Journal of Chromatography B, 879 (2011) 1893–1898

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantitation of desmosine and isodesmosine in urine, plasma, and sputum by LC–MS/MS as biomarkers for elastin degradation

Shuren Ma, Gerard M. Turino, Yong Y. Lin*

Department of Medicine, St. Luke's Roosevelt Hospital Center, Columbia University College of Physicians and Surgeons, 432W 58th St, New York, NY 10019, USA

ARTICLE INFO

Article history:
Received 16 February 2011
Accepted 5 May 2011
Available online 13 May 2011

Keywords:
LC–MS
Elastin degradation
Biomarker
Chronic obstructive pulmonary disease

ABSTRACT

The aim of this study is to develop a standardized LC–MS/MS method for accurate measurement of desmosine (DES) and isodesmosine (IDS) in all body fluids as biomarkers for in vivo degradation of matrix tissue elastin in man and animals. A reproducible three-step analytical procedure: (1) sample hydrolysis in 6 N HCl, (2) SPE by a CF1 cartridge with addition of acetylated pyridinoline as internal standard (IS), and (3) LC/MSMS analysis by SRM monitoring of transition ions; DES or IDS (m/z 526–481 + 397) and IS (m/z 471–128) was developed. The method achieves accurate measurements of DES/IDS in accessible body fluids (i.e. urine, plasma, and sputum). LOQ of DES/IDS in body fluids is 0.1 ng/ml. The % recoveries and reproducibility from urine, plasma, and sputum samples are above 99 ± 8% (n = 3), 94 ± 9% (n = 3) and 87 ± 11% (n = 3), with imprecision 8%, 9% and 10%, respectively. The proposed method was applied to measure DES/IDS in body fluids of patients with chronic obstructive pulmonary disease (COPD) and healthy controls. Total DES/IDS in sputum and plasma is increased over normal controls along with the free DES/IDS in urine in patients. DES/IDS can be used to study the course of COPD and the response to therapy. This practical and reliable LC–MS/MS method is proposed as a standardized method to measure DES and IDS in body fluids. This method can have wide application for investigating diseases which involve elastic tissue degradation.

1. Introduction

Elastin fibers are part of the extracellular matrix and are an essential structural component of lung, skin, and blood vessels. Desmosine (DES) and isodesmosine (IDS) are two unique pyridinium amino acids that serve as crosslinking molecules binding the polymeric chains of amino acids into the 3D network of elastin [1–3]. The degradation of elastin-containing tissues that occurs in several widely prevalent diseases, such as atherosclerosis, aortic aneurysms, cystic fibrosis and chronic obstructive pulmonary disease (COPD) which includes pulmonary emphysema, etc., have been associated with increased excretion in the urine of peptides containing these two pyridinium compounds [4–13].

In lung, elastin degradation occurs with the development of COPD related to smoking or related to α1-antitrypsin deficiency (AATD) [14,15]. COPD currently affects over 18 million Americans and is the fourth leading cause of death in the US. Various techniques including RIA [16,17], HPLC [18–20], and capillary zone electrophoresis [21,22] have been utilized for the analysis of urinary DES and IDS. We have developed a more specific and sensitive LC–MS analysis, which can measure DES and IDS in plasma, urine, and for the first time in sputum. Measurements in COPD show increased levels of DES and IDS in acid hydrolyzed sputum and plasma, along with elevated free DES and IDS in urine without acid hydrolysis [23,24]. The LC–MS analysis which provides increased sensitivity and specificity may be an important method for biomarker analysis of elastin degradation in disease [25]. Subsequent to our publication, three LC–MS/MS analyses of DES and IDS have been reported [26–28]. These studies have been limited to the analysis of DES and IDS content in urine or mouse lung hydrolysates. As we have shown in the study of DES and IDS in COPD [23,24] and a study on the effect of Tiotropium therapy on DES and IDS levels in COPD [29], it is recommended that DES and IDS levels to be evaluated in other body fluids as well; such as sputum, plasma, or bronco alveolar lavage fluid. An accurate and reproducible quantification of DES and IDS in several body fluids may be more useful to characterize elastin degradation in disease and to follow the course of disease and therapies [30]. In addition, two recent FDA workshops sponsored by the COPD Foundation held in May, 2009 and January, 2010 have called for standardization of the analysis to provide practical clinical biomarkers for COPD.

Abbreviations: DES, desmosine; IDS, isodesmosine; DES/IDS, desmosine and isodesmosine; LC–MS, liquid chromatography–mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass spectrometry; COPD, chronic obstructive pulmonary disease; AATD, alpha-1 antitrypsin deficiency; SPE, solid phase extraction; HFBA, hexafluorobutyric anhydride; SRM, selected reaction monitoring; FDA, Food and Drug Administration; LOQ, limit of quantitation; LOD, limit of detection.

