Determination of Free Desmosine and Isodesmosine as Urinary Biomarkers of Lung Disorder using Ultra Performance Liquid Chromatography-Ion Mobility-Mass Spectrometry

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Abstract

The elastin degradation products, desmosine (DES) and isodesmosine (IDES) are highly stable, cross-linking amino-acids that are unique to mature elastin. The excretion of DES/IDES in urine, in the free form and with associated peptide fragments, provides an indicator of lung damage in chronic obstructive pulmonary disease (COPD). A quantitative ion mobility-mass spectrometry (IM-MS) method has been developed for the analysis of free DES/IDES in urine with deuterated IDES as an internal standard.

Resolution of DES/IDES isomers was achieved in less than five minutes using ultra performance liquid chromatography (UPLC) combined with ion pairing. The optimized UPLC-IM-MS method provided a linear dynamic range of 10-300 ng/mL and a limit of quantitation of 0.028 ng/mL for IDES and 0.03 ng/mL for DES (0.55 ng and 0.61 ng on column respectively). The method reproducibility (%RSD) was < 4% for DES and IDES. The UPLC-IM-MS method was applied to the analysis of urine samples obtained from healthy volunteers and COPD patients. The DES/IDES concentrations in healthy and COPD urine showed an increase in DES (79%) and IDES (74%) in the COPD samples, relative to healthy controls. The incorporation of an IM separation prior to *m/z* measurement by MS was shown to reduce non-target ion responses from the bio-fluid matrix.

Keywords: Ion Mobility-Mass Spectrometry, Urinary Analysis, COPD, Desmosine, Isodesmosine.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is defined as a completely irreversible, progressive air flow obstruction, associated with lung inflammation caused by prolonged exposure to noxious particles and gases, particularly tobacco smoke [1]. The term COPD includes a variety of pathological disorders characterized by acute inflammatory processes that present identical symptoms; these include bronchitis, asthma, cystic fibrosis, acute lung injury, emphysema and alpha-1-antiprotease deficient patients [2].

These inflammatory reactions to exogenous [3] and genetic factors [4-6], initiate the recruitment (chemotaxis) of the cells involved in the immune system [7]. COPD is still under-diagnosed with approximately half of severe/very severe patients in the United Kingdom having any form of respiratory diagnosis [8].

The prognosis of COPD at the severe/very severe stages is poor with, currently, no prospect to reverse lung degradation [9]. The early detection and stratification of COPD patients can enable more focused treatment regimes to be employed [9-11] and a variety of biomarker candidates have been tested through multi-center clinical based trials [11].

Biomarkers identified include the elastin degradation product desmosine (DES) and its isomer isodesmosine (IDES). DES and IDES are pyridinium ring containing amino acids (Figure 1) produced by the aggregation of four lysine residues [12], which form the highly stable cross-links unique to elastin. During the lung repair process, DES and IDES molecules are liberated from the extracellular matrix (ECM) in the free form (15%) and bound form, associated with peptide fragments (85%). Concentrations range from 0.5 – 3 μg/g creatinine and 5 – 14 μg/g creatinine for free and total DES/IDES, respectively, in healthy subjects [13, 14], normalized to urinary creatinine to adjust for individual variation in sample volume and rate of urine production [15]. The presence of DES/IDES in the human body is independent

of diet and is elimination route specific to urine [16]. These factors make DES and IDES potentially important biomarkers for detection and monitoring of COPD [17-20].

The analytical determination of DES/IDES has been previously reviewed by Viglio *et al.* [21], with reported methods including amino-acid analysis, radio-immunoassay (RIA) and enzyme-linked immunosorbant assays (ELISA). These methods were of limited clinical practicality due to extensive sample preparation required and false positives resulting from cross-reactivity [19]. The majority of recent studies for the detection and quantitation of DES/IDES, have employed high performance liquid chromatography-mass spectrometry (LC-MS) [13,14, 22-25] with ion-pairing for the separation of DES/IDES on ODS based stationary phases, due to the cationic nature of these analytes.

