patients with cystic fibrosis. Amounts as low as 50 ng of desmosine or isodesmosine were detected by using the amino acid analyzer. Zarkadas *et al*. [50] developed an interesting amino acid analysis method for determining collagen and elastin in tissue hydrolysates from the amount of 5-hydroxylysine and desmosine or isodesmosine and for studying protein hydroxylation, glycosylation, crosslinking formation and the turnover rates of collagen and elastin in normal and diseased tissues. Finally, Chapman *et al*. [51] assayed cellular elastin degradation by measuring the net loss of desmosine/isodesmosine during coculture of human macrophages with extracellular matrices rich in elastin.

Taken together, these reports show that, although more laborious and less sensitive than other techniques that will be presented in the following sections, amino acid analysis should be considered a reliable technique for detection of desmosine. This procedure is still in use in several laboratories whose research is focused on disorders that involve elastin degradation. All chromatographic profiles show that peaks of desmosine and isodesmosine are well separated from each other and can be integrated with precision. This allows to quantitate exactly elastin from small amounts of tissue or to follow the destruction of connective tissue in ongoing disorders that involve its degradation.

3 Immunochemical methods

The preparation by several research groups of polyclonal antibodies against desmosine [24, 28] and isodesmosine [52] allowed to develop a series of immunochemical assays (RIA and ELISA) aimed at quantitating these compounds in tissue and urine samples or at providing insight into the organization and maturation of the elastin macropolymer. The following paragraphs contain a description of the most reliable immunochemical procedures developed for the determination of desmosine and, more rarely, of isodesmosine, and their practical applications.

3.1 ELISA

The first report on an ELISA merely dedicated to the determination of desmosine was described by Gunja-Smith [29]. Based on the use of rabbit antisera directed against a desmosine-BSA conjugate and of microtiter plates coated with desmosine-gelatin conjugate, this method was applied either on tissue specimens (lungs and aortas) and on urine. In both the cases, the method proved to be rapid and sensitive, being able to detect desmosine in the range of 2.5–50 pmol. As indicated by the author, the method could be useful in studying elastin metabolism and for preparing a desmosine-gelatin coating antigen. Nevertheless, few different antibody preparations directed against desmosine exhibited a cross reactivity (15–20%) toward pyridinoline (Pyr; a nonreducible collagen crosslinking compound also present in urine and other tissue samples) that could represent a limitation of the procedure. Matsumoto *et al*. [53] and Verplanke *et al*. [54] described two ELISAs for determining urinary desmosine not very different from that used by Gunja-Smith. Although rather sensitive (standard desmosine could be detected in the range of 0.01–10 ng), the methods showed a complete crossreactivity with isodesmosine. Moreover, the low titer rabbit antiserum used in this inhibition immunoassay suffered for some interferences, since a number of unknown compounds present in urinary hydrolysates could not be completely removed even after the introduction of a chromatographic step to clean up the sample.

The ELISA method developed by Laurent *et al*. [30] was of particular interest. Based on the competition between solid phase-bound desmosine-protein conjugate and free desmosine for binding to monospecific antidesmosine antiserum, it showed greater sensitivity and specificity than those previously described. Briefly, rabbits were immunized with desmosine-BSA and microtiter plates were coated with desmosine-egg albumin. Antidesmosine antibodies bound to the desmosine-protein conjugate (the conjugation of desmosine to protein carriers was obtained by reaction with 1-ethyl-3-(dimethylaminopropyl)carbodiimide; ECDI) were revealed by using an avidin-biotin peroxidase system. To remove the antibodies directed against nonspecific epitopes present on the carrier proteins, antidesmosine antiserum was absorbed on rabbit albumin polymerized with ECDI. Urine samples from 118 normal male volunteers were analyzed by this method, with a sensitivity ranging from 0.07 to 4 ng of desmosine/well. Only a little crossreactivity was observed with lysinonorleucine and isodesmosine. This assay also allowed quantitation of elastin fiber biosynthesis in the connective tissue matrix of cultured rat pleural mesothelial cells. The method developed by Watanabe *et al*. [55] certainly had a series of analogies with that described above. They used rabbit antisera directed to the conjugate of desmosine and desmosine-BSA. The assay was performed in microtiter plates coated with a desmosine-gelatin conjugate. The range of detection was between 0.4 and 400 ng/mL. By adding standard desmosine to urine they calculated a recovery between 90.6 and 117%. Although a crossreactivity with isodesmo-

sine ranging between 13 and 45% was observed, other 19 standard amino acids did not interfere with this determination. Osakabe *et al*. [56] applied an ELISA approach to the determination of desmosine and isodesmosine in human and rabbit aorta. Once compared with those obtained using an HPLC procedure, these data correlated well, the values of r ranging between $r = 0.854$ and 0.938. The LOD for both analytes was 6 pmol/mL.