* Corresponding author. Tel.: +1 212 523 7305; fax: +1 212 523 7442.
E-mail address: yylin@chpnet.org (Y.Y. Lin).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.05.011

In this report we describe a practical, and a reliable LC–MS/MS analysis that can measure DES and IDS in all body fluids including urine, plasma, sputum, and lavages and serve as a standardized method. The analysis utilizes commercially available acetylated pyridinoline as the internal standard to optimize reproducibility and accuracy.

2. Materials and methods

2.1. Chemicals

Desmosine (DES) and isodesmosine (IDS) standard (mixed 50% DES and 50% IDS) were purchased from Elastin Products Company (Owensville, MI), Acetylated pyridinoline was obtained from Quidel (San Diego, CA). CF1 cellulose powders were purchased from Whatman (Clifton, NJ), and all other reagents were from Sigma (St. Louis, MO).

2.2. Samples collection and human subjects

Urine (24 h samples), plasma, and sputum samples were collected as previously described [23,24] from volunteers with informed consent at the James P Mara Center for Lung Disease, St. Luke/Roosevelt Hospital Center, New York. COPD was diagnosed in study patients according to the Global initiative for Chronic Obstructive Lung Disease stages 1–4 [31]. Patients gave informed consent for the study. Control subjects were selected by clinical history free of any specific known disease or significant symptoms, and none have ever smoked. The study was approved by the Institutional Review Board.

2.3. Creatinine and protein measurement

Urine creatinine was measured by the commercially available 555A creatinine kit (Sigma–Aldrich). Total protein in plasma and sputum samples was measured by the commercially available microprotein assay kit (Sigma–Aldrich), which is based on protein-dye (Coomassie blue) binding.

2.4. Standardized LC–MS/MS analysis of DES and IDS in all body fluids

We have developed the following conventional three-step analytical procedure which can be used to measure DES and IDS in all relevant body fluids (urine, plasma, and sputum) or lavage fluids.

2.4.1. Acid hydrolysis

Samples of urine (0.1 ml), plasma or sputum (0.5 ml each) were placed in a glass vial with equal volumes of conc. HCl (37%). Air in the vial was displaced with nitrogen, and was heated at 110 °C for 24 h. The hydrolyzed sample was filtered and evaporated to dryness. For the free (unconjugated) forms of DES and IDS analysis, 0.2 ml of urine was analyzed directly without the HCl hydrolysis.

2.4.2. Cellulose (CF1) cartridge extraction

The acid hydrolyzed samples (after drying under vacuum or nitrogen stream to remove residual acid) or unhydrolyzed urine sample (for free DES and IDS analysis) were added with 1 ng (for urine samples) or 0.5 ng (for plasma and sputum samples) of acetylated pyridinoline as the internal standard. The mixture was dissolved in 2 ml of n-butanol/acetic acid/water (4:1:1), and applied onto a 3 ml cellulose cartridge, which was prepared by introduction of 3 ml of 5% CF1 cellulose powder slurry in n-butanol/acetic acid/water (4:1:1). The cellulose powder slurry must be well dispersed slurry by stirring for 24 h. The cartridge was washed 3 times with 3 ml of n-butanol/acetic acid/water (4:1:1), and the samples retained in the cartridge were eluted with 3 ml of water, dried and dissolved in 200 μl (for urine sample) or 100 μl (for plasma and urine samples) of LC mobile phase and analyzed by LC–MS/MS.

2.4.3. LC–MS/MS analysis

A TSQ Discovery electrospray tandem mass spectrometer (Thermo Fisher Scientific) was used for LC–/MS/S analysis. HPLC conditions used were a 2 mm × 150 mm dC18(3 μm) column (Waters, MA) and the mobile phase A (7 mM HFBA/5 mM NH_4Ac in water) and B (7 mM HFBA/5 mM NH_4Ac in 80% acetonitrile) were programmed linearly from 100% A to 82% A in 12 m.

Quantitation was performed by selected reaction monitoring (SRM) of the transitions of both DES and IDS (m/z 526–481 + m/z 397) and the internal standard (m/z 471–128), with collision energy set at 33 V for both transition, collision gas pressure was 1.5 mTorr, tube lens at 132 V, with sheath gas pressure set at 45 and auxiliary gas pressure at 6 units and ion spray voltage at 3.8 kV. The scan time set at 1.00 ms and both quadrupoles (Q1 and Q3) were at 0.7 Da FWHM.