Ma *et al.* [13, 14] applied LC-MS and LC-MS/MS to the separation of DES/IDES in healthy urine, with a partial separation of DES/IDES peaks achieved in 35 minute LC run. The LDR of their method (0.1 – 50 ng on column; based on standards), indicated the potential of applying an ion pairing liquid chromatography approach to the quantification of free DES/IDES in urine. Recently, Albarbarawi *et al.* [24] reported the LC-MS/MS analysis of total (free and bound) DES/IDES to predict the extent of lung damage based upon the combined DES/IDES response, but without chromatographic separation of the isomers. An ultra performance liquid chromatography (UPLC) approach has been reported by Shiraishi *et al.* [25], with a chromatographic run time of 15 minutes for the determination of total DES/IDES after bound DES/IDES was released by acid hydrolysis. These studies reported DES/IDES levels to increase (approximately 100%) in COPD affected individuals, relative to healthy patients. However, the liberation of free DES/IDES from associated peptide fragments requires a lengthy acid hydrolysis procedure, typically taking over 24-48 hours.

This paper reports an evaluation of the potential of ion mobility-mass spectrometry (IM-MS) combined with UPLC, for the quantitative determination of free DES/IDES in urine. The

incorporation of ion mobility (IM) spectrometry, [26-28] allows gas-phase separation of ions based on their rotationally-averaged collisional cross-section (i.e. size and shape) prior to *m/z* measurement using time-of-flight mass spectrometry. IM-MS has been shown to enhance the separation of complex samples such as urine [29] and the resolution of isomeric compounds, [30] but the future potential of the technique for the quantitative bioanalysis of small molecules has received little attention. The IM-MS method developed gives baseline chromatographic separation of free DES and IDES without a lengthy hydrolysis sample preparation step and demonstrates good quantitative performance. The determination of free DES/IDES at physiological levels, as biomarkers for discrimination between healthy and COPD-affected individuals, is demonstrated for disease *vs* control pooled urine samples.

2. Experimental

2.1. Chemicals

Elastin soluble DES, IDES and deuterated D9-IDES were provided by AstraZeneca R&D (Charnwood, Loughborough, UK). Acetonitrile (analytical grade), methanol (analytical grade), water (analytical grade), ammonium formate (>99.9%) and sodium acetate (analytical grade) were purchased from Fisher Scientific (Loughborough, UK). Heptafluorobutyric acid, HFBA, (>99%) was purchased from Acros Organics (Geel, Belgium). HPLC grade formic acid (>99.9%) and creatinine (>99%) was purchased from Sigma-Aldrich (Gillingham, UK).

2.2. Preparation of DES, IDES and D9-IDES Standards

Standards of DES and IDES with concentrations 10, 25, 50, 100, 200, 300, 400 and 500 ng/mL were prepared, each containing 25 ng/mL of D9-IDES. All solutions were freshly prepared in 85:15 H_2O : H_2O:MeOH (60:40) (v/v) containing 5 mmol/L ammonium formate, 0.1% (v/v) HFBA and pH buffered to 2.5 using formic acid.

2.3. UPLC Chromatographic Conditions

The chromatographic separations were performed using a Waters AQUITY UPLC (Waters, Manchester, UK). DES/IDES separation was achieved on an AQUITY BEH C18 column (2.1 x 100 mm, 1.7 µm Waters, Manchester, UK) under isocratic conditions with a flow rate of 600 µL/min and an injection volume of 20µL. Mobile phase A consisted of 5 mmol/L ammonium formate in H_2O with 0.1% (v/v) HFBA. Mobile phase B consisted of 5 mmol/L ammonium formate in 60:40 H_2O: MeOH with 0.1% (v/v) HFBA, each buffered to a pH of 2.5 using formic acid. A 5 minute isocratic flow of 85:15 mobile phase A: mobile phase B was used to elute DES and IDES. The mobile phase composition was then raised to 100% of

mobile phase B for 0.5 min before returning to the starting conditions. The total run time was 6 minutes.