Using this ELISA procedure more recently the same authors measured the urinary levels of desmosine and elastin peptides in 23 patients with aneurysm [57] to estimate elastin metabolism in this disorder. The amount of analytes in the aneurysm group was significantly increased compared with that of a control group containing subjects ($n = 33$) > 20 years. A specific indirect competitive ELISA method based on the use of rabbit antiserum to desmosine-hemocyanin conjugate and microtiter plates in which wells were coated with desmosine-albumin conjugate was developed by Cocci *et al*. [31]. They applied this procedure to COPD subjects and found that urinary excretion of desmosine was significantly higher in patients than in controls (294 ± 121 *vs.* 183 ± 93 μg).

3.2 RIA

Several RIAs have been developed by different research groups to quantitate desmosine. In particular, Starcher and his group (G. S. King; R. P. Mecham; M. Scott; C. Kuhn) have described a variety of applications of this procedure in a series of stimulating papers [24, 25, 28, 58–60]. They developed the method by injecting desmosine, conjugated with ECDI to BSA, into rabbits which developed useful titers of serum antibodies after 6 months. Radioiodination of desmosine was accomplished with ^{125}I Boltan-Hunter reagent. The sensitivity of the assay was in the range of 1–50 pmol of desmosine and the antibody was highly selective, reacting less than 1% with other known crosslinks [24, 25]. This RIA was considered as a means of studying elastogenesis in cell culture based on the observation that elastin is synthesized by fibroblasts and chondroblasts following two different processes [58]. In fact, if fibroblasts secrete elastin into the medium as soluble tropoelastin molecules, which form desmosine crosslinks and become constituents of the cell layer only after 3 wk, crosslinking occurs immediately in the chondroblast cell layer forming stable, insoluble elastic fibers. Thus, the determination of desmosine could be useful for understanding elastogenesis. Another interesting application concerned the study on the progression of experimental emphysema produced in hamsters by a single intratracheal injection of elastase [59]. Elastin catabolism "*in vivo*" was followed by measuring the urinary excretion of desmosine through the RIA approach. The release of soluble desmosine could also be used as a measure of the active elastase in the tissue. The former RIA procedure was also modified to obtain a rapid estimation of lung elastin catabolism in urine of patients with cystic fibrosis [60]. Using an antibody bound to magnetic particles, the RIA was adapted to handle large numbers of samples, only 50 μL of urine being required to perform this procedure. Their results showed that it was not necessary to extract or hydrolyze the urine prior to assay, although in a further study they re-evaluated the method having observed that other compounds in hydrolysates of human urine competed for desmosine in the RIA. By submitting urine from nine normal adults to an extraction procedure with chloroform/ethanol (60:40) they realized that an average of 45% of the RIA-competing material was not desmosine. Their conclusion was that fractionation of urine was necessary to obtain higher precision of analysis. Harel *et al*. [26, 61] have applied the RIA approach to the urine of 23 nonsmoking subjects and of 20 smokers with evidence of COPD. The amount of desmosine excreted was of 47 ± 15 μg/24 h in the former group and of 40–400 μg/24 h in the latter. The RIA applied for these determinations did not show substantial crossreactivity with isodesmosine and other amino acids and the LOD for desmosine was of 200 pg.

Of particular interest were the applications of the RIA procedure proposed by Janoff and coworkers [62–64]. One of these concerned the detection of desmosine in urine of several burned adult males [62]. The RIA applied allowed to observe urinary desmosine levels significantly higher in burned patients than in controls (250–1411 *vs.* 82–142 nmol/24 h). Urinary hydroxyproline was also higher in burned patients than in healthy adults used as controls (56–471 *vs.* 31 ± 6 mg/24 h). The same RIA was used as a reliable index of pulmonary elastin breakdown in investigations that involved male sheep and subjects with PiZZ α_1-antitrypsin (AAT) deficiency [63, 64].

In the former case, 30 male sheep were treated with varying doses of endobronchial elastase to induce lung injury [63]. Urinary excretion of elastin peptides was then measured by desmosine RIA. Elevation in urinary desmosine excretion occurred 48 h after elastase administration and increase of analyte was positively correlated with enzyme dose. In the latter case [64], excretion of desmosine in 17 homozygous AAT-deficient (PiZZ) patients with emphysema was compared with that in 27 patients with interstitial lung diseases (16 sarcoid, 5 idiopathic pulmonary fibrosis, 6 other interstitial lung diseases) and 26 healthy subjects. Both smokers and nonsmokers were present in all groups. They found that the amount of desmosines excreted by patients was not significantly higher than that of controls. Skinner *et al*. [52] were interested in the quantitation of isodesmosine and developed a RIA for its determination as the tetraacetyl derivative. Isodesmosine tetraacetate conjugated with bovine albumin was injected into rabbits which developed useful titers of antibodies. The radioligand for the