2.5. Statistical analysis

A t test adjusted for unequal variance was used to test the null hypothesis. The level of significance was 0.05. The p values were calculated based on the summed values of DES and IDS using the unpaired t test (The used software is "GraphPad Prism 4 (2)").

3. Results

3.1. DES and IDS are stable toward HCl hydrolysis

The assay of total DES and IDS in biological fluids requires HCl hydrolysis at 110 °C to release DES and IDS from their crosslinked or peptide conjugates. We examined the stability of DES and IDS in three different concentrations (10, 5, and 1 ng/ml) during HCl hydrolysis at 110 °C for 24 h. The DES/IDS solutions resulting from the HCl treatment was subjected to LC–MS/MS measurements of DES and IDS, which were compared with the measurements of the same concentrations of untreated DES and IDS to calculate the recovery by the acid treatment. The results show that DES and IDS are stable through acid hydrolysis with virtually complete recoveries at all three concentrations. The recoveries were 104 ± 5% (n = 3), 100 ± 4% (n = 3), and 98 ± 19% (n = 3) at the concentrations of 10, 5, and 1 ng/ml, respectively.

3.2. Recovery of DES and IDS in human body fluids

Three known amounts of DES/IDS were spiked into the control urine, plasma, and sputum samples at concentration ranges expected to be encountered in biological samples (10, 20 and 40 ng/ml in urine samples; 0.2, 0.4 and 0.8 ng/ml in plasma and sputum samples). The mixtures were subject to HCl hydrolysis at 110 °C for 24 h, addition of IS, CF1 cartridge chromatography, and LC–MS/MS analysis under the established procedure to measure DES/IDS levels. The recoveries of DES/IDS are above 99 ± 8% (n = 3) in urine, 94 ± 9% (n = 3) in plasma, and 87 ± 11% (n = 3) in sputum samples; with the imprecision 8%, 9%, and 10%, respectively. Limit of quantitation (LOQ) in all relevant body fluids are determined as 0.1 ng/ml of samples, which is the minimum DES/IDS concentration of recovery study that can achieved with <10% imprecision.

3.3. Acetylated pyridinoline as internal standard

Acetylated pyridinoline is an acetylated derivative of 3-hydroxy pyridinoline which serves as a trifunctional crosslink in collagen. The compound has been used as an internal standard (IS) in HPLC

