ELSEVIER

Journal of Chromatography B, 668 (1995) 199–207

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

# Enrichment and analysis of desmosine and isodesmosine in biological fluids

Wayne R. Cumiskey, Edward D. Pagani, Donald C. Bode*

*Sterling Winthrop Pharmaceuticals Research Division, Department of Pharmacology, Collegeville, PA, USA*

First received 10 October 1994; revised manuscript received 15 February 1995; accepted 15 February 1995

**Abstract**

A method has been developed for the enrichment and analysis of the elastin crosslinks, desmosine and isodesmosine, in biological fluids and tissues. It is adapted from published methods, offering improved recovery, sensitivity, resolution, and speed of analysis. Samples were hydrolyzed in 6 *M* HCl, after which the desmosines were enriched by CF1 cellulose chromatography and analyzed by HPLC with a $C_{18}$ column. Isodesmosine and desmosine were quantitated based on absorbance at 275 nm, with a limit of detection of approximately 30 pmol and recovery of approximately 66% in urine. Their $t_R$ values on our HPLC system were approximately 9 and 12 min, respectively. This method was used to evaluate the daily and weekly variation in the concentrations of desmosine and isodesmosine in human urine. The results suggest that this method can be used to process large numbers of biological samples for analysis of desmosine and isodesmosine.

## 1. Introduction

Neutrophil elastase (EC 3.4.21.37) is a serine protease whose physiological substrates include elastin and extracellular matrix components such as collagen and fibronectin. There is at least suggestive evidence for the involvement of elastase in the pathogenesis of a variety of diseases, including chronic obstructive pulmonary disease [1,2], cystic fibrosis [3–5], and adult respiratory distress syndrome [6]. Thus, considerable effort has been devoted to measuring biochemical markers of in vivo elastase activity, with the aims of (1) verifying the involvement of elastase in specific pathological conditions, and (2) demonstrating the efficacy of potential elastase inhibitors in animal models and in humans.

Perhaps the most direct biochemical marker of in vivo elastin degradation is the concentration of desmosine and/or its isomer isodesmosine, rare amino acids that are unique to elastin. Due to the fact that, in mammals, they are only found in elastin [7], the appearance of desmosines in solution, either free or in peptides, must be indicative of elastin breakdown. Other positive attributes of desmosines as markers are the limited absorption of dietary desmosines [8–10] and their metabolic and chemical stability [8] (Cumiskey, unpublished observations). Furthermore, desmosines and desmosine-containing peptides appear to be filtered completely by the kidney and excreted almost exclusively by this route [11]. Thus, urine is a convenient source of

* Corresponding author.

0378-4347/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
*SSDI* 0378-4347(95)00092-5

metabolically generated desmosines, the concentration of which is believed to reflect the amount of elastase activity in the body. A disadvantage of urinary desmosines as a marker of elastase activity in a particular organ (e.g. the lung) is that it is not an organ-specific marker. However, elevation of urinary desmosines associated with organ-specific damage or disease, in the clinic or in an animal model, could be presumed to reflect an increase in elastase activity in that organ.

Several methods have been reported for the assay of desmosines in biological fluids and tissues. In general, desmosines are assayed together or separately, in peptides or free (after acid hydrolysis), by HPLC [12–16] or radioimmunoassay (RIA) [2,3,17–21]. In some cases, there has been a concern with the specificity of the antibody and interference by other sample components [16,22]. HPLC methods, which generally require hydrolysis of peptides to free desmosines followed by enrichment prior to analysis, generally suffer from low recovery of desmosines and are more labor-intensive than RIA methods.

We have combined aspects of two published methods [12,23] into one that can be used to analyze large numbers of samples with good recovery and reproducibility. The results suggest that, with this analytical method, urinary desmosines can be used as an index of in vivo elastase activity, either in clinical or pre-clinical studies.

## 2. Experimental

All procedures involving animals described in this manuscript were performed in accordance with the Sterling Winthrop Pharmaceuticals Research Division (SWPRD) Policy on Animal Use and all federal regulations. The SWPRD animal facilities and programs are accredited by the American Association for Accreditation of Laboratory Animal Care.