2.4. IM-MS Conditions

IM-MS analyses were carried out on a Waters Synapt HDMS spectrometer (Waters, Manchester, UK) using an ESI source operated in positive ionisation mode with enhanced duty cycle (EDC) set at *m/z* 526 to transport the DES/IDES and D9-IDES ions. The source and desolvation temperatures were set to 150°C and 450°C, gas flow (N_2) rates were set to 50 L/h and 1000 L/h respectively. The tri-wave drift cell conditions were set at 20 mL/min drift gas (N_2) with a travelling wave height/velocity of 5 V and 300 m/s. IM-MS allowed nested mass spectra (0.065 ms/scan) and ion mobility spectra (13 ms/scan) to be acquired within the timescale of a typical UPLC peak, width a 2 s full with at half maximum (FWHM). The acquired UPLC-IM-MS data was processed using DriftScope and MassLynx 4.1 (Waters, Manchester, UK).

2.5. Urine Sample Preparation

Urine samples obtained from healthy volunteers and COPD patients were provided by AstraZeneca R&D Charnwood. To 1 mL of a combined (male and female) urine sample, 80 µL of a 3.8 nmol/mL D9-IDES solution and 120 µL of 10% HFBA solution were added. The solution was then subjected to SPE clean-up on an Oasis HLB 30 mg cartridge (Waters, Manchester, UK). The cartridge was conditioned with 1 mL of acetonitrile (ACN) containing 1% (v/v) HFBA and then equilibrated using 1 mL of aqueous 1% (v/v) HFBA. Urine samples (1.2 mL) were loaded onto the Oasis HLB solid phase, the column was washed with 1 mL of aqueous 1% (v/v) HFBA and eluted with 1 mL of 1% (v/v) HFBA in ACN. The extracts were then frozen (-80 °C) until required.

Prior to analysis, the urine extracts were thawed, evaporated to dryness using a TurboVap LV system (Biotarge AB, Uppsala Sweden) and reconstituted in 30 µL of freshly prepared H_2O:MeOH (94:6, v/v) containing 0.1% (v/v) HFBA ion pairing reagent, buffered to pH 2.5 using formic acid and ammonium formate.

2.6. Creatinine Determination

The creatinine concentration in pooled urine samples was determined using an Agilent 1200 HPLC system with isocratic mobile phase (80:20 (v/v) H_2O:MeOH and 10 mmol/L sodium acetate) flowing at 1.8 mL/min through a SCX HYPERSIL column (4.6 x 100 mm, 5 µm, Capital HPLC Ltd).UV detection was carried out (230 nm) with a total run time of 2 minutes. Urine samples were diluted (1:4, v/v) with 80:20 (v/v) (H_2O:MeOH) mobile phase prior to injection (5 µL). Urinary creatinine concentrations were calculated based on a calibration curve ($R^2 = 0.99$) of known creatinine standards, prepared at concentrations of 0.1, 0.5, 1, 2, 3 and 5 mmol/L.

3. Results and Discussion

3.1. UPLC-IM-MS analysisof DES/IDES standards.

The rapid UPLC chromatographic analysis of DES/IDES was achieved using isocratic elution with methanol/water containing HFBA ion pairing reagent, buffered using formic acid/ammonium formate (Figure 2a) The separation of DES/IDES was achieved with a total chromatographic run time of 6 minutes, compared to a 15 minute run time previously reported [25] which is more suitable for high-throughput screening than reported previously [24, 25]. Optimization of the mobile phase combined with the UPLC (5 µm) stationary phase enabled baseline resolution (Rs = 1.9) of the DES/IDES isomers with good inter-day retention time reproducibility (%RSD) of 2.6% and 3.1% respectively. The DES/IDES ion drift time *vs.* retention time profile is shown in Figure 2b. The mobility range for DES (TOF scans/bins 96-126, t_d 6.24-8.19 ms) was higher than that of IDES (TOF scans/bins 89-121, t_d 5.78-7.86 ms), indicating small differences in the rotationally averaged cross-sections, but the DES/IDES isomers were not resolved by ion mobility drift time. The drift time (bin) window selected for the maximum response of both DES and IDES isomers was in the range 7.22-7.35 ms, indicated by the dotted line in Figure 2b, which provided the best compromise between analytical sensitivity and mobility selectivity, and was used in subsequent analyses. Optimized UPLC conditions were tested using a series of DES/IDES standards (10-500 ng/mL), each spiked with deuterated D9-IDES (25 ng/mL) to determine linear dynamic ranges (LDR), limit of quantitation (LOQ) and the reproducibility in IM-MS mode. The LDR of the UPLC-IM-MS method was determined to be 10-300 ng/mL (r^2 = 0.99) for DES and IDES, respectively (Figure 3).