assay was prepared by acetylating isodesmosine with ^{3}H-acetic anhydride. The bound was separated from free ligand by coprecipitation with human γ-globulin in 46% saturated ammonium sulfate solution. The sensitivity of the assay was 2 ng isodesmosine. The specificity of antiserum was good, a partial crossreactivity (4%) being evident only with desmosine tetraacetate. Uitto *et al*. [65] combined the determination of desmosine by RIA with computerized digital image analysis to obtain an accurate quantitation of the elastic fibers in punch biopsy specimens of human skin. With this procedure the elastin fibers, visualized by elastin-specific stain, were examined through a camera unit attached to the microscope obtaining binary images that allowed determination of their volume fraction. As an independent measure of these fibers, desmosine was assayed on tissue sections. Once applied to the skin of ten healthy controls and five PXE patients, the procedures allowed to observe that the volume fractions occupied by the elastic fibers in the lesions of patients were six-fold increased in comparison with those of controls. The desmosine RIA allowed Desai *et al*. [66] to assess elastin maturation in the developing human lung. Their findings indicated that there was a tendency for higher desmosine concentration in prematurely born growth retarded infants compared to normals. Tenholder *et al*. [67] have investigated the adult respiratory distress syndrome (ARDS) by determining urinary desmosine excretion with RIA. Given that different desmosine concentrations were detected for different groups of patients, the authors concluded that substantial increases in urinary desmosine excretion may favor a diagnosis of ARDS. Based on their results Fill *et al*. [68] have suggested that acute lung injury (ALI) is associated with an increase of urinary desmosine excretion. Desmosine levels from ten ALI patients were monitored (with and without acid hydrolysis) over 10 days using a RIA. Baseline urinary desmosine was increased in two of ten patients but the concentration of this crosslink did not appear to be related to age, gender, neutrophil elastase/α_1-antiprotease complex concentration.

In keeping with the content of the articles indicated above, it seems possible to conclude that both RIA and ELISA are assays very helpful in detecting desmosines as surrogate markers of connective tissue disorders. It is not a surprise that this determination has been mostly performed on urine that can be considered the preferred matrix chosen by the majority of scientists for detection of desmosines. Urine in fact is extremely easy to collect, can be transported in an intact state simply by using preservatives and freezing and, in many cases, can be utilized without any pretreatment. Moreover, the fact that some analytes become significantly more concentrated in urine than in other fluids, making measurement easier, supports the firm believe that this is an important matrix for performing analyses of those coumpounds that are excreted in very low amounts. Most of the immunochemical methods described use antisera directed against a desmosine-conjugate and, although a partial crossreactivity of isodesmosine and a few other amino acids have been observed in many cases, all authors agree that desmosine and/or isodesmosine measurement is a reliable index of elastin breakdown, especially in pulmonary disorders.

4 HPLC

Decisive methodologic progress achieved with the advent of HPLC and the combination of HPLC with MS allowed the development of rapid and sensitive methods for quantitating desmosines from different sources. Aim of this section is to offer a survey of these publications in the last 25 years.

A comprehensive attempt to determine the amount of desmosines (Des and Ides) in hamster aortas and lungs was performed by Faris *et al*. [69] who used HPLC equipped with an anion-exchange column and a gradient of K_2HPO_4 to achieve this goal. Desmosines were separated from other amino acids and absorbance monitored at 275 nm. The detection limit of crosslinks was 0.1 nm. This method was later modified by Soskel [70] who made it more rapid and sensitive by using an anionic exchange column and KH_2PO_4 buffers containing a high percent of ACN (buffer A) or of trimethylamine (buffer B). The pH was adjusted to 6.3. This approach allowed the author to obtain an LOD of less than 100 pmol and a linearity from 0.1 to 5 nmol. The content of desmosines in aorta (human) was also investigated by Fujimoto [71] who, however, performed the separation of the hydrolysate on RP-HPLC. Amino acids were trinitrophenylated prior to separation with an ACN-water gradient containing 0.1% w/v oxalic acid and 0.1% w/v sodium lauryl sulfate (SDS). Also Covault *et al*. [47] used an RP method with a C_{18} column to separate desmosines from other components of canine and human aortic hydrolysates. The amino acids were eluted with various proportions of methanol/water containing 10 mmol of sodium heptane sulfonate at pH 3.0. Although results agreed well with those obtained from amino acid analysis (see Section 2), this HPLC method was much more sensitive than the former, an amount of 100 pmol desmosines *per* sample being detectable. Likewise, to study the alterations of elastin induced by atherosclerosis, Yamaguchi *et al*. [33] have applied an RP-HPLC method to determine desmosines in hydrolysates of aorta, lung, and liver from rats treated with β-aminoproprionitrile and an atherogenic diet. Using 0.1 M phosphate buffer-ACN containing 20 mM SDS (pH 4.5) the detection limit of these amino acids was 0.1 μg/mL. Particularly interesting was the method developed by Lunte *et al*. [72] who derivatized desmosines with

