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Micellar electrokinetic chromatography for the determination of urinary desmosine and isodesmosine in patients affected by chronic obstructive pulmonary disease

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Abstract

The presence in urine of desmosine (DES) and isodesmosine (IDES), two crosslinked amino acids unique to the elastic fiber network, can be used as a specific indicator of degradation of mature elastin. Compared to methodologies so far available, the capillary electrophoretic technique reported here seems to be suitable and convenient for determining desmosines in urine of patients affected by chronic obstructive pulmonary disease (COPD). By using 35 m*M* sodium tetraborate pH 9.3 containing 65 m*M* SDS as the background electrolyte, the peaks of DES and IDES could be detected in hydrolyzed urine samples from controls and patients. Owing to the simultaneous determination of endogenous urinary creatinine used as appropriate internal standard, the amount of these amino acids could be accurately quantified. The results obtained were of the same order of magnitude as the data already reported in the literature for COPD patients. Thus micellar electrokinetic chromatography (MEKC) may be considered as a reliable technique for studying the turnover of the elastic fiber in clinical conditions. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Desmosine; Isodemosine

1. Introduction

Pulmonary emphysema and chronic bronchitis, usually included in the mutually embracing term "Chronic Obstructive Pulmonary Disease" (COPD) are clinical conditions characterized by disabling air flow limitation and dyspnoea [1]. The more common form of emphysema (centrilobular emphysema) is usually associated with cigarette smoking and, although direct evidence of increased elastin degradation in smokers or in subjects with COPD is still limited, most investigators accept that irreversible destruction of elastic fibers within the lung interstitium is central to the development of this disease [2,3]. The extent of degradation of crosslinked elastin in the body can be monitored by measuring the urinary excretion of desmosine (DES) and isodesmosine (IDES), two crosslinked aminoacids that are unique to elastin and which are formed upon oxidative deamidation of lysyl residues in tropoelastin followed by the spontaneous condensation of adjacent aldehydes [4]. Gottlieb et al. [5] have recently demonstrated that the quantity of these specific markers is higher in urine of apparently

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healthy smokers and of COPD patients than in that of healthy nonsmokers. The method they developed for measuring DES and IDES in the urine also allowed simultaneous detection of two specific markers for mature collagen degradation, i.e. hydroxylysylpyridinoline and lysylpyridinoline [6,7]. DES and IDES were purified from most contaminants by gel permeation chromatography and separated by reversed-phase high-performance liquid chromatography (RP-HPLC). The amount of cross-linked amino acids recovered from the various chromatographic steps was quantified exactly only upon addition of known amounts of labeled [^{14}C]DES and [^{14}C]IDES to the original urine samples. Moreover, as the concentration of these two amino acids in urine is usually expressed as a ratio to endogenous creatinine, it was necessary to calculate the quantity of the latter through an independent assay [7]. Although the reliability of this method was considerably higher than the previously reported radioimmunoassay (RIA) [8–11] or the enzyme-linked immunosorbent assay (ELISA) [12,13], nevertheless it was still labour-intensive and may have severe limitations in terms of complexity and sensitivity.

The aim of this paper is to demonstrate the feasibility of high-performance capillary electrophoresis (HPCE) as an alternative technique for estimating urinary DES and IDES. We used micellar electrokinetic chromatography (MEKC) for simultaneous detection of creatinine and desmosines. This approach is simple and reliable and has proved to be useful because it has the advantage of automated processing of large numbers of samples. Owing to its reproducibility it could become an efficient aid for routine diagnostic screening of diseases involving degradation of elastic fibers.

2. Experimental

2.1. Chemicals

Standard desmosine and isodesmosine were obtained from Elastin Products (Owensville, MO, USA) and standard creatinine was purchased from Sigma (St. Louis, MO, USA). Doubly distilled water utilized for all CE experiments was obtained from a Millipore (Bedford, MA, USA) Milli-Q purification system. All other chemicals were of analytical reagent grade and were used without further purification.

2.2. Origin of urine samples and their treatment

Urine from eight healthy adult controls (nonsmokers, volunteer laboratory workers) and from eight COPD patients was collected for 24 h and stored at −20°C until further use.

Aliquots of 500 ml were removed and reduced to a volume of 50 ml with a rotary evaporator (the temperature of the sample was raised to 50°C under reduced pressure). Urine was then treated for 10 min at room temperature with activated charcoal (Norite A) to decolorize it, filtered on 0.45 μm Millipore filters and centrifuged for 10 min at 12 000 *g*. Aliquots (2 ml) of each urine sample were used for direct injection onto the capillary electrophoretic system or transferred to hydrolysis pyrex tubes, evaporated to dryness in vacuo and hydrolyzed by refluxing with twice distilled constant boiling HCl (5.9 *M*) at 106°C for 24 h. The hydrolyzed samples were dried under a nitrogen stream, the residue was diluted with deionized water and the evaporation repeated twice. Complete removal of HCl was accomplished by keeping the samples overnight under reduced pressure over NaOH. The samples were finally taken up with 0.5 ml of the background electrolyte and processed as above.