### *2.1. Materials*

Desmosine and isodesmosine standards, and porcine pancreatic elastic were from Elastin Products (Owensville, MO, USA); Nucleosil $C_{18}$ analytical HPLC columns (150 × 4.6 mm I.D., 5 $\mu$m particle size) from Alltech Associates (Deerfield, IL, USA); WISP 710B autosampler, Model 991 photodiode array detector, and Bondapak $C_{18}$, 10-$\mu$m precolumns from Waters Chromatography Division, Millipore (Milford, MA, USA); Model HPX HPLC pump from Rainin (Woburn, MA, USA); Model LC-95 UV absorbance detector from Perkin Elmer (Norwalk, CT, USA); CF1 cellulose from Whatman (Clifton, NJ, USA); Econo-Pac and Poly-Prep columns and funnels for CF1 chromatography from Bio-Rad Laboratories (Richmond, CA, USA); conical polypropylene centrifuge tubes and microfuge tubes from Sarstedt (Newton, NC, USA); chemicals (analytical or HPLC grade) from J.T. Baker (Phillipsburg, NJ, USA); and reagents for the creatinine assay from Sigma Diagnostics (St. Louis, MO, USA).

### *2.2. Collection of urine samples*

Urine samples (of 24 h) were collected from healthy human volunteers in 3-l plastic containers without a preservative. The urine collection for any given day consisted of all urine after the first morning urine of that day, including the first urine from the following morning. The container was stored at 4°C until it was received in our laboratory. Upon receipt, it was mixed well by inversion, and aliquots were removed for analysis of creatinine and desmosines. The aliquots were stored at −20°C.

Hamster urine was collected over a 24-h period in metabolism cages, centrifuged at 1000 *g* for 10 min to pellet any particulate material, and the supernatant was stored at −20°C. Typically, 4 to 8 ml of urine were collected per hamster per day.

### *2.3. Preparation and assay of hamster lung homogenates*

After administering a lethal dose of pentobarbital i.p., hamster lungs were removed rapidly and rinsed well in 0.9% NaCl at room temperature. One pair of lungs was used per experiment. The lungs were homogenized at

room temperature, with a motor-driven Teflon/glass tissue grinder, in ten volumes of buffer containing 0.1 *M* Tris–HCl (pH 7.5 at 37°C) and 0.5 *M* NaCl. Aliquots of the homogenate were incubated, with gentle oscillation, at 37°C with porcine pancreatic elastase (300 $\mu$g/g lung) for various times. At the end of the incubation, the homogenate was centrifuged at 200 000 *g* for 15 min at 4°C, the surface of the pellet was rinsed gently (and the rinses combined with the supernatant), and the pellet and supernatant fractions were separately processed for analysis of desmosines as described below.

### 2.4. Processing of samples of biological fluids and tissues

The sample (urine or a tissue homogenate) was mixed well prior to dispensing an aliquot into the hydrolysis tube, and an equal volume of concentrated HCl added (final concentration 6 *M* HCl). For human urine, a convenient sample size was 24 ml. Hamster urine samples were smaller in volume, and were used as is or after dilution with water to a convenient volume. The hydrolysis tubes were 50-ml conical polypropylene tubes. The contents were overlayed with a stream of argon, then inverted several times to mix and placed in an oven at 92°C overnight (approximately 16 h). The recovery of desmosine and isodesmosine under these conditions was approximately 100%.

After hydrolysis, the tubes were allowed to cool to room temperature (this is critical prior to the subsequent centrifugation step) and mixed by inverting. All subsequent operations were performed at room temperature, unless indicated otherwise. After centrifugation at 2000 *g* for 15 min, the supernatant was transferred to a glass-stoppered Erlenmeyer flask and the following reagents added, *in order, with mixing after each addition*: one volume glacial acetic acid (reagent grade), and 4 volumes *n*-butanol (reagent grade). This mixture is referred to as the "sample cocktail".