napthalenedialdheyde/cyanide (NDA/CN) using electrochemical detection to determine the cyano[*f*]benzoisoindole (CBI) derivatives. The combination of this derivatization with electrochemical detection was found to be linear over three orders of magnitude and detection limit was 100 fmol. This method allowed to quantitate amino acids in elastin yielding results well-correlated with those previously published in the literature. A general strategy for the determination of desmosines in tissues (rat aorta and liver) was also developed by Guida *et al*. [34] who submitted tissue hydrolysates purified by cellulose minicolumns to precolumn derivatization with dansylchloride (DNS-Cl). Using a C_{18} column, the desmosine derivatives (the two amino acids could not be separated from each other) were eluted in 15 min using a linear gradient of ACN in phosphate buffer. Owing to the concentration and purification pretreatment of the samples, they could determine elastin in tissue samples of low elastin content such as fetal lungs, the LOD being as little as 3 pmol. Elastin-related skin disorders were studied by Schwartz *et al*. [73] by quantitating desmosines in small amounts of skin using isocratic HPLC. Biopsies were obtained from normal, nonsolar exposed skin, and from the lesional skin of patients with PXE, cutis rhomboidalis nuchae (AE), and cutis laxa (CL). The analyses on the disorders of elastolysis (PXE and AE) demonstrated a two- to five-fold increased content of desmosines with respect to controls; in contrast the CL patients had only 20% of the normal content of desmosines.

A great deal of research on desmosines was done by Stone *et al*. [27, 74] who developed a new HPLC method to measure urinary desmosines and applied it extensively for the study of collagen and elastin degradation in a series of conditions including COPD and scleroderma [9, 11 – 13, 75 – 77]. In this procedure urine samples were spiked with a known amount of [^{14}C]Des and submitted to hydrolysis in 6 N HCl. Gel-filtration on a Sephadex G-15 column was performed on hydrolyzed specimens to remove contaminants and desmosines were quantified by HPLC and by amino acid analysis. The amount of isotope recovered was used to determine losses during the overall procedure and the isotope dilution to calculate the amounts of endogenous desmosines originally present in the urine. Since the two methods gave similar results, the more rapid HPLC method was chosen for applicative purposes. The content of desmosines in urine of hamsters (controls and treated with human neutrophil elastase or porcine pancreatic elastase) and of eight men who were never-smokers was determined by using this method [27, 74]. The procedure proved to be useful and was successfully applied to the detection of desmosines in (i) urine of never-smokers (n = 22), smokers without airflow obstruction (n = 13), and 21 COPD patients [9]; (ii) urine of 22 controls and 20 patients affected by systemic sclerosis [11]; (iii) urine of volunteers submitted to different diets [75]; (iv) urine of three pregnant women before and after parturition [76]; (v) urine of current smokers with (n = 10) and without (n = 8) rapid decline of lung function [12]; (vi) urine of 16 healthy subjects (men and women) collected at different hours to determine whether there is circadian variation in excretion of desmosines [77]; and (vii) urine of 12 patients (men and women) with emphysema due to severe, congenital deficiency of AAT [13].

Human elastin, rat aorta, and bovine ligamentum nuchae were the sources of desmosines chosen by Hanis *et al*. [78] to develop a new method for their quantitation in tissues. Collagen was removed by treating tissues with 10% cold TCA and samples were submitted to hydrolysis in 6 N HCl. A cellulose column first eluted with *n*-butanol-acetic acid-water and then with water, was used to preseparate desmosines from other amino acids of hydrolysates. The crosslinks were finally derivatized with phenylisothiocyanate (PITC) and detected by RP-HPLC using a gradient of sodium acetate (pH 6.4)-ACN. The same derivatization procedure of desmosines with PITC was adopted by Salomoni *et al*. [79] to study elastin in small human biopsies. They also applied a chromatographic purification step prior to submitting samples to derivatization. In contrast, their procedure of separation consisted of an isocratic process on a C_{18} column. The detection limit of this method was of 0.5 ng for both compounds. Surprisingly, Nakamura and Suyama [80] determined desmosines in bovine ligamentum nuchae using normal phase (NP) HPLC. They treated elastin hydrolysates with an SEP-PAK disposable column and separated desmosines on a silica-gel NP column using *n*-propanol-water-25% ammonia (96 : 3 : 0.06 v/v/v) as the eluent. To the best of our knowledge, this is the only report in which NP-HPLC is indicated as the method employed to separate desmosines. Another very interesting method that should be useful to process large numbers of biological samples (urines) for analysis of desmosines was developed by Cumiskey *et al*. [81]. The peculiarity of this method consisted in the enrichment in desmosines of hydrolyzed samples through a step with CF1 cellulose chromatography followed by a classical procedure on a C_{18} column. The run was very rapid (elution time between 9 and 12 min) and the LOD was of approximately 30 pmol. The method proved to be particularly suitable to evaluate the daily and weekly urinary variation in the concentration of desmosines [81] and to compare desmosines excreted in patients with COPD and cystic fibrosis [82].