2.3. Capillary electrophoretic instrumentation and running conditions

All runs were performed using a Biofocus 3000 system (Bio-Rad, Richmond, CA, USA) equipped with a high-speed UV–Vis scanning detector. Samples were injected by pressure (5 s, 0.07 MPa) onto an uncoated fused-silica capillary of 57 cm (50 cm effective length)×50 μm I.D..

Separations were performed using 35 m*M* sodium tetraborate, pH 9.3 containing 65 m*M* sodium dodecyl sulfate (SDS) as the running buffer. Temperature was maintained at 15°C, the applied voltage was 10 kV and the typical operating current was 42±0.5 μA. Migration was positive to negative polarity.

Analytes were monitored at 200, 214 and 268 nm and data were recorded at the same wavelengths. In addition, spectra were acquired at 5 nm intervals from 190 to 320 nm in parallel. Between runs the capillary was flushed for 2 min with electrolyte buffer.

3. Results and discussion

When elastic fiber network is destroyed, peptides containing desmosine and isodesmosine, two elastin-specific crosslinked aminoacids whose structures are shown in Fig. 1, are excreted in the urine in amounts

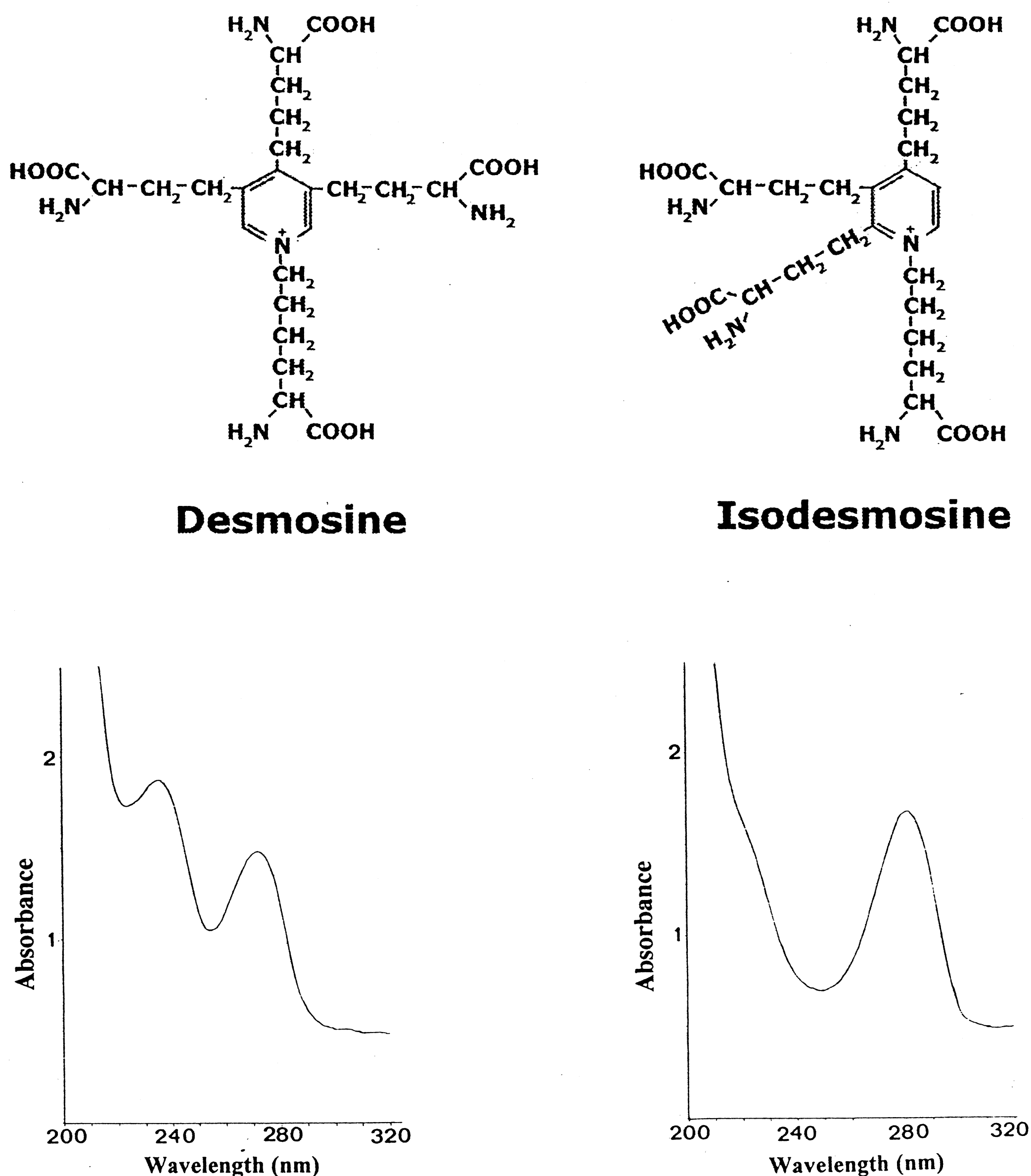

Fig. 1. Molecular structures and ultraviolet absorption spectra of desmosine and isodesmosine.