A slurry of fibrous cellulose powder (Whatman CF1) was prepared in "CF1 mobile phase" (water–glacial acetic acid–*n*-butanol, 1:1:4), in a ratio of 1 g cellulose powder to 20 ml CF1 mobile phase, then degassed by brief sonication or by standing overnight. An individual CF1 column was prepared for each sample by carefully pipetting 10 ml of stirred CF1 slurry into 20-ml polypropylene disposable columns (Bio-Rad Econo-Pac columns) fitted with funnels and top frits. Care must be taken to avoid introducing air bubbles into the resin bed by (1) individually pipetting the resin for each column; (2) never interrupting the flow of mobile phase at any time after the resin has been added, to avoid disturbing the continuity of the resin bed; (3) adding a top frit just above the bed of each column, after most of the CF1 cellulose has settled but before the sample is added; and (4) never allowing the top of the bed to dry out. If air spaces appear during the procedure, the processing generally slows down considerably, with a concomitant reduction in the recovery of desmosines. The CF1 columns were run by gravity.

The sample cocktail was applied to the column (in several aliquots due to sample volume and column capacity), mixing prior to the addition of each aliquot to ensure homogeneity. After the sample had run into the column bed, the column was washed with 15 slurry volumes (150 ml) of CF1 mobile phase. Smaller wash volumes were less effective in terms of removing contaminants, and did not improve the recovery of desmosines. Finally, desmosines were eluted with 7.0 ml of water; larger elution volumes did not increase recovery (data not shown). Several minutes were allowed for separation of water and upper organic phase, which was then aspirated (taking care not to aspirate any of the lower aqueous phase containing the desmosines).

For human urine samples, a second CF1 chromatographic step was required; this served to remove many residual interfering components with minimal loss of desmosines (data not shown). To the water eluate containing desmosines was added an equal volume of glacial acetic acid, then 4 volumes *n*-butanol, mixing after each addition in a glass-stoppered Erlenmeyer flask. This second "sample cocktail" was applied to a second, smaller CF1 column, prepared by adding 5 ml CF1 slurry (described above) to 10-ml polypropylene disposable col-

umns (Bio-Rad Poly-Prep; no top frit is available) fitted with funnels. After most of the sample had run into the column bed, the column was washed with 10 slurry volumes (50 ml) of CF1 mobile phase, after which desmosines were eluted with 5 ml of water. The upper organic phase was aspirated carefully, and the aqueous phase evaporated to dryness under vacuum. The residue was stored at 4°C in capped tubes prior to analysis by HPLC.

### 2.5. Analysis of processed biological samples

The residue from CF1 chromatography was reconstituted (on the day of analysis) in 150 μl of 0.1 *M* sodium phosphate, pH 3.75, the main constituent of the HPLC mobile phase (see below). After vortex-mixing well, the contents were transferred to a 400-μl polypropylene microfuge tube and centrifuged to pellet any sediment.

The HPLC apparatus consisted of a single Rainin HPX pump delivering isocratic mobile phase, of which 80% was 0.1 *M* dibasic sodium phosphate (analytical grade) adjusted to pH 3.75 with phosphoric acid; the remaining 20% was acetonitrile. Sodium dodecyl sulfate (SDS) was added to a final concentration of 10 m*M*. The pH was re-adjusted after the addition of acetonitrile and SDS. The flow-rate was 0.8 ml/min through a Waters Bondapak $C_{18}$ precolumn coupled to a 150 × 4.6 mm I.D. Nucleosil $C_{18}$, 5-μm analytical column. Injection volumes of 1 to 100 μl were typically used; 100 μl of human urine extract (prepared as above from 24 ml of urine and reconstituted in 150 μl) was suitable for detection of desmosines in samples of human urine. Injections were performed manually or with a Waters WISP 710B autosampler. Detection of desmosines was by absorbance at 275 nm, using a Perkin-Elmer LC-detector. Peak purity was checked with a Waters 991 photodiode array detector, scanning from 190 to 600 nm with 3 nm resolution. In the case of pure peaks, the UV absorbance spectra were identical throughout the peak. Isodesmosine eluted at approximately 9 min and desmosine at approximately 12 min.

### 2.6. Quantitation of desmosine and isodesmosine concentrations

Peak height and appropriate external standard calibration curves were used to quantitate desmosine and isodesmosine in unknown samples. Standard peak-height response curves were constructed by HPLC injections representing 10 to 5000 pmol, which bracket the values expected from test samples. The limit of detection is approximately 30 pmol (300 pmol/ml in 100 μl of reconstituted extract), equivalent to 2 pmol/ml in unhydrolyzed urine.