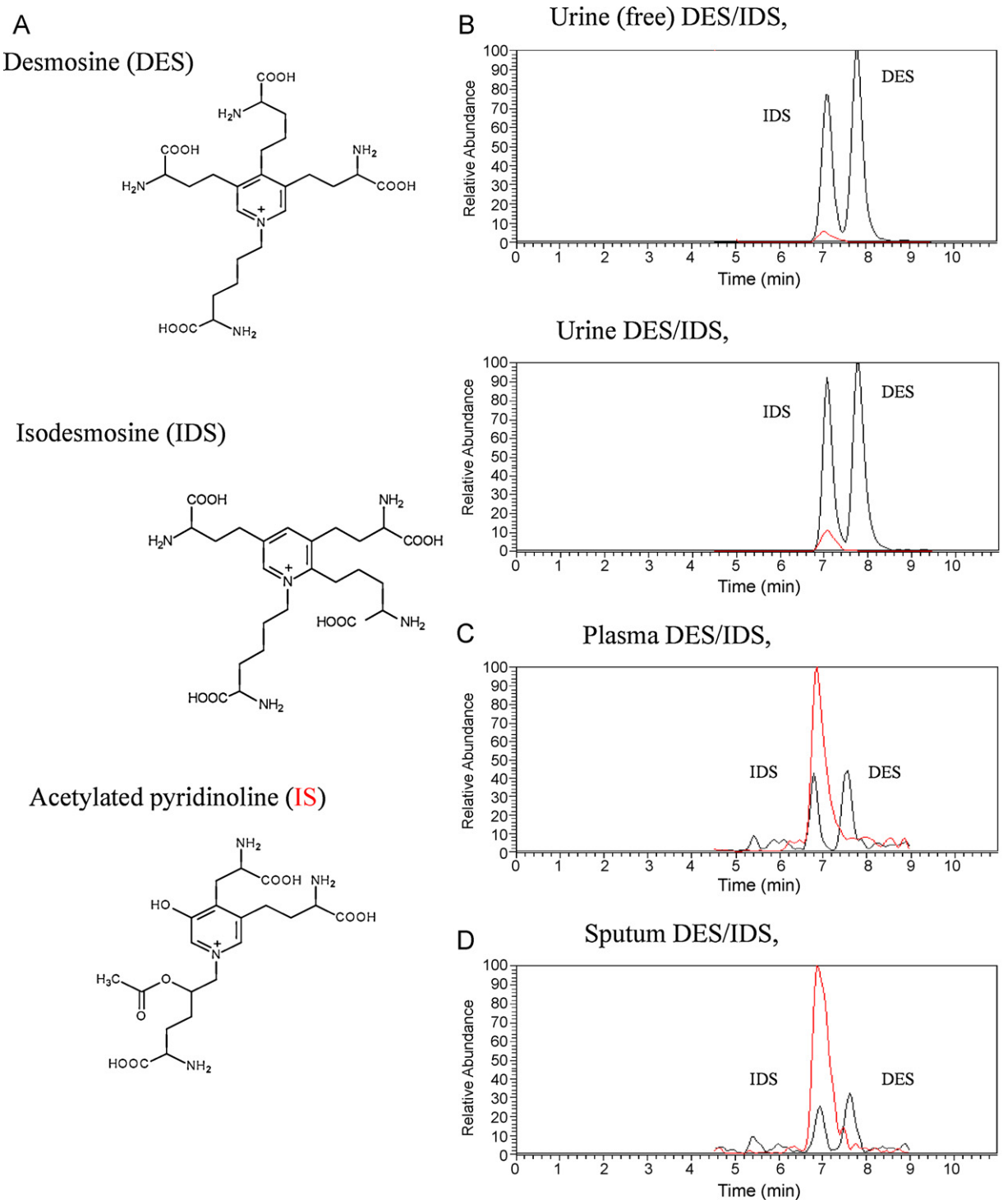

Fig. 1. LC/MSMS analysis of desmosine (DES) and isodesmosine (IDS) using acetylated pyridinoline as internal standard (IS). (A) Molecular structures of desmosine (DES), isodesmosine (IDS), and acetylated pyridinoline (IS). (B) LC–MS/MS chromatograms of DES/IDS and acetylated pyridinoline (IS) in urine (free and total), plasma, and sputum samples.

analysis of pyridinium crosslinks of collagen in urine or tissue [32–34].

Since acetylated pyridinoline has a similar molecular structure and polarity to that of DES and IDS (Fig. 1A), the use of the pyridinoline as an IS for the LC–MS/MS analysis of DES and IDS was explored. The elution characteristics of acetyl pyridinoline in both a CF1 cartridge and HPLC column are closely similar to DES and IDS. It also exhibits a similar linear response in mass spectrometric analysis. Since DES and IDS have been found stable without losses through HCl hydrolysis, we introduce acetylated pyridinoline as IS after the HCl hydrolysis for the DES and IDS quantitation to determine recovery from the subsequent analytical procedure. The HPLC can effectively separate the two DES and IDS isomers, thus, the isomers can be conveniently quantified separately or as the sum of DES + IDS. Good linear responses of DES/IDS (from 1.25 to 125 pg) using acetylated pyridinoline as the IS for the analytical procedure were obtained: the regression lines for DES $y = 0.1464x - 0.3187$ ($R^2 = 0.9992$), for IDS $Y = 0.1546X - 0.2146$ ($R^2 = 0.9990$), and for