proportional to the degree of destruction [14]. The measurement of these fragments can therefore be used as a method for estimating the degradation of body elastin. The presence of desmosine-containing peptides in the urine has been documented by several previous publications [2,7,15–17] and attempts to establish whether CE is well suited as a replacement for traditional methods have been carried out in our laboratory. Experiments performed using 35 m*M* sodium tetraborate, pH 9.3 containing 65 m*M* SDS as the background electrolyte allowed identification of some elastin-derived peptides (peaks 1 to 4 of Fig. 2) in urine of COPD patients. As shown in the electropherogram of Fig. 2, which is representative of separation profiles obtained from patients' urine, although ultraviolet absorption spectra (inset of Fig. 2) indicated the presence of these crosslinked amino acids in some peaks, interference from contaminants was heavy and careful optimization of experimental conditions was required to achieve better separation. However, since elastin-derived peptides are not specific markers of mature elastin degradation [3] and because isolation of peptides went beyond the aim of the present investigation, no further attempts were made to improve their separation. Our attention was concentrated on CE determination of urinary DES and IDES, surrogate markers which are released upon hydrolysis of elastin breakdown products.

Although a HPCE method for detecting these compounds in solutions of hydrolyzed elastin has already been reported [18], the free solution CE mode of separation employed prevented accurate quantification of DES and IDES, the two peaks being very close to each other. As previously suggested by Yamaguchi et al. [19] who developed a HPLC method for the determination of desmosines in tissues, the addition of a micellar buffer modifier such as SDS to the background electrolyte, provided enhanced resolution and thus almost complete separation of the two compounds was achieved under the same electrophoretic conditions as those described above for the separation of peptides. As shown in the profile in Fig. 3 (panel A) the two peaks of standard IDES and DES (peaks 1 and 2) were near-baseline separated in a relatively short analysis time, the calculated retention times being 13.65 ± 0.15 min for IDES and 14.1 ± 0.15 min for DES. This method guaranteed high reproducibility of separation over more than fifty repeated injections of different amounts of standard components with the relative standard deviation (R.S.D.) for migration times being about 1%. Calibration curves for both compounds in a range of concentration from 0.1 to 10 m*M* showed excellent linearity of peak area response vs. amount of analyte injected with correlation coefficients of 0.998 and 0.997 for IDES and DES, respectively (inset of panel A). Their calculated detection limit in terms of amount of analyte introduced into the capillary was as low as 0.5 pmol for a 1-s injection at a signal-to-noise ratio of 3.5. The absorbance vs. retention time vs. wavelength relationship for standard desmosines is shown in the three-dimensional electropherograms of panel B.

On the basis of the CE conditions established for the reference compounds, all urine samples were analyzed by MEKC. In accordance with data reported in the literature [3,5,7], the IDES and DES concentrations in urine specimens were normalized to endogenous urinary creatinine to adjust for variability in urine dilution. To evaluate the loss of urinary creatinine upon hydrolysis, we determined the exact content of this compound before and after sample hydrolysis. To this purpose, aliquots of each urine sample were divided into two portions of equal volume, one of which was used as the reference and introduced directly into the anodic end of the capillary; the other, after drying, was combined with 6 *M* HCl, hydrolyzed and processed as above. As shown in Fig. 4, a representative example of electrophoretic profiles obtained from the urine of healthy controls before (panel A) and after hydrolysis (panel B), creatinine (peak 1, retention time 10.8 ± 0.05 min), appeared well resolved from the other analytes and easy to quantify in both electropherograms. Comparable results were also obtained from patients' urine (data not shown). The quantification of urinary creatinine was easily accomplished by comparing the peak area value with that of the standard. A calibration curve (inset of panel A) was obtained by applying scalar amounts (from 20 to 300 pmol) of the authentic standard to the capillary. To assess the accuracy of the determination, two parallel analyses were performed on each urine sample from controls ($n=8$) and from patients ($n=8$). The results obtained were in excellent agreement with the values reported