To calculate recoveries, selected samples (e.g., urine from control subjects) were spiked with a known amount of both desmosine and isodesmosine; the net recoveries were determined by comparison with external standards. The recovery is independent of the amount spiked (data not shown). The recovery standard was added to the sample prior to hydrolysis and carried through the entire hydrolysis and isolation procedure. The corresponding unspiked sample must be processed and analyzed at the same time as the spiked sample in order to correct for endogenous desmosines, the concentration of which did not affect the calculated recovery (data not shown). The amounts of desmosine and isodesmosine in spiked and unspiked samples were determined by comparison with a standard curve. The raw net recovery, i.e. the difference between the aliquots from spiked and unspiked samples, was then corrected for the fraction of the extract that was analyzed by HPLC (e.g., 100 μl out of 150 μl total) and for the fraction of the original urine sample that was processed. The result, the corrected net recovery, was applied to each unknown that was processed at the same time.

### 2.7. Creatinine assay

The concentration of creatinine in urine was measured with a Roche Cobas Fara II automated analyzer (Sigma Diagnostics Procedure No.

557). All urinary desmosine and isodesmosine concentrations were normalized for the concentration of creatinine in the same sample, to account for any variation in glomerular filtration rate within and between individual subjects. The concentration of creatinine in human and hamster urine did not change over a seven-day period at room temperature, 4°C, or −20°C (data not shown).

## 3. Results

Representative HPLC chromatograms are shown in Fig. 1, illustrating the resolution of isodesmosine from desmosine, the separation of each from other components of human and hamster urine, and the co-elution of authentic standards of each with peaks in spiked and unspiked urine.