The use of IM-MS in quantitative bioanalysis presents a challenge because of the reduced dynamic range of the IM technique and the LDR in IM-MS mode is significantly lower than in MS mode, because of detector saturation at higher concentrations resulting from the pulsed injection of ions into the drift cell. Nevertheless, the LDR is compatible with the expected range of concentrations for free DES/IDES in urine from COPD patients and healthy controls, making IM-MS suitable for clinical studies. The limit of quantitation (10:1 signal/noise ratio) was 0.028 ng/mL and 0.03 ng/mL (0.55 ng and 0.61 ng on column) for IDES and DES respectively. The LOQ values are equivalent to 0.98 μg/g creatinine and 1.07 μg/g creatinine for IDES and DES respectively assuming a standard creatinine concentration of 5 mmol/L. Sensitivity levels achievable using UPLC-IM-MS allows quantitation of free DES/IDES in urine, removing the requirement for the lengthy hydrolysis process (24-48h), used previously for the determination of total DES/IDES [13, 14, 23, 24]. Method reproducibility was determined for a DES/IDES concentration in the middle of the LDR (100 ng/mL) over three consecutive days giving inter-day % RSDs (n=9) of 3.4% and 3.9% for DES and IDES, respectively, comparable with previously reported data [24]. The comparable precision and improved run time of the UPLC-IM-MS method demonstrates that there is no reduction in analytical quality associated with separation of the DES and IDES and doing so allows an assessment of DES/IDES variation across subjects. These data demonstrate that the linearity, sensitivity and reproducibility of the UPLC-IM-MS method are satisfactory for the quantitation of free DES/IDES in a clinical based setting, in accordance with the FDA guidance for industry bioanalytical method validation [31] and at physiologically relevant levels [13].

3.2. UPLC-IM-MS analysis of healthy and COPD urine.

The UPLC-IM-MS determination of free DES/IDES was assessed using pooled samples of healthy male urine, healthy female urine and COPD patient urine. Pooled healthy urine samples (three male and three female subjects) were selected based upon their creatinine content (3-15 mmol/L and 3-8 mmol/L for males and females respectively). These were chosen to represent the variation of urinary creatinine between individuals [32]. The pooled COPD urine samples (5.8 mmol/L) consisted of six individuals (both male and female) with a range of COPD diagnoses ranging from moderate to severe. The extracted ion chromatograms (*m/z* 526) for the analysis of a pooled COPD urine sample are shown in Figure 4. The retention times for free DES/IDES in the urine extract were slightly shorter than observed for DES/IDES standards under identical conditions. The assignment of the peaks at retention times of 3.0 and 3.3 minutes for DES and IDES respectively was confirmed by a standard addition of DES/IDES to a healthy urine sample. The DES/IDES peaks in the spiked healthy urine sample eluted from the column earlier (by approximately 30 seconds) than DES/IDES standards that were run before and after the spiked urine (data not shown). The result indicates that the urine matrix has a significant effect on free DES/IDES retention. However, the DES and IDES retention times were consistent for all the urine samples analyzed, with co-elution of IDES/ D9- IDES, and the analyte resolution was still achieved, making quantitative measurements possible using the IDES-D9 internal standard. The selected ion chromatogram shown in Figure 4a represents an acquisition where the ion mobility data (TOF scans/bins 1-200) have been combined, generating a response equivalent to UPLC-MS without ion mobility separation. Figure 4c illustrates the selected ion response in UPLC-IM- MS mode, where a five bin window (TOF scans/bins 115-120, t_d 7.36-7.68 ms) was selected, showing a comparable signal to noise ratio. The effectiveness of the ion-mobility separation is illustrated in Figure 4 (b) and (d) which shows the mass spectra corresponding to UPLC-MS (Figure 4(b)) and UPLC-IM-MS (Figure 4(d)) analyses at the

retention time for DES. The intensity of the DES ion (*m/z* 526) in the IM-MS spectrum is enhanced relative to matrix ions, demonstrating that ion mobility based separation combined with MS can improve mass spectral quality.