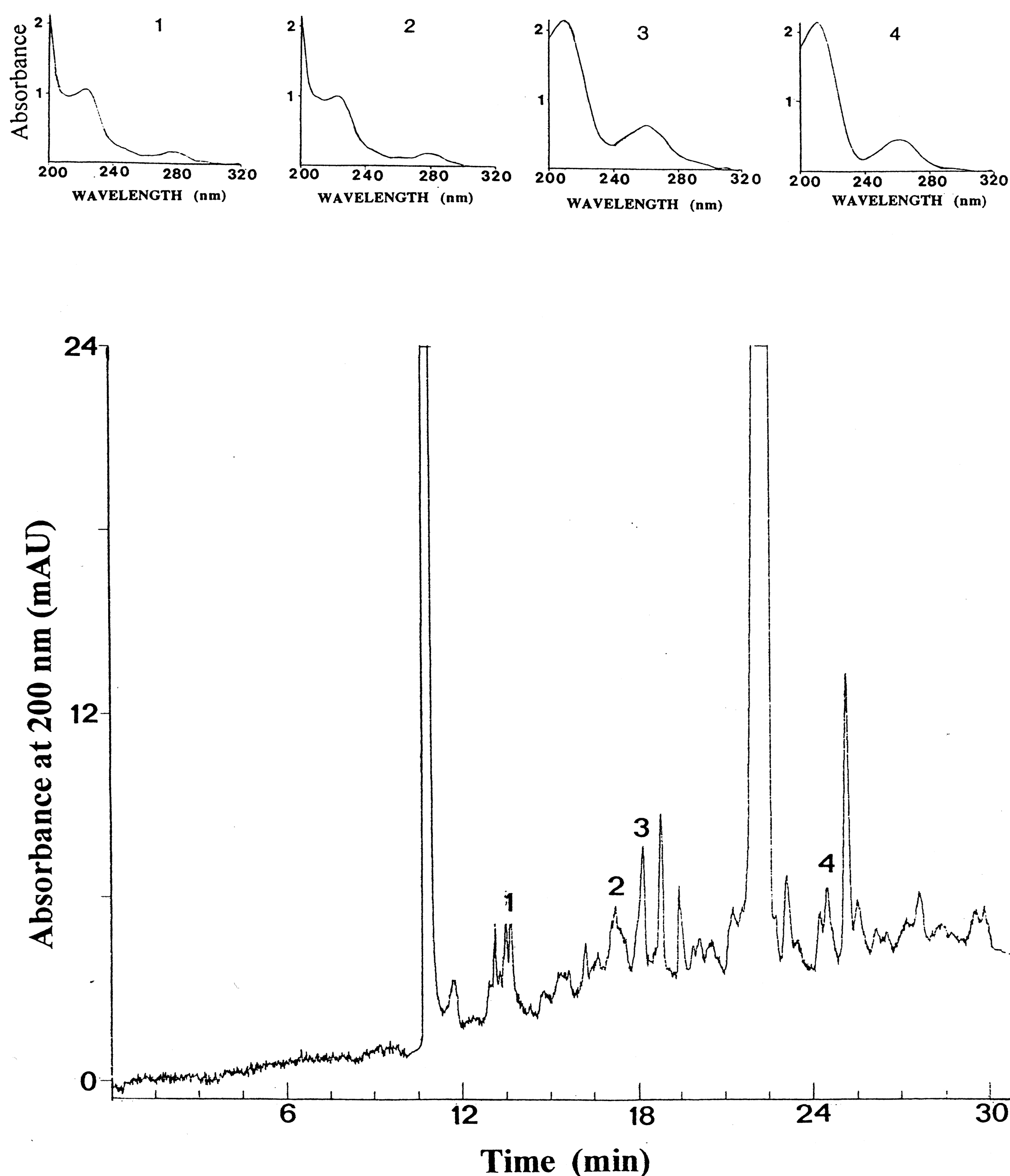

Fig. 2. Micellar electrokinetic chromatography of urine from a healthy control performed in 35 m*M* sodium tetraborate pH 9.3 containing 65 m*M* SDS. Peaks indicated 1 to 4 were identified as desmosine-containing peptides. The relative UV-absorption spectra acquired during separation are shown above. Experimental conditions: fused-silica capillary 50 cm effective length×50 μm I.D.; voltage: +10 kV; detection: UV absorbance at 200 nm; temperature: 15°C. Positive to negative polarity.

elsewhere and determined with similar CE methods [20–22], and evidenced small loss of creatinine upon hydrolysis, the recovery ranging from 85 to 91% in all subjects considered (Table 1). The calculated standard deviation was typically between 1.2 and 1.6%. To evaluate the precision of the MEKC