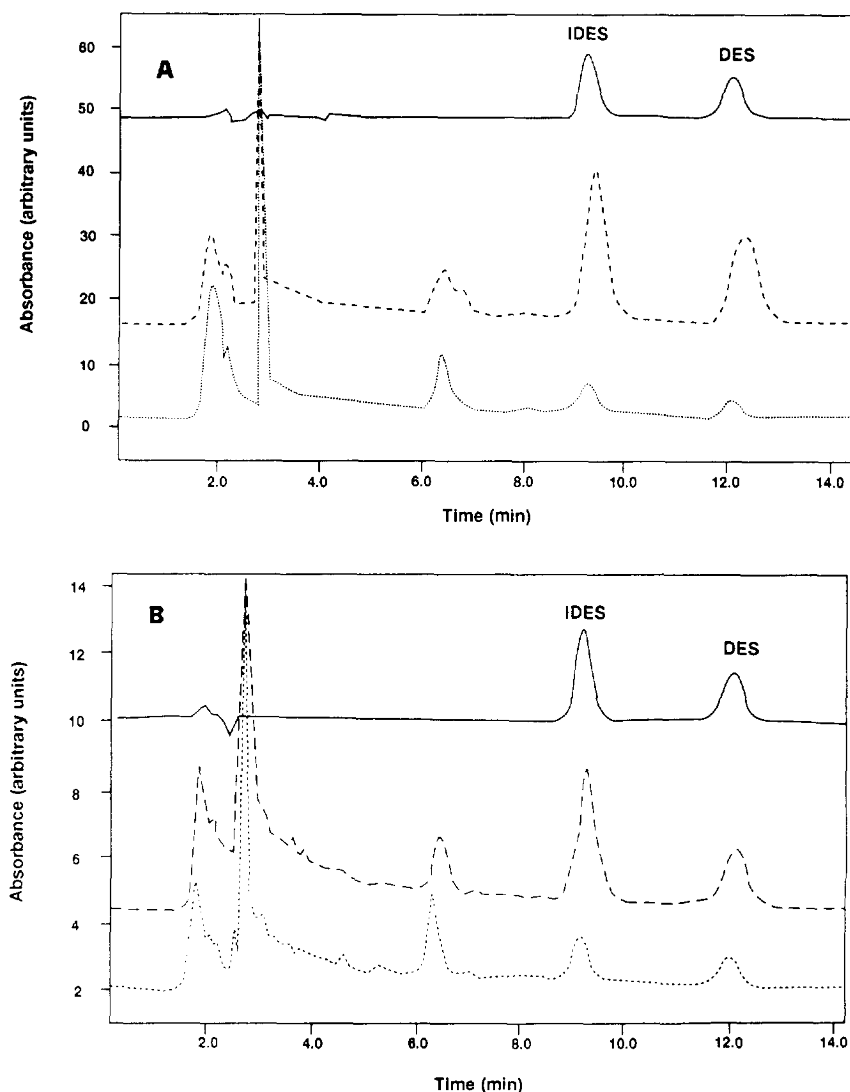


Fig. 1. Chromatograms of (1) a mixture (300 pmol each) of isodesmosine (IDES) and desmosine (DES) standards (top tracing in each panel), (2) extract of urine spiked with isodesmosine and desmosine standards (middle tracing), and (3) extract of unspiked urine (bottom tracing). The peaks of interest are indicated. (A) Human urine; (B) hamster urine.

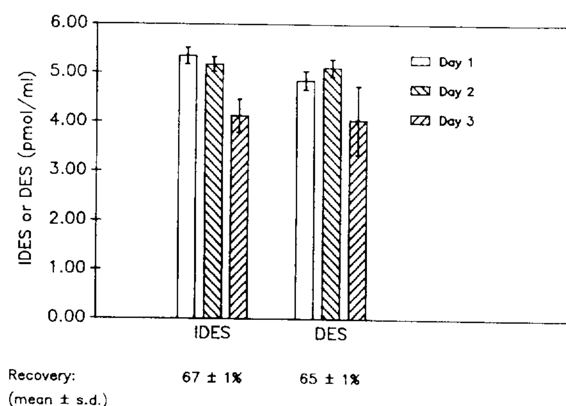


Fig. 2. Reproducibility of the processing and analysis of isodesmosine (IDES) and desmosine (DES) in human urine. Aliquots of the same sample were separately processed and analyzed in triplicate on each of three different days.

To demonstrate the reproducibility of the method for processing and analyzing desmosines, nine aliquots of a sample of human urine were frozen. Three aliquots were thawed, processed, and analyzed on each of three different days over a two-week period. The results, shown in Fig. 2, are expressed as raw concentrations (not normalized for the creatinine concentration). On the third day of analysis, all of the values were lower and more variable, for reasons that are not known. The day-to-day variability was not due to differences in recovery. The recoveries shown in Fig. 2, 67% for isodesmosine and 65% for desmosine, are typical values.

Another illustration of inter-assay variability is presented in Table 1, in which six different samples of human urine (from three subjects) were each analyzed on two separate occasions. The data are normalized for the creatinine concentration and expressed as pmol of isodesmosine or desmosine per mg creatinine. The ratios of the isodesmosine or desmosine concentration determined on day 1 to the value determined on day 2 ranged from 0.74 to 1.42, with a mean ratio of 0.94 ± 0.05 (S.E.M.).

The identities and purity of the peaks, in human and hamster urine, which co-elute with authentic isodesmosine and desmosine standards, were confirmed using a photodiode array detector (data not shown). The absorbance spectrum of the 9-min peak from hamster or human urine was identical to the corresponding peak for an authentic isodesmosine standard. Likewise, the spectrum of the 12-min peak from hamster or human urine was identical to the corresponding peak for an authentic desmosine standard. This strongly suggests that the peaks in urine extracts that co-elute with standards represent isodesmosine and desmosine, and that they are pure. Changing the mobile phase composition to 75% sodium phosphate and 25% acetonitrile, which shifted the $t_R$ of isodesmosine and desmosine to 6 and 8 min, respectively, resulted in a pure desmosine peak but a contaminated isodesmosine peak from human urine. Both peaks in hamster urine remained pure under these chromatographic conditions.

In order to determine the degree of intra-

Table 1
Inter-assay variability in desmosine and isodesmosine concentration in urine samples from healthy subjects

| Subject | Sample | Concentration (pmol/mg creatinine) | | | | | |
|---|---|---|---|---|---|---|---|
| | | IDES | | DES | | IDES + DES | |
| | | Day 1 | Day 2 | Day 1 | Day 2 | Day 1 | Day 2 |
| 11 | A | 7.06 | 7.06 | 10.43 | 7.34 | 17.49 | 14.40 |
| 11 | B | 6.99 | 8.00 | 7.58 | 9.55 | 14.57 | 17.55 |
| 12 | C | 10.27 | 9.67 | 7.92 | 8.12 | 18.19 | 17.79 |
| 12 | D | 12.74 | 14.03 | 10.12 | 12.08 | 22.86 | 26.11 |
| 13 | E | 4.52 | 6.10 | 5.06 | 6.47 | 9.58 | 12.57 |
| 13 | F | 7.61 | 8.08 | 8.52 | 8.88 | 16.13 | 16.96 |