An on column LOQ of 0.61 ng and 0.55 ng of DES/IDES equivalent to 0.98 μg/g creatinine and 1.07 μg/g creatinine (assuming 5 mmol/L creatinine) in urine was achieved for all three urine samples following the SPE pre-concentration and clean-up described in Section 2.5, and UPLC-IM-MS analysis. Data were normalized to creatinine concentrations in pooled urine samples (to adjust for individual glomular filtration rates), which were determined by UV ion-exchange chromotography (Section 2.6). Concentrations of free DES/IDES (μg/g creatinine) are shown in Figure 5. The quantitative UPLC-IM-MS analysis shows comparatively higher levels of free DES/IDES in COPD urine than control (male and female) urine with increases in concentration of 79% and 74% respectively. These results indicate the potential clinical applicability of the UPLC-IM-MS method for the distinction of healthy and COPD-affected individuals using DES and IDES as urinary biomarkers, potentially enabling targeted treatment strategies based on individual patient need.

4. Conclusions

The development of a UPLC-IM-MS method is reported for the rapid separation (Rs > 1.5) and determination (6 minute analysis time) of the elastin cross-linking molecules DES and IDES, using ion-pair chromatography under isocratic conditions, with good precision (< 4% RSD, *n* =9). The method has been applied to determination of free DES/IDES in pooled urine samples obtained from healthy and COPD-affected individuals, using isotope dilution with D9-IDES and minimal sample preparation. Ion mobility-based separation enhanced the mass spectral ion intensities of DES/IDES and D9-IDES, relative to matrix ions, compared to UPLC-MS without ion mobility separation, reducing non-target interferences and allowing urine samples from healthy and COPD-affected individuals to be distinguished based solely upon free DES and IDES levels. The method shows increased throughput and selectivity, applicable to the rapid, non-invasive biomarker screening in a clinical setting, and demonstrates the future potential of IM-MS for quantitative bioanalysis.

Acknowledgements

The authors wish to acknowledge the BBSRC and AstraZeneca for funding this research.

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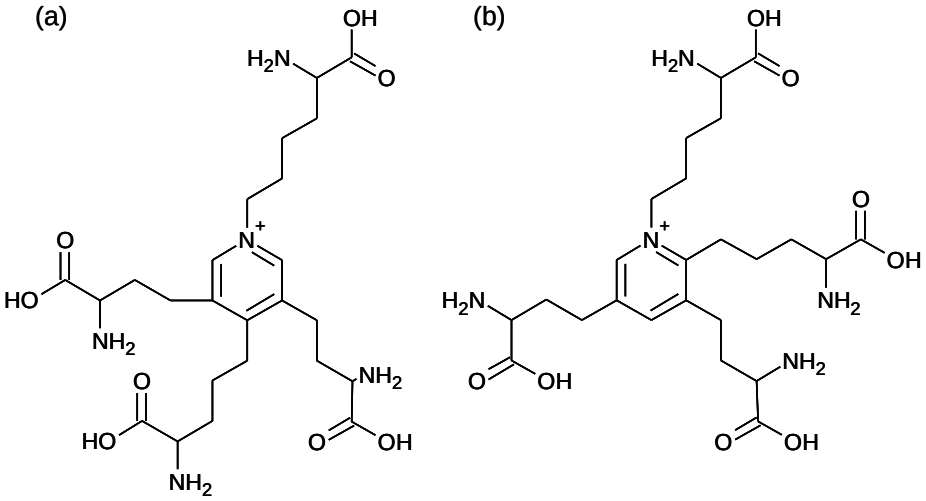

Figure. 1. Chemical structures of the elastin cross-linking isomers (a) desmosine and (b) isodesmosine, $[M]^+$ = 526.2877.

