Development of a Robust LC-MS/MS Method for Determination of Desmosine and Isodesmosine in Human Urine

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Abstract: Desmosine (DES) and isodesmosine (IDES) are both pyridinium amino acid isomers that serve as cross-linking molecules binding the polymeric chains of amino acids into elastin. Found in urine, they are markers for the degradation of elastin which occurs in chronic obstructive pulmonary disease (COPD). In this study, a robust method using ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) with selected reaction monitoring (SRM) mode was developed for the analysis of DES and IDES in human urine. Pyridylethyl-cysteine (PE-Cys) as internal standard (I.S.) was employed for the quantification of DES and IDES. The analytes and I.S. were extracted by solid-phase extraction with Oasis MCX cartridges and separated on an AccQ-TagTM Ultra column. The assay was accurate (−6.8% to 14.5%) and precise (2.8% to 13.8%) within the concentration range of 1 to 250 pmol/mL. Moreover, the recovery and stability (working/ I.S. solution, urine samples with added elastin, and pretreated sample) was investigated, and these parameters were found acceptable. The UPLC-MS/MS method was validated and had good reproducibility and stability for the quantification of DES and IDES, which requires only 100 μ L of human urine. This assay will be a useful means for measuring DES and IDES levels in urine with robustness and characterizing patients with COPD.

Key words: desmosine, isodesmosine, elastin, biomarker, tandem mass spectrometry

1 INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a respiratory disorder generally caused by smoking^{1,2)}. It is widely thought to be a result of an imbalance of elastin-degrading proteases and protease inhibitors in the lungs, the degradation of elastin³⁾. It progresses of pulmonary emphysema which is a major component of COPD^{4,5)}. The specific markers for the degradation of mature cross-linked elastin are cross-link amino acids, desmosine (DES) and isodesmosine (IDES), which are endogenous substances. These structures are shown in Fig. 1.

In the steady state, urinary excretion of DES and IDES should reflect the rate of degradation of cross-linked elastin in the body. To prove out the value of DES and IDES as candidate markers of COPD, laboratories around the world have developed different methods for their screening and quantification. Cocci *et al.* have reported higher levels of urinary DES in patient with COPD compared to the con-

Fig. 1 Chemical Structures of A) Desmosine and B) Isodesmosine.

trols⁶⁾. In another study, Viglio *et al.* have also reported high levels of urinary DES and IDES⁷⁾. Moreover, Ma *et al.* have reported an analytical method for the determination

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of DES and IDES in human urine using high-performance liquid chromatography coupled mass spectrometry (HPLC-MS), which showed higher levels of unbound DES and IDES in patients with COPD compared to the controls^{8,9}. These studies suggested that DES and IDES can be useful biomarkers for COPD, therefore the determination of DES and IDES is important. In recently study, Boutin *et al.* have reported a high sensitive analytical method using nanoflow liquid chromatography tandem mass spectrometry (nanoLC-MS/MS), after the sample derivatization¹⁰. None of these studies have suggested the robustness of their methods as they have evaluated only the reproducibility and the recovery of the methods. Additionally, the method reported by Boutin *et al.* can not be versatile as they need a complicated procedure (derivatization with propionic anhydride) in pretreatment, and a homemade precolumn in measurement. To characterize patients with COPD in clinical level, it is necessary to develop not only robust but also a versatile analytical method for the quantitation of DES and IDES. In addition, it would be applicable for other destructive lung diseases and elastin-degrading diseases such as atherosclerosis.

In this study, we have undertaken to develop and validate a highly sensitive and also a robust method using ultra-performance liquid chromatography coupled to an electrospray ionization quadrupole tandem mass spectrometry (UPLC-MS/MS) for the quantitation of DES and IDES, without derivatization, in human urine. Validation was performed to evaluate the reproducibility as well as the stability under various conditions.