subject variability over time as well as the degree of variability between subjects, the following study was conducted. Twenty-four-hour urine samples were collected from three normal human volunteers over fourteen consecutive days, and once a week for twelve consecutive weeks. The data, normalized for urinary creatinine concentration, are presented in Figs. 3 and 4. The mean data (shown in the top half of each figure) indicate that the urinary desmosine and isodesmosine concentrations for normal humans are fairly consistent, suggesting that this may be a good biochemical marker for in vivo elastase activity. The data for individual days (shown in bottom half of each figure) illustrate the intra-subject variability inherent in this marker, and suggest that measurement of desmosines in a single urine sample from a subject may not necessarily provide an accurate assessment of ongoing elastase activity.

Another application of the analysis of desmosines in biological tissues is illustrated in Fig. 5. Hamster lungs were removed, rinsed well, and homogenized. Aliquots of the lung homogenates were incubated with porcine pancreatic elastase for varying time intervals, after which the particulate and soluble fractions were analyzed for desmosines. The disappearance of isodesmosine and desmosine from the pellet was mirrored by their appearance in the supernatant. This provides an in vitro assay for the degradation of lung elastin that is more specific than, for example, monitoring the increase in absorbance at 280 nm in the supernatant, which could reflect the

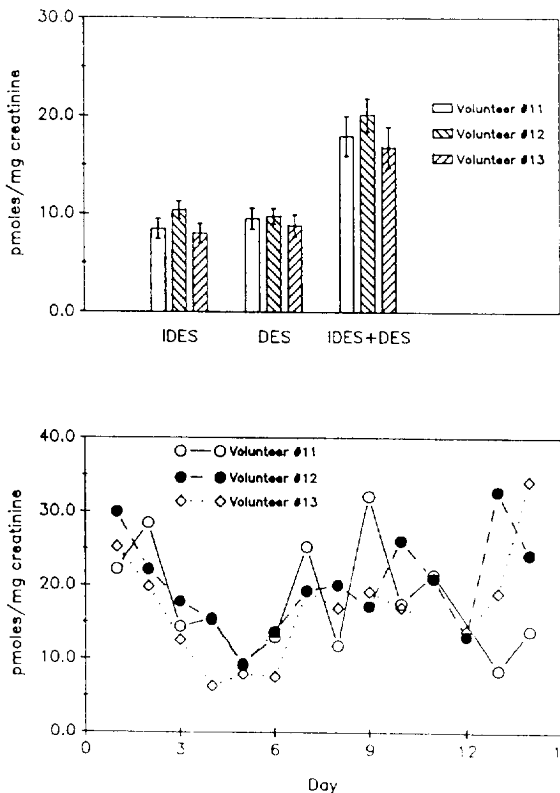


Fig. 3. Inter- and intra-subject variability in the daily excretion of isodesmosine (IDES), desmosine (DES), and their sum (IDES + DES) in human urine over fourteen consecutive days. The data are expressed as pmoles excreted in 24 h, and are normalized to creatinine.