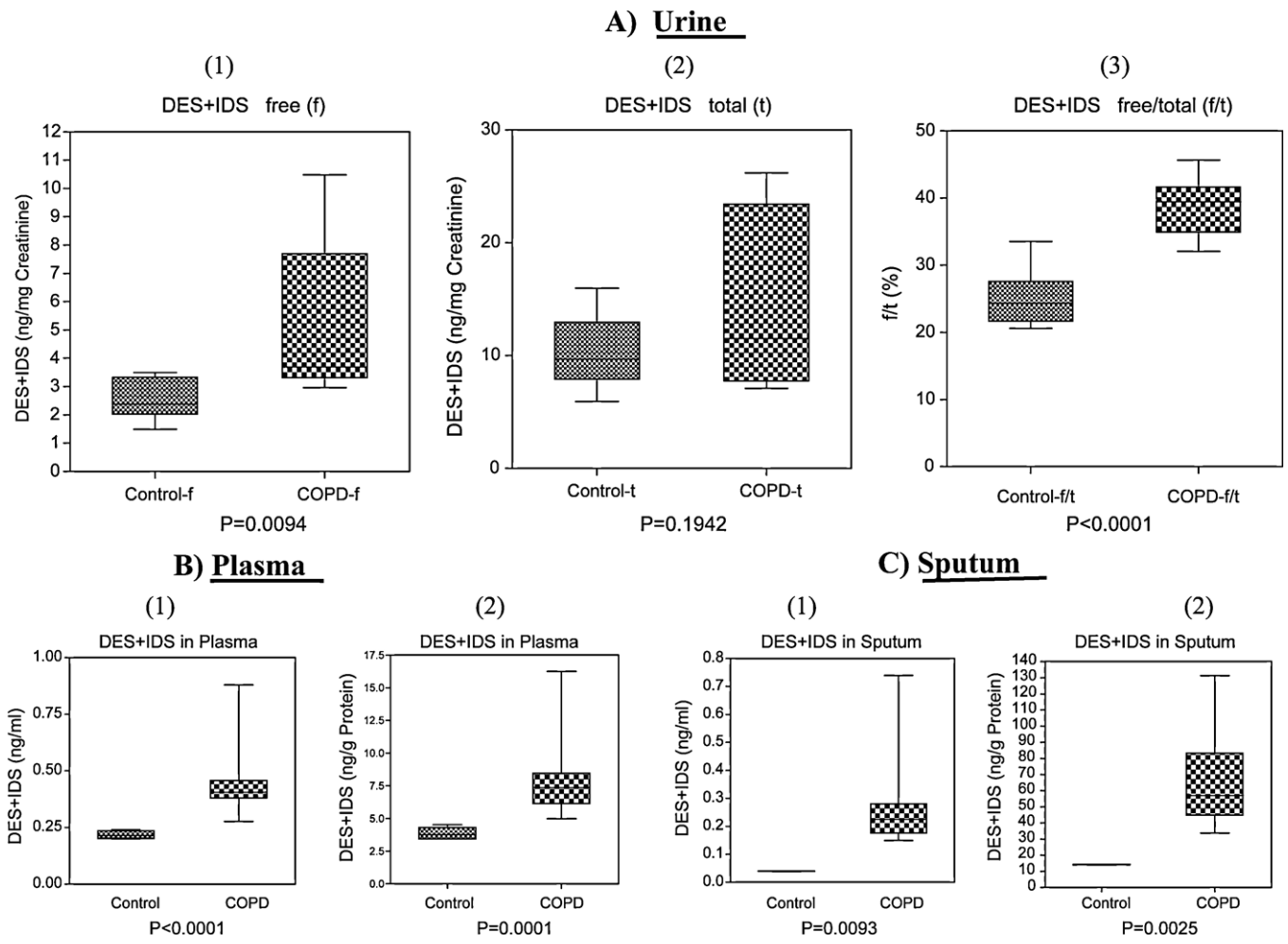

Fig. 2. DES/IDES levels in COPD patients and healthy controls: the boundaries of the box indicate between the 25th and 75th percentile, and the line within the box is the median. The level (mg/g creatinine) of urinary DES + IDES in COPD patients ($n = 11$) and healthy controls ($n = 9$): (1) free DES + IDS of COPD (median 3.69) versus controls (median 2.39), $p = 0.0098$; (2) total DES + IDS of COPD (median 11.50) versus controls (median 9.67), $p = 0.1147$; (3) ratio of free DES + IDS to total DES + IDS of COPD (median 39.5) versus controls (median 24.3), $p = <0.0001$. (B) The levels of plasma total DES + IDS in COPD ($n = 14$) and healthy controls ($n = 4$): (1) total DES + IDS (ng/ml) of COPD (median 0.40) versus controls (median 0.21), $p < 0.0001$; (2) total DES + IDS (ng/g protein) of COPD (median 7.37) versus controls (median 3.76), $p = 0.0001$. (C) The levels of sputum of total DES + IDS in COPD ($n = 8$) and healthy controls ($n = 5$): (1) total DES + IDS (ng/ml) of COPD (median 0.22) versus controls (below LOD limit 0.04 ng/ml), $p = 0.0093$; (2) total DES + IDS (ng/g protein) of COPD (median 56.94) versus controls (below LOD 14.29 ng/g protein), $p = 0.0025$.

DES + IDS $y = 0.1509x - 0.5520$ ($R^2 = 0.9995$). LC–MS/MS analysis of DES/IDS in urine, plasma, and sputum samples using acetylated pyridinoline as IS are shown in Fig. 1B.