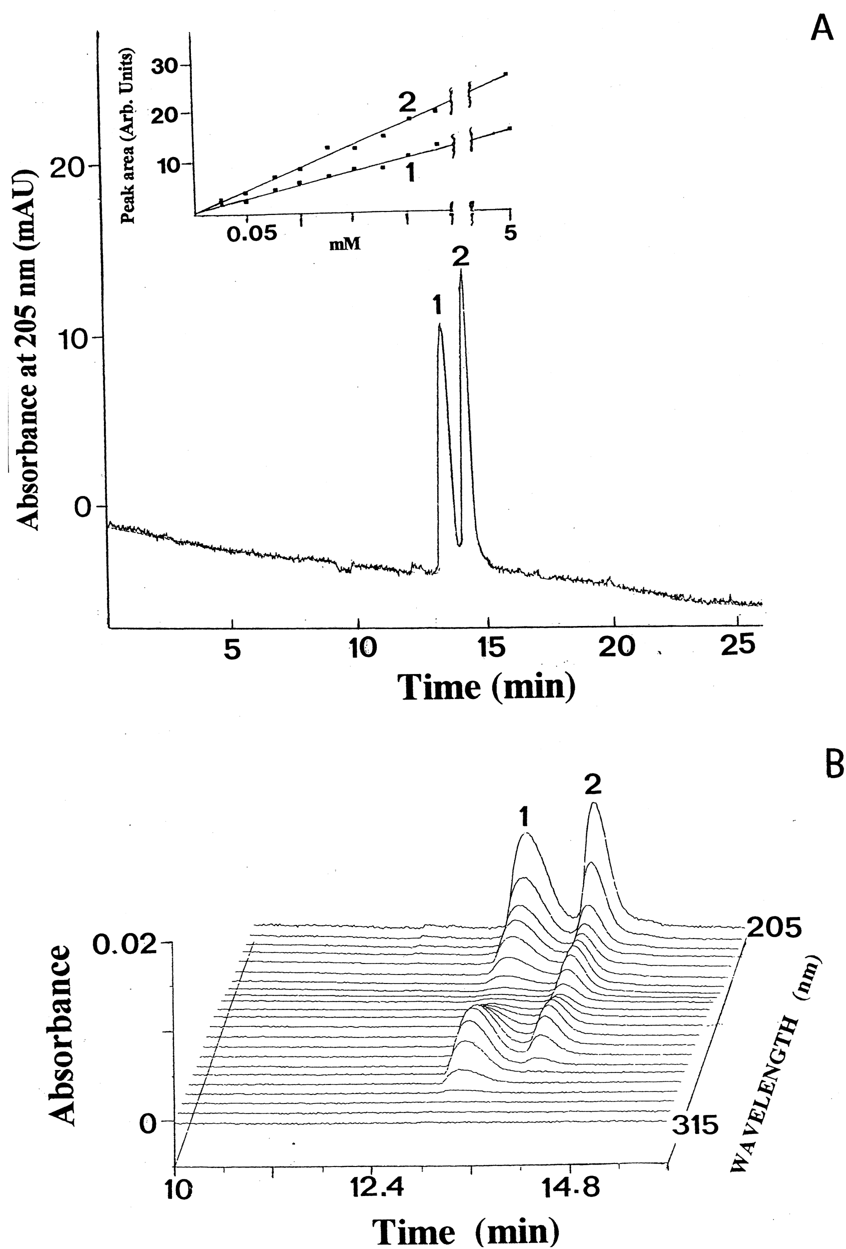

Fig. 3. Panel A: micellar electrokinetic chromatography of a mixture of standard IDES (peak 1) and DES (peak 2). Inset: calibration curves for the determination of IDES (curve 1) and DES (curve 2) concentration based on the peak area. Panel B: three dimensional MEKC electropherogram of standard IDES and DES. Experimental conditions are the same as those in Fig. 2.

technique, recovery experiments were also carried out by injecting several dilutions of standard creatinine to aliquots of urine. The mean recovery was 97.4±1.5%. These data indicate that MEKC is a reliable quantitative technique for measuring urinary creatinine.

In the light of these results all electropherograms obtained from hydrolyzed urine of controls and