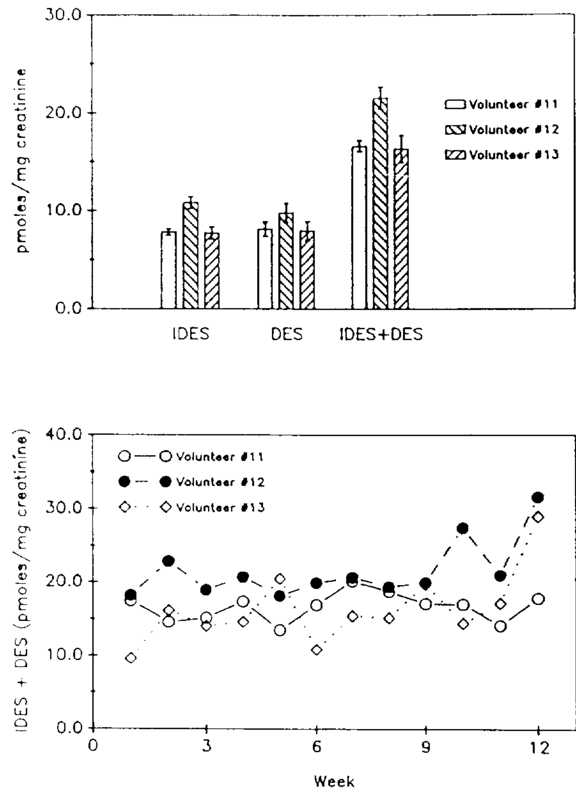


Fig. 4. Inter- and intra-subject variability in the daily excretion of isodesmosine (IDES), desmosine (DES), and their sum (IDES + DES) in human urine over twelve consecutive weeks. The data are expressed as pmoles excreted in 24 h, and are normalized to creatinine.

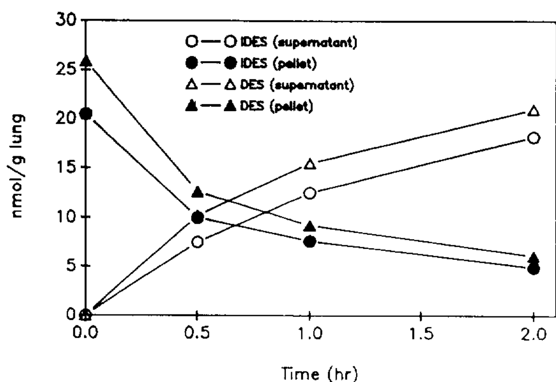


Fig. 5. Time course of the movement of isodesmosine (IDES) and desmosine (DES) from the pellet to the supernatant fraction of hamster lung homogenates following exposure to porcine pancreatic elastase in vitro.

degradation of particulate proteins other than elastin (e.g., collagen).

## 4. Discussion

Elastin, a major component of elastic connective tissue, is one of several endogenous substrates of neutrophil elastase, as well as the elastases from the pancreas and macrophages. In addition, elastin appears to be refractory to hydrolysis by other enzymes such as collagenase and stromelysin. Thus, it seems reasonable to focus on the elastinolytic activity of neutrophil elastase as a therapeutic target in certain pulmonary diseases.

With this in mind, our goal was to develop an improved procedure for the analysis of desmosine and isodesmosine, the elastin cross-links. Due to the potential for non-specific interference with RIAs, HPLC was our analytical tool of choice. Using the method detailed herein, which is based on the work of Skinner [23] and Yamaguchi et al. [12], we have obtained reasonably high (upwards of 65%) and reproducible recoveries of desmosines. We have attempted to provide sufficient detail to enable others to reproduce our results; in many cases, seemingly minor changes drastically affected the recovery and analysis.

With biological samples such as lung homogenates and urine, it was necessary to enrich the samples by means of an initial CF1 cellulose chromatographic cleanup step. At the final stage of analysis by HPLC, desmosine and isodesmosine were eluted separately from each other and from other interfering substances within five to fifteen minutes, allowing their individual quantitation and the analysis of a fairly large number of samples (at least three to four per hour), which can be run overnight with an autosampler. The results shown for representative samples of human and hamster urine demonstrate the resolution obtained by the combined enrichment and analytical chromatographic steps.

One advantage of HPLC (vs. RIA) for analysis is that the purity and identity of the peak of interest in a biological sample can be verified using a photodiode array detector. We used this tool to verify the purity and identity of the peaks in hamster and human urine that co-eluted with authentic desmosine and isodesmosine standards. In human urine, a minor change in the composition of the mobile phase resulted in detectable contamination of isodesmosine with an unidentified impurity. Such a purity check would have been impossible with an RIA.

Prior to initiating clinical studies to determine whether or not urinary desmosines are elevated in particular patient populations, we determined the inter- and intra-subject variability in urinary desmosines in a limited number of healthy subjects. Based on the results of these studies, we concluded that the precision of the analytical method is adequate and that the mean levels in healthy subjects are consistent enough to use urinary desmosines as a biochemical marker. However, the day-to-day variability in a given subject is such that urine samples may need to be collected on multiple days in order to reliably assess ongoing elastase activity.