3.4. Standardized assay procedure

Our study resulted in development of a highly specific and reproducible LC–MS/MS method that can be used as a standardized assay of DES/IDS in urine, plasma, and sputum as biomarkers for elastin degradation. The method consists of the following three major steps:

Step 1: Sample (urine 0.1 ml, plasma 0.5 ml, sputum 0.5 ml) hydrolysis in 6 N HCl at 110 °C for 24 h [for Free DES/IDS assay skip this hydrolysis step].
Step 2: Addition of acetylated pyridinoline (IS) to the acid hydrolyzed sample and dissolve in a mixture of butanol/acetic acid/water (4:1:1). The mixture is passing through a 3 ml CF1 cellulose cartridge to extract DES/IDS + IS.
Step 3: Quantitation of DES/IDS by HPLC separation (a 3 μm dC18, 2 mm × 150 mm column) combined with tandem MS/MS detection, using selected reaction monitoring (SRM) to measure transition ions of both DES and IDS (m/z 481 + m/z 397) and IS (m/z 128). Details of the assay procedure are described in Section 2.

3.5. Measurement of DES and IDS in urine, plasma, and sputum of COPD patients and healthy subjects

The analytical procedure we have developed was used to compare the levels of DES and IDS in urine, plasma, and sputum obtained from COPD patients and that of healthy normals. The results are shown with urine samples in Fig. 2A, plasma samples in Fig. 2B, and sputum samples in Fig. 2C. The urinary DES/IDS levels after HCl hydrolysis (total DES/IDS) in COPD patients are slightly higher than that of healthy controls, but the difference is not statistically significant ($p = 0.1942$). On the other hand, the urinary DES/IDS levels without HCl hydrolysis (free DES/IDS) in COPD patients are statistically significantly higher than that of healthy controls ($p = 0.0094$). The ratios of free DES/IDS to total DES/IDS are significantly higher in COPD patients than that of controls ($p < 0.0001$) (Fig. 2A). This result is in agreement to our previous data [23,24]. Also the data indicates that the ratio of total DES/IDS to free DES/IDS levels in urine samples are good biomarkers for showing increased elastase activity in COPD patients. In plasma, DES/IDS levels in COPD patients are statistically higher than that of

the healthy controls ($p < 0.0001$) (Fig. 2B). The DES/IDS in sputum of COPD patients shows the median of 0.24 ng/ml, while the levels in induced sputum from healthy subjects are below detection limit (0.04 ng/ml) (Fig. 2C). Validation of the developed method can be made by comparison of the results on DES/IDS in urine, plasma, and sputum of COPD patients and healthy subjects to that of previously published study by LC–MS analysis [24]. In both studies, similar results were observed with DES/IDS levels in COPD and healthy controls.

4. Discussion

A strength of DES/IDS as biomarkers in COPD is the recognition that matrix elastin is a structural target of the disease. Among the many techniques developed to measure DES/IDS, the LC/MSMS technique which provides higher sensitivity and specificity appears to be an improved choice for biomarker analysis [25]. Several modifications of LC–MS/MS methods for DES/IDS analysis have been reported recently, but they are all developed for the measurements of DES/IDS in urine [26–28]. Our previous studies on the measurements of DES/IDS in COPD patients [23,24] and DES/IDS levels in response to Tiotropium treatment of COPD patients [29] demonstrate that DES/IDS levels in sputum and plasma are effective indicators of elastin degradation in patients in the body as a whole and the lung per se. Development of a sensitive, accurate, and reproducible method which can measure DES/IDS levels in all body fluids can be clinically meaningful especially related to parameters such as lung structure analyzed by computed tomography quantitative, lung function or genomic analysis.

Previously published LC–MS or LC–MS/MS methods [23–28] all have disadvantages of either lack of a reliable internal standard or the method not standardized for the analysis of all relevant body fluids (i.e. urine, plasma, and sputum), which are important for assessing clinical meaning of elastin degradation. In this report we have developed a practical and simplified LC–MS/MS analytical procedure that can universally utilized for the analysis of DES and IDS in all relevant body fluids; including urine, plasma, and sputum.

We confirm that DES/IDS are stable under the conditions of hydrolysis in 6 M HCl at 110 °C for 24 h, the acid hydrolysis generally used to release DES/IDS from their peptide conjugate. We introduce acetylated pyridinoline as the internal standard after the HCl hydrolysis step to correct for losses occurring from the subsequent steps. Acetylated pyridinoline has a closely similar molecular structure and chromatographic mobility to that of DES and IDS molecules, which enables the development of a reproducible and accurate LC–MS/MS measurement of DES/IDS in urine, plasma, and sputum. The LC–MS/MS analysis can also effectively separate the two DES/IDS isomers, thus two DES/IDS isomers can be conveniently quantified either separately or as combined DES + IDS in all body fluids.