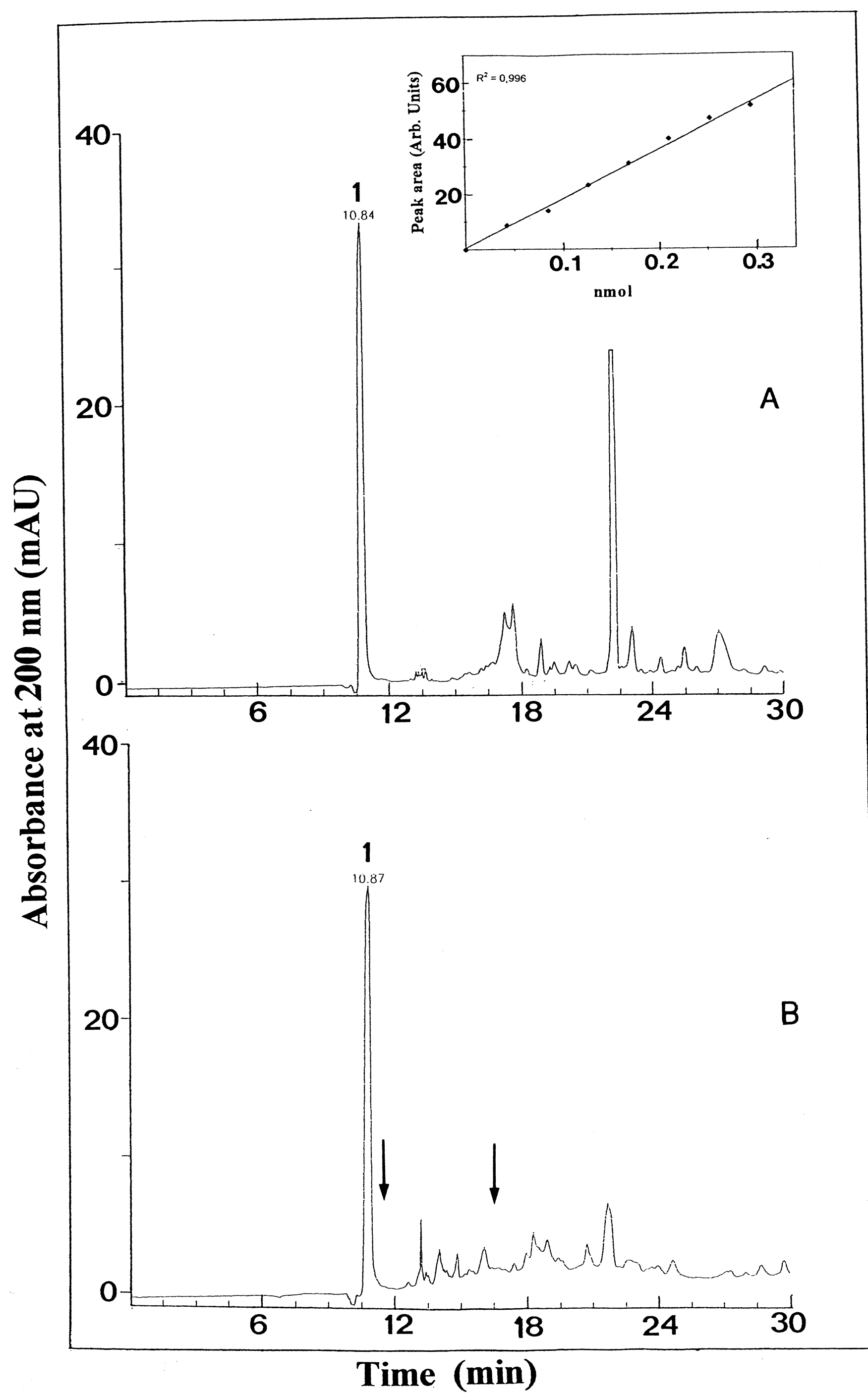

Fig. 4. Micellar electrokinetic chromatography of urine from a healthy control before (panel A) and after (panel B) hydrolysis. Peak 1: endogenous creatinine. Inset: calibration curve for the determination of creatinine content based on the peak area. Arrows in panel B indicate the desmosine-containing region. Experimental conditions are the same as those in Fig. 2.

Table 1
Determination of creatinine in urine of controls and COPD patients

Urine samples	Creatinine concentration (g/24 h)[a]	
	Unhydrolyzed urine	Hydrolyzed urine
U_1	2.48	2.11
U_2	2.15	1.79
U_3	1.39	1.27
U_4	1.93	1.72
U_5	2.93	2.50
U_6	1.57	1.35
U_7	1.67	1.63
U_8	2.90	2.61
U_9	2.82	2.26
U_{10}	1.50	1.25
U_{11}	1.40	1.25
U_{12}	1.04	1.00
U_{13}	1.77	1.56
U_{14}	1.61	1.39
U_{15}	1.80	1.58
U_{16}	1.27	1.20

$U_1 \rightarrow U_8$: urine samples from controls.
$U_9 \rightarrow U_{16}$: urine samples from COPD patients.
[a] Each value is the mean of two independent determinations.

patients were accurately analyzed to identify desmosines. Due to a high baseline noise at 200 nm (the chemistry involved may have contributed to this noise), the wavelength chosen for detection of desmosines was 268 nm despite the fact that at this wavelength absorbance of DES and IDES was 3.3- and 3.6-fold lower, respectively, than at 200 nm. However, as can be seen from the ultraviolet spectra shown in Fig. 1, desmosine has an absorption maximum at 268 nm and isodesmosine also has a high absorbance in this region. Moreover, to gain further insight into each profile, the desmosine-containing region (indicated by arrows in panel B of Fig. 4 and included between 11 and 16 min of the electropherogram) was expanded as shown in Fig. 5. The lower trace of Fig. 5 is in fact a representative example of the profiles, in the above mentioned region, obtained from all eight healthy controls studied. The identification of the two peaks (numbered 1 and 2 in the figure) with retention times of 13.64±0.15 min and 14.15±0.16 min (the R.S.D. being about 1.1 percent), respectively, as IDES and DES was established: (i) by coinjection of authentic compounds; the height of peaks 1 and 2 increased when standard desmosines (2 pmol each) were coinjected with urine samples (upper trace); and (ii) by comparison of ultraviolet absorption spectra acquired during the separation with the corresponding set of data obtained by analyzing authentic compounds (inset of Fig. 5). Thus, multiwavelength scanning of peaks permitted a quick and reliable confirmation of the analytes, whose peak area was integrated to obtain the amount of urinary desmosines. In order to accurately evaluate losses occurring during hydrolysis, desmosines were quantified following a procedure similar to that previously reported for creatinine. In separate experiments known amounts of standard desmosines were directly hydrolyzed or added to urine and processed as above. By comparing the amount of desmosines recovered in both cases with those obtained from corresponding unhydrolyzed samples it was possible to find that absolute losses of these analytes were small, the recovery ranging from 85.6 to 92.3%. Thus, after adjustment for losses, the mean value of IDES was 8.9±1.2 μg/g creatinine and that of DES 9.5±1.4 μg/g. These data, although of the same order of magnitude as those reported elsewhere [7], were approximately 15% higher than the values determined from healthy adults. However, it should be noted that, while a reduction in the number of steps involved in sample pretreatment is an obvious advantage for increasing the recovery of these compounds, overestimation of peak areas, due to the badly shaped peaks observed, cannot be excluded. Conversely, the assumption that CE measurements enable more sensitive detection than the isotope dilution method [7] remains to be proven.