A couple of interesting papers dealing with possible alteration of crosslinking amino acids of human aorta in relation with age [83] or in association with dissecting aneurysm [84] have been published by Watanabe *et al.* In both cases areas of thoracic aorta were submitted to acid hydrolysis and the hydrolyzed material purified on an SEP-PAK silica-gel column followed by Fe^{3+}/activated char-

coal columns. This treatment allowed them to obtain, with a classical RP-HPLC procedure, not only the separation of desmosine and isodesmosine but also of neodesmosine, oxodesmosine, isooxodesmosine, and aldosine. Also Pyr, a major crosslinking amino acid of collagen, which represents an index of fibrosis, could be analyzed by this approach. A direct method to obtain rapid and sensitive detection of all collagen and elastin crosslinks (Pyr; deoxypyridinoline, Dpyr; pentosidine, Pen; desmosine; and isodesmosine) in the hydrolysate of human yellow ligament was described also by Chen *et al*. [85]. Using a C_{18} column eluted with a binary gradient in which mobile phase A was 20 mM phosphate buffer containing 0.2% SDS (pH 3.5) and MeCN (19 : 1 v/v), and mobile phase B was the same mixture but in the proportion 3 : 2 v/v they separated all the components cited above in about 23 min. Des and Ides were detected in the UV at 275 nm, while a fluorescence detector (excitation at 305 nm and emission at 397 nm) was used for the other three analytes. Based on their results, the authors considered this method very useful for investigating the content of these crosslinks in both collagen and elastin under various conditions. That elastin contained also pyridine crosslinks was shown by Umeda *et al*. [37] who isolated and determined the structure of two new amino acids, indicated as desmopyridine (Desp) and isodesmopyridine (Idp), from bovine ligamentum nuchae. These two crosslinks were measured by RP-HPLC with UV detection (at 275 nm) and were found in a variety of bovine tissues. Moreover, the content of Desp and Idp in human aorta was found to be gradually increased with age and the Desp/Des and Idp/Ides ratios in aorta elastin were found to be higher than in other tissues. The authors concluded that the assay of these pyridine crosslinks may be useful for determining the damage of aortic elastin caused by ammonia.

To verify the stability of Pyr, Pen, Des, and Ides, Abe *et al*. [86] used a classical RP-HPLC method for determining their concentration in formalin-fixed tissues. Pyr and Pen were not significantly affected by or related to the duration of formalin fixation. By contrast, the concentration of Des and Ides were significantly lower in the formalin-fixed yellow ligament compared to the frozen samples, indicating that they were not preserved by this treatment and suggesting that formalin may mask or alter elastin crosslinks. To obtain a diagnostic tool applicable on children (aged 4 wk to 12 years), Winfield *et al*. [87] have produced a method in which the extraction technique of urinary desmosines was improved in comparison with the procedures previously published. This goal was achieved by increasing hydrolysis time, temperature, and cellulose column size and separating hydrolysate on RP-HPLC. This method had small intra- and interassay variability and allowed to verify that there was no significant diurnal or day-to-day variability in total desmosine levels. The authors were able to establish a reference range of desmosine concentration for healthy prepubertal children.

Owing to the salient features of sophisticated techniques, such as HPLC-MS, several authors have been able to detect desmosines in complex mixtures in which these crosslinks could not be measured before. In a very interesting paper, Ma *et al*. [88] have described a method for quantitating free desmosines in urine of patients with COPD. Using HPLC followed by ESI-MS, they have detected free desmosines in unhydrolyzed urine of these patients. It should be emphasized that, up to that moment, these amino acids were found to occur in urine only as high molecular weight peptides. The sensitivity of the method was 0.1 ng. Obviously also peptide-bound desmosines in urine and sputum of patients above indicated were successfully measured using this procedure. That HPLC-MS could be a useful procedure for quantitating desmosines in complex matrices was confirmed by Kaga *et al*. [89], who worked on hydrolysates of rat lungs. Using an RP-HPLC procedure (mobile phase A was 7 mM pentafluoropropionic anhydride (PFPA) and phase B was 7 mM PFMA in 80% methanol) coupled with MS, they determined the concentration of these two amino acids in the lung. The reliability of this LC/ESI-MS procedure allowed Getie *et al*. [90] to study the effect of irradiation, by UVA; UVB; and IR radiations, on Des and Ides solutions. A significant time-dependent degradation of these two amino acids was observed upon exposure of their solution to UVA and UVB radiations. By contrast IR radiation (520 W) did not cause significant degradation of desmosines.

To underline the versatilty of these chromatographic procedures for the determination of desmosines, we would like to consider, in conclusion of this section, a few applications that are of particular interest owing to their peculiarity. Baxter *et al*. [91] have used HPLC to quantify elastin crosslinks and to determine if elastin mRNA was detectable in the disease-prone infrarenal aorta from patients with abdominal aortic aneurysm in comparison with a control group. They found that the proportion of insoluble elastin was markedly decreased in abdominal aortic aneurysm tissue, but the desmosine concentration in this elastin was not different for pathological and normal tissues. In an effort to find a biochemical explanation to the development of varices, Venturi *et al*. [92] determined by HPLC the content of 4-L-hydroxyproline (HYP) and of desmosines in saphenous veins collected from 20 patients with varices. Desmosine levels were reduced in dilated segments of varicose vein *versus* nondilated segments. They concluded that dilation of the varicose vein wall may be related to some defect in elastin metabolism. The same group investigated the biochemical basis of alterations present in upper esophagical sphincter of patients with Zenker's diverticulum [93]. They found that in samples of cricopharyngeal muscle (CPM) Ide to Des and collagen to elastin ratios were signif-

icantly higher in patients than in controls thus suggesting that a primary disease of CPM may be the cause of Zenker's diverticulum.