In addition to the application of this analytical method to human and animal urine samples, it can be utilized to quantitate desmosines in other tissues of interest such as the lung. For example, the movement of desmosines from the particulate to the soluble fraction during incubation of hamster lung homogenates with porcine pan-

creatic elastase in vitro is a specific assay of elastinolytic activity.

The method described for enrichment and analysis of desmosine and isodesmosine in biological fluids and tissues is specific, reproducible, and versatile. Its use should help to define the role of elastase-mediated elastin degradation in in vitro and in vivo animal models, as well as in human diseases.

## References


[1] G.L. Snider, Ann. Rev. Med., 40 (1989) 411.
[2] E.E. Schriver, J.M. Davidson, M.C. Sutcliffe, B.B. Swindell and G.R. Bernard, Am. Rev. Respir. Dis., 145 (1992) 762.
[3] M.C. Bruce, L. Poncz, J.D. Klinger, R.C. Stern, J.F. Tomashefski and D.G. Dearborn, Am. Rev. Respir. Dis., 132 (1985) 529.
[4] C.M. O'Connor, K. Gaffney, J. Keane, A. Southey, N. Byrne, S. O'Mahoney and M.X. Fitzgerald, Am. Rev. Respir. Dis., 148 (1993) 1665.
[5] K.C. Meyer and J. Zimmerman, J. Lab. Clin. Med., 121 (1993) 654.
[6] M.F. Tenholder, K.R. Rajagopal, Y.Y. Phillips, T.A. Dillard, L.L. Bennett, T.G. Mundie and C.J. Tellis, Chest, 100 (1991) 1385.
[7] S.M. Partridge, Fed. Proc., 25 (1966) 1023.
[8] B.C. Starcher and R.A. Goldstein, J. Lab. Clin. Med., 94 (1979) 834.
[9] S.S. Yu, S.K. Ruthmeyer and J.W. Shepard, Proc. Soc. Exp. Biol. Med., 161 (1979) 239.
[10] V. Pai, A. Guz, G.J. Phillips, N.T. Cooke, D.C.S. Hutchinson and T.D. Tetley, Metabolism, 40 (1991) 139.
[11] R.A. Goldstein and B.C. Starcher, J. Clin. Invest., 61 (1978) 1286.
[12] Y. Yamaguchi, J. Haginaka, M. Kunitomo, H. Yasuda and Y. Bando, J. Chromatogr., 422 (1987) 53.
[13] J.N. Manning, G.S. Sullivan and P.F. Davis, J. Chromatogr., 487 (1989) 41.
[14] E. Guida, M. Codini, C.A. Palmerini, C. Fini, C. Lucarelli and A. Floridi, J. Chromatogr., 507 (1990) 51.
[15] F. Nakamura and K. Suyama, J. Chromatogr. Sci., 29 (1991) 217.
[16] P.J. Stone, J. Bryan-Rhadfi, E.C. Lucey, D.E. Ciccolella, G. Crombie, B. Faris, G.L. Snider and C. Franzblau, Am. Rev. Respir. Dis., 144 (1991) 284.
[17] G.S. King, B.C. Starcher and C. Kuhn, Bull. Eur. Physiopath. Resp., 16 (suppl.) (1980) 61.
[18] S. Harel, A. Janoff, S.Y. Yu, A. Hurewitz and E.H. Bergofsky, Am. Rev. Respir. Dis., 122 (1980) 769.
[19] S.F. Davies, K.P. Offord, M.G. Brown, H. Campe and D. Niewoehner, Am. Rev. Respir. Dis., 128 (1983) 473.
[20] F. Pelham, M. Wewers, R. Crystal, A.S. Buist and A. Janoff, Am. Rev. Respir. Dis., 132 (1985) 821.
[21] T.J. Dillon, R.L. Walsh, R. Scicchitano, B. Eckert, E.G. Cleary and G. McLennan, Am. Rev. Respir. Dis., 146 (1992) 1143.
[22] B. Starcher and M. Scott, Ann. Clin. Biochem., 29 (1992) 72.
[23] S.J.M. Skinner, J. Chromatogr., 229 (1982) 200.