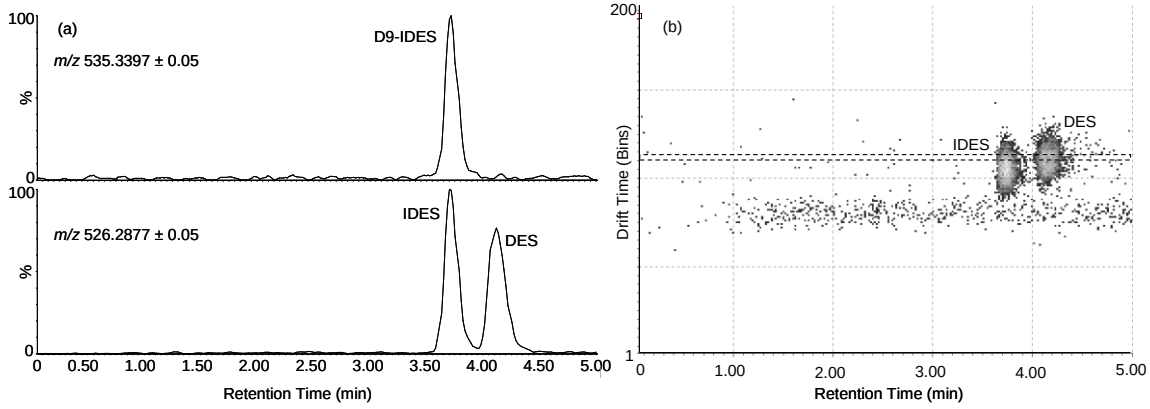

Figure. 2. UPLC-IM-MS separation of DES/IDES standards (Rs = 1.9) (a) selected ion chromatograms for DES, IDES, *m/z* 526.29 ± 0.05 (300 ng/mL), and D9-IDES, *m/z* 535.34 ± 0.05, (25 ng/mL). (b) Mobility *vs m/z* profile for DES and IDES (TOF scans/bins 1-200).

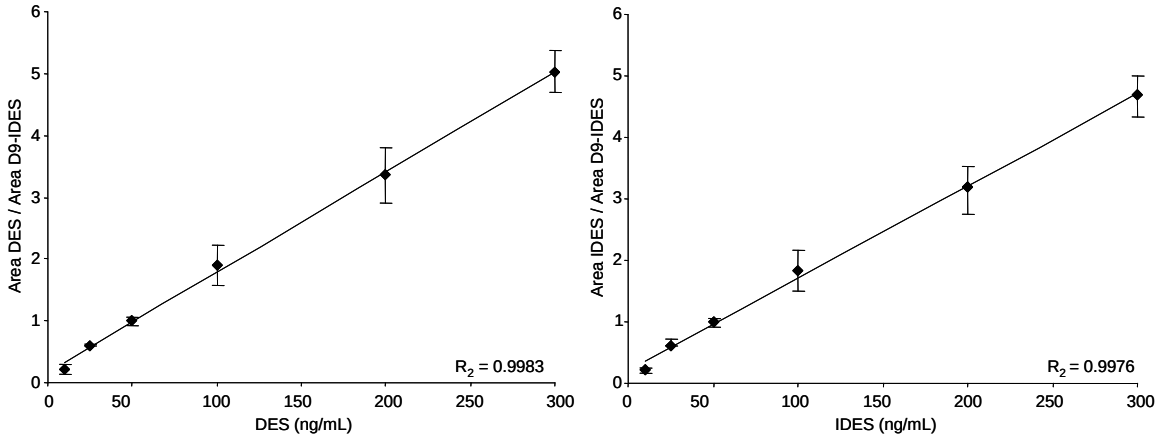

Figure. 3. Quantitative UPLC-IM-MS response for (a) DES and (b) IDES normalized to D9-IDES internal standard.