Since the first report on the use of HPLC to detect Des and Ides in biological specimens, this technique has been refined, optimized, and expanded to the detection of a variety of other elastin and collagen crosslinks. Advances in the production of stationary phases or in the mobile phase composition and the advent of sophisticated detection strategies allowed to carry out efficient determinations in complex matrices. The innovative modifications and expanded repertoire of applications that occurred during 2004 and 2005 also point to continued developments in the future. The HPLC-MS on-line coupling in fact has opened new perspectives in the analysis of biological samples allowing the investigators to achieve information not only on desmosines but on a series of other crosslinks simultaneously, thus enhancing the efficiency of the system. Based on the number of reports listed in this section it can be concluded that HPLC has an evident role in the analysis of these crosslinks and that, owing to its versatility, it can be tailored for a number of interesting applications.

5 Electrophoresis/CE

Determination of desmosines in biological samples has been successfully performed also by electrophoresis. Keller *et al*. [94] applied 1-DE to rapidly separate the commonly encountered crosslinking amino acids in hydrolysates of ligamentum nuchae elastin. However, to obtain a more complete separation of desmosine, isodesmosine, and merodesmosine, a 2-D technique was developed in which ascending chromatography on silica gel G plates was performed in the first dimension, followed by electrophoresis in the second. These "fingerprints" of elastin digests could be useful to detect differences in elastins from various sources. The same thin-layer electrophoresis procedure was applied by Cantor *et al*. [95] to measure crosslinked elastin synthesis in bleomycin-induced pulmonary fibrosis. They noted that crosslinked elastin synthesis was significantly elevated in controls at 1 – 3 wk after exposure to bleomycin. The increase observed could suggest that this tissue component was an important part of the fibrotic response of the pulmonary parenchyma.

Since the high potential of CE to resolve complex mixtures of analytes meets most of the demands of desmosine analysis (high efficiency; fast analyses; powerful flexibility in changing the selectivity of the separation), currently it is widely applied as a useful approach for this determination. Giummelly *et al*. [36] have developed a CZE procedure based on the use of 90 mM phosphoric acid to determine desmosines in elastin hydrolysates. Although the peaks of the two crosslinks were not completely separated, analysis was fast (less than 15 min) and linear over the range of 1 – 500 pmol injected, the minimal amount detected at 254 nm being 2 pmol. This method was applied by Norman *et al*. [96] to investigate aortic elastin content and aortic function in rats exposed to Vitamin D during gestation and in the postnatal period. Although they could not reproduce this separation they observed that the mean content of desmosines in the abdominal aorta was higher in control rats than in those treated with low/high-dose Vitamin D using the area of the combined peaks for integration. The drawbacks mentioned above prompted studies in our laboratory to evaluate the feasibility of using other CE modes for obtaining a better separation of these two amino acids. As shown in Fig. 2a, MEKC, with its combination of high efficiency and selectivity, was suitable for overcoming this problem since it allowed to obtain their adequate resolution (peaks 1 and 2 represent Des and Ides, respectively) and subsequent quantification [35]. Separations were performed in 35 mM sodium tetraborate (pH 9.3) containing 65 mM SDS and detection limit was 0.5 pmol for a 1-s injection at an S/N of 3.5. This method was applied to the determination of urinary desmosines in eight controls and eight COPD patients and it evidenced that all patients considered, except one, had higher urinary levels of desmosines than controls. To verify the reliability of this approach the levels of urinary desmosines were determined in a variety of other individuals including: (i) patients with stable COPD (n = 11); (ii) patients with an acute exacerbation of COPD (n = 19); (iii) patients with AAT deficiency (n = 9); (iv) patients with bronchiectasis (n = 13); and (v) patients with cystic fibrosis (n = 11). All data were compared with those from healthy subjects (n = 24), used as controls [97 – 99]. The results of these determinations clearly evidenced that urinary desmosine excretion was increased in all conditions characterized by airway inflammation.

However, since pretreatment and preconcentration steps of urine were needed to meet the requirement of sensitivity that CE with UV detection could offer, we developed a more sensitive approach in which samples, derivatized with FITC, were detected using an LIF detection system. Although the two FITC-labeled desmosines could not be resolved owing to the similarity of their structures (see peak 3 in Fig. 2b), nevertheless the method was reliable and allowed the crosslinks to be quantified as the sum of the two isomers. In the conviction that the higher the sensitivity and precision of the method employed, the more reliable will be the answer to the question of whether desmosines can actually be adequate markers of elastin degradation, we have applied this approach to the investigation of a large number of patients affected by a variety of connective tissue disorders [15, 100, 101]. Our data clearly indicated that altered levels of desmosines were present in urine and plasma of