Two previous reports have introduced deuterated compounds as internal standards to improve LC–MS/MS analysis of DES/IDS [27,28]. However the origin of the deuterium compounds are not stated, and appeared to be obtained through catalytic proton exchange reactions with DES/IDS. The structures and the stability of the introduced standard were not demonstrated. In this regard, acetylated pyridinoline is a commercially available compound with defined structure, which can be readily added as the internal standard. The accuracy and reproducibility of the developed LC–MS/MS analysis was further tested by recovery studies of DES/IDS in a series of known contents of DES/IDS in urine, plasma, and sputum samples. The recoveries from urine, plasma, and sputum samples are above 99%, 94%, and 87%, respectively, with good reproducibility.

This proposed method was used to measure DES and IDS in urine, plasma, sputum of a cohort of COPD patients as compared to their healthy controls (Fig. 2). The results confirm our previous reports [24] that the DES/IDS levels are useful biomarkers to characterize elastin degradation in COPD.

The degradations of elastin-containing tissues also occur in aorta [35,13], skin [36–38], and liver [39], etc. The developed LC–MS/MS analysis of DES/IDS can have wide application for investigating diseases which involve in those elastic tissues.

5. Conclusion

We have developed a sensitive, reproducible, and practical method using tandem mass spectrometric LC–MS/MS analysis to measure DES and IDS using acetylated pyridinoline as the internal standard. This procedure can serve on a standardized LC–MS/MS method to measure DES and IDS in all relevant body fluids, which are important for the clinical assessment of elastin degradation in diseases. The developed method demonstrated increased DES/IDS levels in urine, plasma, and sputum samples of patients with COPD over healthy controls. This analytical method can be applied to investigate diseases which induce elastic tissue degradation in vivo.

Acknowledgement

This work was supported by funds from James P. Mara Center for Lung Diseases, the Flight Attendant Medical Research Institute, the Charles A Mastronardi Foundation, the Ned Doyle Foundation, the Alpha-1 Foundation, and by Funds from Ethel Kennedy, John Kennedy, and Judith Sulzberger.

References

[1] J. Thomas, D.F. Elsden, S.M. Partridge, Nature 200 (1963) 651.
[2] W. Shimada, A. Bowman, N.R. Davis, R.A. Anwar, Biochem. Biophys. Res. Commun. 37 (1969) 191.
[3] M. Akagawa, K. Suyama, Connect. Tissue Res. 41 (2000) 131.
[4] L.B. Sandberg, Int. Rev. Connect. Tissue Res. 7 (1978) 159.
[5] J. Rosenbloom, Connect. Tissue Res. 10 (1982) 73.
[6] E.E. Schriver, J.M. Davidson, M.C. Sutcliffe, B.B. Swindell, G.R. Bernard, Am. Rev. Respir. Dis. 145 (1992) 762.
[7] M.F. Tenholder, K.R. Rajagopal, Y.Y. Phillips, T.A. Dillard, L.L. Bennett, T.G. Mundie, C.J. Tellis, Chest 100 (1991) 1385.
[8] P.J. Stone, D.J. Gottlieb, G.T. O'Connor, D.E. Ciccolella, R. Breuer, J. Bryan-Rhadfi, et al., Am. J. Respir. Crit. Care Med. 151 (1995) 952.
[9] S. Viglio, P. Iadarola, A. Lupi, R. Trisolini, C. Tinelli, B. Balbi, et al., Eur. Respir. J. 15 (2000) 1039.
[10] P.J. Stone, M.W. Konstan, M. Berger, H.L. Dorkin, C. Franzblau, G.L. Snider, Am. J. Respir. Crit. Care Med. 152 (1995) 157.
[11] D.C. Bode, E.D. Pagani, W.R. Cumiskey, R. von Roemeling, L. Hamel, P.J. Silver, Pulm. Pharmacol. Ther. 13 (2000) 175.
[12] E. Schwartz, F.A. Cruickshank, M. Lebwohl, Exp. Mol. Pathol. 52 (1990) 63.
[13] M. Watanabe, T. Sawai, Tohoku J. Exp. Med. 187 (1999) 291.
[14] R.R. Wright, Am. J. Pathol. 39 (1961) 355.
[15] T. Yoshida, R.M. Tuder, Physiol. Rev. 87 (2007) 1047.
[16] G.S. King, V.S. Mohan, B.C. Starcher, Connect. Tissue Res. 7 (1980) 263.
[17] S. Harel, A. Janoff, S.Y. Yu, A. Hurewitz, E.H. Bergofsky, Am. Rev. Respir. Dis. 122 (1980) 769.
[18] P.J. Stone, J. Bryanrhadfi, E.C. Lucey, D.E. Ciccolella, G. Crombie, B. Faris, et al., Am. Rev. Respir. Dis. 144 (1991) 284.
[19] W.R. Cumiskey, E.D. Pagani, D.C. Bode, J. Chromatogr. B 668 (1995) 199.
[20] J.R. Chen, M. Takahashi, K. Kushida, M. Suzuki, K. Suzuki, K. Horiuchi, A. Nagano, Anal. Biochem. 278 (2000) 99.
[21] P. Giummelly, P. Botton, R. Friot, D. Prima-Putra, J. Atkinson, J. Chromatogr. A 710 (1995) 357.
[22] S. Viglio, G. Zanaboni, M. Luisetti, R. Trisolini, R. Grimm, G. Cetta, P. Iadarola, J. Chromatogr. B 714 (1998) 87.
[23] S.S. Ma, S. Lieberman, G.M. Turino, Y.Y. Lin, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 12941.
[24] S. Ma, Y.Y. Lin, G.M. Turino, Chest 131 (2007) 1363.
[25] M. Luisetti, S. Ma, P. Iadarola, P.J. Stone, S. Viglio, B. Casado, et al., Eur. Respir. J. 32 (2008) 1146.
[26] N. Kaga, S. Soma, T. Fujimura, K. Seyama, Y. Fukuchi, K. Murayama, Anal. Biochem. 318 (2003) 25.
[27] M. Boutin, C. Berthelette, F.G. Gervais, M.B. Scholand, J. Hoidal, M.F. Leppert, et al., Anal. Chem. 81 (2009) 1881.
[28] O. Albarbarawi, A. Barton, Z. Lin, E. Takahashi, A. Buddharaju, J. Brady, et al., Anal. Chem. 82 (2010) 3745.