As expected, all COPD patients considered, except one (urine samples $U_9 \rightarrow U_{15}$), showed higher urinary levels of desmosines. As can be seen in panels B→H of Fig. 6 the peak heights of IDES (peak 1) and DES (peak 2) in each electropherogram are significantly higher than in controls (panel A). Unexpectedly the urine sample indicated as U_{16} (panel I) contained amounts of desmosines comparable to those found in healthy controls (10.2 and 9.7 μg/g creatinine for DES and IDES, respectively); further experiments performed by injecting different aliquots of urine from this patient confirmed the reliability of this result. The discrepancy found for this patient may be explained on the basis of the well known hetero-

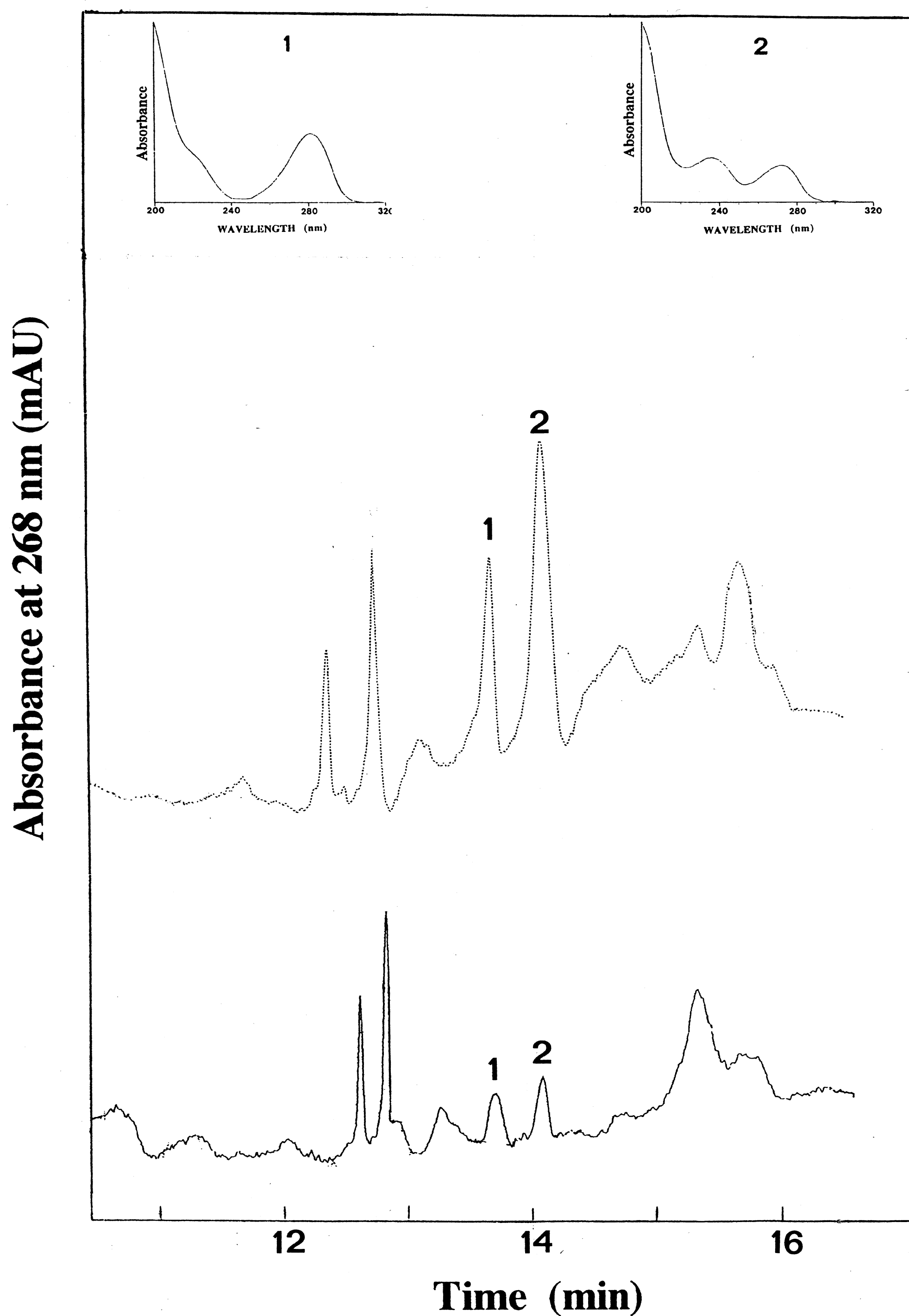

Fig. 5. Micellar electrokinetic chromatography of hydrolyzed urine from a healthy control. Lower trace: expansion of the region of the electropherogram of Fig. 4 included between 11 and 16 min. Peaks 1 and 2 are IDES and DES, respectively. Upper trace: coinjection of 2 pmol of standard desmosines to the urine sample above reported. Inset: UV spectra obtained from desmosines in the urine sample above reported. Detection: UV absorbance at 268 nm. All other experimental conditions are the same as those in Fig. 2.