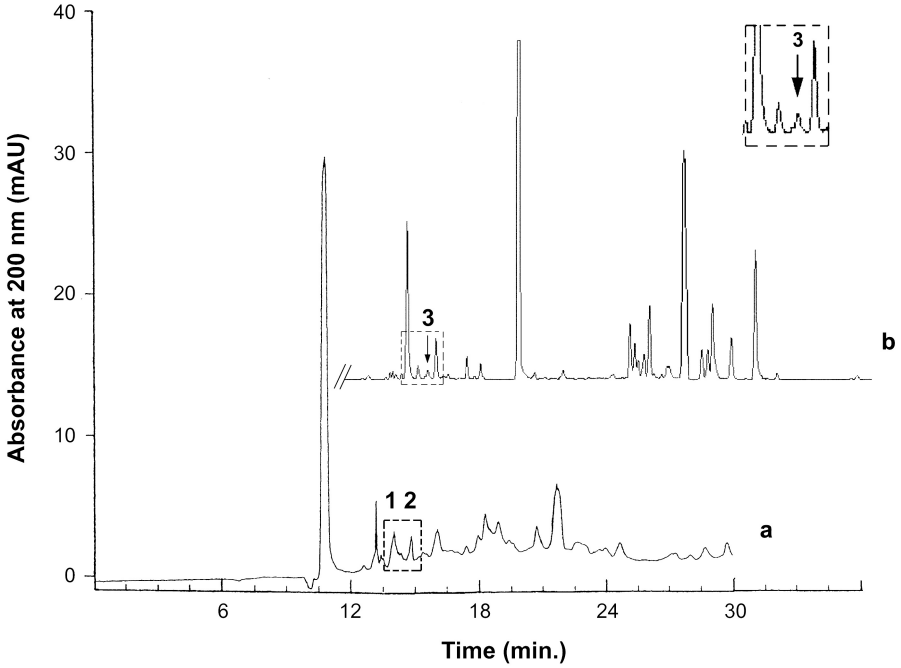

Figure 2. (a) Electrophoretic pattern indicating the peaks of isodesmosine and desmosine (peaks 1 and 2, respectively) in the hydrolyzed urine of a COPD patient, obtained using MEKC with UV detection. Separation was performed in 35 mM sodium tetraborate (pH 9.3) containing 65 mM SDS. For other experimental details see ref. [35]. (b) Electropherogram showing the peak (peak 3, expanded in the inset) of the FITC-desmosine isomers obtained by submitting (in 20 mM sodium tetraborate (pH 9.0) containing 60 mM SDS and 15% v/v methanol) the urine sample cited above to MEKC, after its derivatization with FITC. For other experimental details see ref. [38].

Table 1. A complete list of biological fluids analyzed in our laboratory, from 1998 up to date, using CE with UV or LIF detection systems, and levels of desmosines determined for each class of disorder investigated

Source	Total number of samples analyzed (n = 1017)	Date of analysis	Technique applied	Level of desmosines (Des + Ides) detected for each class of disorder investigated					
				Healthy controls (n = 215)	Smokers with normal lung function (n = 240)	Cystic fibrosis patients (n = 107)	COPD patients (n = 185)	AAT deficient patients (n = 160)	PXE patients (n = 112)
Urine[a)]	281	1998/1999	CE/UV	18.4 ± 2.2	20.7 ± 1.9	41.53 ± 8.5	34.7 ± 4.7	39.7 ± 5.1	
	40	2000/2001		20.1 ± 3.1	22.1 ± 3.0	38.75 ± 6.8	35.1 ± 4.1		
	50	2002/2003		19.6 ± 2.5	22.1 ± 3.0	42.14 ± 8.7	37.3 ± 5.0	39.7 ± 5.1	
	140	2004/2005	CE-LIF	26.19 ± 2.1			38.66 ± 6.5	45.1 ± 6.2	53.55 ± 13.38
	154	2006		28.10 ± 3.0			40.21 ± 5.8	48.0 ± 7.1	55.21 ± 14.30
Plasma/ serum[b)]	183	2004/2005	CE-LIF	54.14 ± 16.34			45.6 ± 12.4	65.1 ± 9.7	70.11 ± 15.2
	135	2006		55.21 ± 18.20			40.2 ± 15.74	62.8 ± 7.3	79.24 ± 15.3
Sputum[b)]	34	2006	CE-LIF	6.31 ± 2.8			12.21 ± 4.6		

[a)] Expressed as μg/g creatinine.
[b)] Expressed as ng/mL.

type Z AAT-deficient patients with clinically significant emphysema [15] or in patients affected by PXE [100] or in subjects with severe COPD [101], compared to healthy individuals. In the case of subjects with severe COPD, CE-LIF was applied for the first time also to the analysis of 34 sputum specimens producing results that well correlated with the data from urine and plasma of the same patients and with their clinical parameters (forced espiratory volume, FEV1).