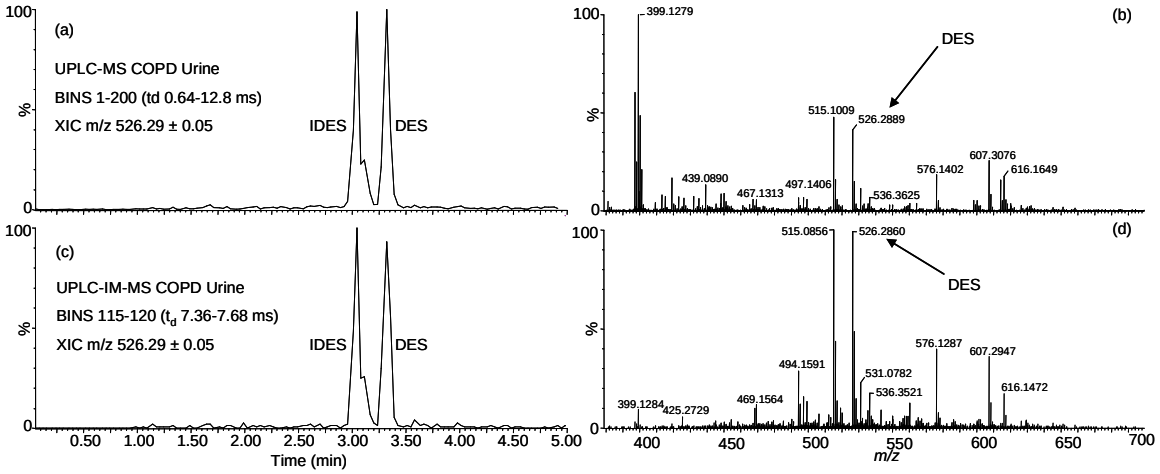

Figure. 4. Analysis of pooled COPD urine (a) selected ion response for *m/z* 526.29 ± 0.05 in UPLC-MS mode, (b) associated MS spectrum at FWHM of DES peak, (c) selected ion response for *m/z* 526.29 ± 0.05 in UPLC-IM-MS mode (TOF scans/bins 115-120), (d) associated IM-MS spectrum at FWHM of DES peak.

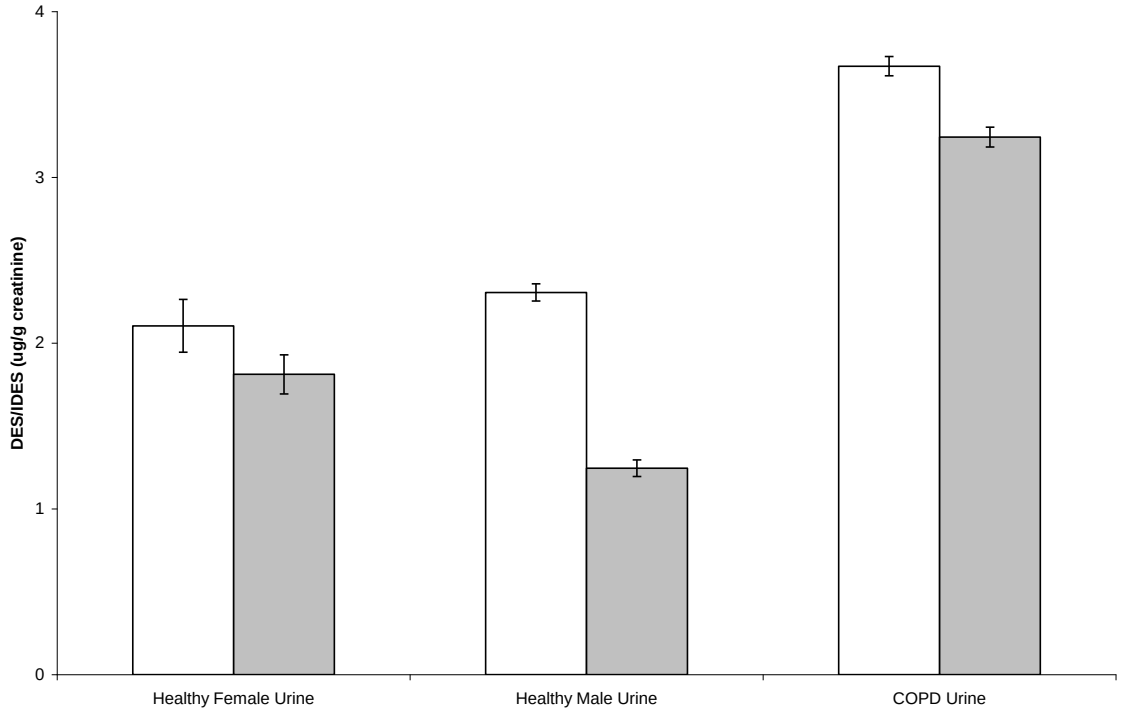

Figure. 5. Free DES/IDES concentrations (µg/g creatinine) in pooled healthy female, pooled healthy male and pooled COPD urine, error bars indicate standard error of the mean.