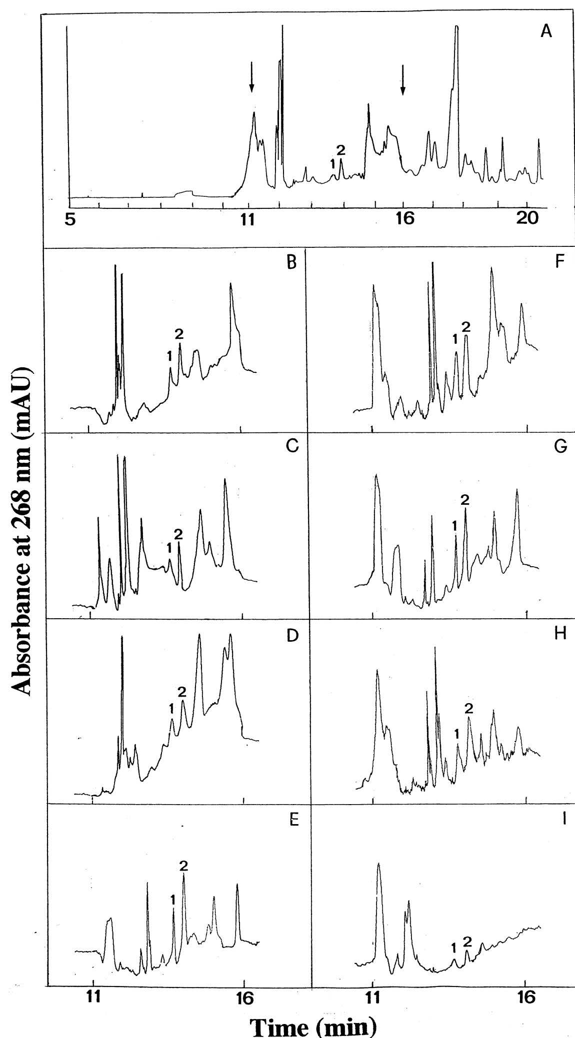

Fig. 6. Micellar electrokinetic chromatography of urine samples from a control (panel A) and from all eight COPD patients studied (panels B→I). Only the expanded region included between 11 and 16 min indicated by arrows in panel A are shown for the patients' electropherograms. Peaks 1 and 2 in all panels are IDES and DES, respectively. Detection: UV absorption at 268 nm. All other experimental conditions are the same as those in Fig. 2.

geneity among patients in the rate of elastin degradation related either to the age of the subject or to the stage of the disease [5,23].

Preliminary quantitative results reported in Table 2 show that the mean urinary excretion of desmosines determined by the CE method was higher by a factor of as much as 3-fold in urine of COPD patients than in controls, the differences found between the two groups being greater than those determined by Stone et al. [7]. As discussed above, we cannot exclude that IDES and DES could also have been slightly overestimated in the patients' urine although the variability of values reported in the literature [3,5,7,16,24,25] makes it difficult to understand how accurate the methods are which have been used so far to measure these elastin-specific crosslinks. Thus, data from a larger number of clinical samples certainly need to be accumulated in order to have greater understanding of the selectivity and sensitivity of the CE measurements, however the possibility of detecting and quantifying desmosines with a minimum of sample manipulation once again indicates that MEKC is an attractive confirmation method for urine analysis.

4. Conclusions

The MEKC method described is highly effective in determining the desmosines content in urine; in comparison with the methodologies so far available which are generally quite labour-intensive, it is relatively rapid and inexpensive and has the advantage of being automated. Although the use of SDS as surfactant seems to be useful because it gives satisfactory separation efficiency of desmosines, a variety of other micellar agents or the addition of organic solvents, such as acetonitrile or methanol, to the running buffer to maximize the resolution between the two peaks and/or to improve the peak shape should be further investigated.

Moreover, the possibility of determining both endogenous creatinine (as an internal standard) and urinary desmosines in a single run, is a way of monitoring the performance of the CE system. In fact any disturbance which might result in changes of the observed peak areas leading to errors in quantification would also be reflected in changes of the internal standard control.

Although additional information from experiments on a larger number of controls and patients are needed to obtain an absolutely reliable determination

Table 2
DES and IDES excretion in urine of controls and COPD patients

Compound (concentration)	Controls $U_1C \rightarrow U_8$	Patients							
		U_9	U_{10}	U_{11}	U_{12}	U_{13}	U_{14}	U_{15}	U_{16}
Creatinine[a] (mg/24 h)	1.87	2.26	1.25	1.25	1.00	1.56	1.39	1.58	1.20
Desmosine (μg/24 h)	17.7±1.4	27.3	23.8	22.3	22.4	27.7	25.3	26.9	12.2
Desmosine (μg/g)	9.5±1.1	12.1	19.0	17.9	22.4	17.8	18.2	17.0	10.2
Isodesmosine (μg/24 h)	16.6±1.2	23.9	23.0	21.2	20.2	26.6	24.9	26.6	11.6
Isodesmosine (μg/g)	8.9±1.0	10.6	18.5	17.0	20.2	17.0	17.9	16.8	9.7

[a] The value reported here is the mean of the values shown in Table 1 for hydrolyzed urine samples.
[b] Expressed as μg/g creatinine.

of IDES and DES, this technique appears appropriate for providing precise information on elastin degradation products in biological fluids. Owing to the simplicity of sample preparation, MEKC could become a primary analytical technique for assessment of lung destruction in COPD patients.

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