The efficiency of the CE procedure was so good that, in few years, we have been able to analyze more than 1000 specimens (urine, plasma, and sputum). To the best of

Table 2. List of approaches described in the literature for determination of desmosines in biological fluids and tissues; indication of the LOD for each approach and reference to original literature

Method	Matrix	LOD	Reference
Amino acid analysis	Elastin hydrolysates	–	[39 – 41]
	Young animals, human uterus, and skin	0.1 nmol	[42]
	Hamster urine	0.5 nmol	[43]
	Bovine ligamentum nuchae elastin	–	[44]
	Human urine	0.1 nmol	[45, 49]
	Hamster and human lung		[46]
	Canine and human aorta	0.1 nmol	[47]
	Rat liver	0.1 nmol	[48]
	Tissue hydrolysate	0.1 nmol	[50]
	Cultured extracellular matrix	–	[51]
ELISA	Lungs, aortas, urine	2.5 pmol	[29]
	Human urine	0.02 pmol	[53, 54]
	Human urine	0.1 pmol/well	[30]
	Human urine	0.7 pmol/mL	[55]
	Human and rabbit aorta, human urine	6 pmol/mL	[56, 57]
	Human urine	0.1 pmol/well	[31]
RIA	Extracellular matrix, human urine	1 pmol	[24, 25, 28, 58–60]
	Human urine	0.4 pmol	[26, 61]
	Human urine	1 pmol	[62–64]
	Standard isodesmosine	4 pmol	[52]
	Human skin	1 pmol	[65–68]
HPLC	Hamster aortas and lungs	0.1 nmol	[69]
	Bovine and nuchae ligamentum; hamster lung and aorta	$\leq$0.1 nmol	[70]
	Human aorta	–	[71]
	Canine and human aorta	0.1 nmol	[47]
	Rat aorta, lung, and liver	0.2 nmol/mL	[33]
	Elastin	0.1 pmol	[72
	Rat aorta and liver	$\leq$3 pmol	[34]
	Human skin	0.1 nmol	[73]
	Human and hamster urine	0.1 nmol	[9, 11–13, 27, 74–77]
	Human tissues	0.1 pmol	[78]
	Elastin fron human biopsies	0.1 pmol	[79]
	Bovine ligamentum nuchae	0.1 nmol	[37, 80]
	Human urine	30 pmol	[81, 82, 87]
	Human aorta	0.1 nmol	[83, 84, 99]
	Human yellow ligament hydrolysate	0.1 nmol	[85]
	Formalin-fixed tissues	0.2 nmol	[86]
	Human sputum	0.2 pmol	[88]
	Rat lung hydrolysates	0.2 pmol	[89]
	Des and Ides solutions	0.1 pmol	[90]
	Human saphenous veins	0.1 nmol	[92]
	Human cricopharyngeal muscle	0.1 nmol	[93]
Electrophoresis	Hydrolysates of ligamentum nuchae elastin	2 nmol	[94]
	Human lung elastin	–	[95]
CE	Elastin hydrolysates	2 pmol	[36]
	Rat aorta	2 pmol	[96]
	Human urine	0.5 pmol	[35, 97–99]
	Human urine and plasma	0.5 fmol	[15, 38, 100, 101]

our knowledge, this is the largest desmosine screening ever performed on biological fluids from individuals with connective tissue disorders.

A list of all samples analyzed in our laboratory and of desmosine levels determined for each class of disorder investigated is reported in Table 1.

The approaches applied for the determination of desmosines in biological fluids and tissues; the indication of the LOD for each approach and references to original literature have been summarized in Table 2.

6 Conclusions

Reliable analytical methods are obviously needed to carry out efficient monitoring of biological markers of human

disorders. In an effort to provide the whole picture of the techniques so far described for determining elastin cross-linking amino acids, this article reports a survey of the literature concerning the utilization of different procedures in this field. The numerous publications cited show the interesting progression achieved in the past 20 years with regard to the strategies adopted for the detection of surrogate markers of elastin degradation. In addition to immunochemical and chromatographic methods, in recent years CE has attracted considerable attention for their determination in different matrices. In particular, also in the light of our own experience, MEKC may be considered not only complimentary to other techniques but certainly mature and adequate to be addressed as the method of choice for a routine use in the determination of these amino acids in real samples. The greatest merit of CE over other approaches is that it provides simple and efficient separation, in a short time, with minimum consumption of reagent and at a relatively high cost. Moreover, most methods applicable to biological matrices require sample purification and extraction procedures which are often time-consuming and may introduce external contamination and extra operational errors. With the MEKC-LIF method, no sample cleanup or extraction procedures are required and it is possible to directly analyze desmosines in the stored sample following a simple derivatization procedure and working on nanoliter volume samples.

In spite of the large number of papers published, the question whether desmosines are a valid surrogate end point of elastin degradation is still unresolved. A common misconception is to mistake a correlate with a given clinical outcome for a surrogate end point [102]. Given that an ideal biological surrogate end point should provide information on the clinical course of the disorder reliably predicting the overall clinical outcome, future studies on larger and more defined populations will be aimed at correlating the estimates of excreted desmosines with the severity of the disorder responsible for elastin destruction.

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