[29] S. Ma, Y.Y. Lin, L. Tartell, G.M. Turino, Respir. Res. 10 (2009) 12.
[30] Desmosine and Isodesmosine analysis as possible biomarkers for COPD has been among the topics of two recent FDA workshops (2009 and 2010 at Bethesda): (a) G.M. Turino, Y.Y. Lin, Desmosine and isodesmosine as biomarkers of elastin degradation in COPD and Alpha-1 antitrypsin deficiency, FDA Study Group on "Improving Endpoints, Improving Care: Alpha-1 Antitrypsin Augmentation Therapy and Clinical Trials" Center for Biologics Evaluation and Research, FDA, Bethesda, Maryland, March 23–24, 2009; (b) M. Svartengren, M. Anderson, Hallberg, N. Pedersen (Karolinska Institutet Stockholm); P. Wollmer (Clinical Physiology Malmö University Hospital, Malmö Sweden); A. Dirksen, S. Shaker (Gentofte University Hospital, Hellerup, Denmark); C. Lindberg, K. Forsman-Semb, A. Lloyd (Astra-Zeneca Respiratory and Inflammation Clinical and Discovery Groups); G.M. Turino, Y.Y. Lin (James P. Mara Center for Lung Disease, St. Luke's-Roosevelt Hospital Centre); I. Kilty, N. Thompson, C. Palmer (Pfizer), F. Gervais, Boutinn et al. (Merk & Co), Study Group on "Desmosine and Isodesmosine as Biomarkers for COPD" FDA, Bethesda, Maryland, January 26–27, 2010.
[31] R.A. Pauwels, A.S. Buist, P.M.R. Calverley, C.R. Jenkins, S.S. Hurd, Am. J. Respir. Crit. Care Med. 163 (2001) 1256.
[32] D.A. Pratt, Y. Daniloff, A. Duncan, S.P. Robinsn, Anal. Biochem. 207 (1992) 168.
[33] I.T. James, A.J. Walne, D. Perrett, Anal. Biochem. 240 (1996) 29.
[34] E. Kindt, D.T. Rossi, K. Gueneva-Boucheva, H. Hallak, Anal. Biochem. 283 (2000) 71.
[35] P.A. Abraham, A.J. Perejda, W.H. Carnes, J. Uitto, J. Clin. Invest. 70 (1982) 1245.
[36] P.J. Stone, J.H. Korn, H. North, E.V. Lally, L.C. Miller, L.B. Tucker, et al., Arthritis Rheum. 38 (1995) 517.
[37] L. Annovazzi, S. Viglio, D. Gheduzzi, I. Pasquali-Ronchetti, C. Zanone, G. Getta, P. Ladarola, Eur. J. Clin. Invest. 34 (2004) 156.
[38] E. Schwartz, F.A. Cruickshank, M. Lebwhol, Exp. Mol. Pathol. 52 (1990) 63.
[39] P.J. Stone, Acta Trop. 77 (2000) 97.