2 EXPERIMENTAL

2.1 Chemicals and reagents

Elastin soluble, DES, and IDES standards were purchased from Elastin Products Company, Inc. (Owensville, MO, USA). S-[β -(4-Pyridyl)ethyl]L-cysteine (PE-Cys) was used as an internal standard (I.S.) and purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Concentrated hydrochloric acid (amino acid analysis grade), methanol (HPLC grade), acetonitrile (HPLC grade), and 25% ammonia water (special grade) were purchased from Wako Chemicals, Inc. (Osaka, Japan). Heptafluorobutyric acid (ion-pair reagent for LC-MS) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). MCX cation exchange columns (150 mg/6 cc) were purchased from Waters Corp. (Milford, MA, USA). Water was obtained from a Millipore Milli-Q purification system (Bedford, MA, USA).

2.2 Preparation of calibration standards and quality control samples

DES and IDES stock standard solutions were prepared in water at the concentration of 1 $\mu\text{mol/mL}$ each. These

stock standard solutions were mixed and diluted with water to prepare the working standard solutions. These working standard solutions were spiked in human urine. The spiked concentrations of calibration standard samples were: 1, 2, 5, 10, 50, 100, and 250 pmol/mL, and named STD.1 to STD.7 samples, respectively. The lower limit of quantification (LLOQ) is 1 pmol/mL and the upper limit of quantification (ULOQ) is 250 pmol/mL. Also, STD.0 sample (urine sample with I.S.) was prepared to determine the content of endogenous substances.

Quality control (QC) samples were prepared with the working standard solutions, and spiked in human urine. The spiked concentrations are: 1 pmol/mL for QC-LLOQ; 2 pmol/mL for QC-L; 50 pmol/mL for QC-M; 250 pmol/mL for QC-ULOQ.

These solutions and QC samples were stored at -80°C .

The accuracy of QC samples was evaluated by comparing determined concentration and nominal concentration, but nominal concentration is arranged as the total content of endogenous substance and spiked concentration in QC sample. Nominal concentrations of DES and IDES were calculated from STD.0 samples as follows.

Nominal concentration = Determined concentration in STD.0 sample + Spiked concentration in QC samples.

2.3 Preparation of urine samples with added elastin

Elastin stock solution was prepared in saline at the concentration of 100 $\mu\text{mol/mL}$. This stock solution was diluted with saline to prepare the working elastin solutions. These working elastin solutions were spiked in human urine. The spiked concentrations of urine samples with added elastin were: 100, 200, 500, and 1,000 pmol/mL. These samples were used to evaluate the acid hydrolysis.

2.4 Sample preparation

A volume of 100 μL of urine sample was placed into a glass vial. A 20- μL of water and 120 μL of concentrated hydrochloric acid were added. For the preparation of urine samples with added elastin, the vial was sealed and hydrolyzed at 110°C for 48 h. A 2 mL of water and 10 μL of I. S. (0.04 $\mu\text{g/mL}$) were added and the samples were centrifuged (4°C , 3,000 rpm [about $1,910 \times g$], 2 min). Their supernatants were applied to an MCX cation exchange column, which had been pre-washed by centrifugation (4°C , 1,000 rpm [about $210 \times g$], 2 min) with 2 mL of methanol and 2 mL of water. These columns were then washed by centrifugation (4°C , 1,000 rpm [about $210 \times g$], 2 min) with 3 mL of water (twice) and 3 mL of methanol. The analytes and I.S. were eluted with 3 mL of 25% ammonia water / methanol (1: 1, v/v) by free-fall. These eluates were evaporated to dryness under nitrogen gas flow at 60°C . The residues were dissolved in 100 μL of the mobile phase A and the solution was transferred into polypropylene vials to be used as injection samples.

2.5 Chromatographic conditions

Ultra-performance LC system (ACQUITY, Waters, Milford, MA, USA) was used. AccQ-TagTM Ultra column (2.1 mm I.D. × 100 mm L., 1.7 µm particle size, Waters) and Van Guard Precolumn (2.1 mm I.D. × 5 mm L., 1.7 µm particle size, Waters) were selected for analytical column and guard column. The mobile phase A was 5 mM heptafluorobutyric acid in water and B was acetonitrile. The gradient was as follows: 2% of B for the run time from 0 to 1 min, followed by a linear gradient from 2 to 10% of B for 1 to 6 min, a linear gradient from 10 to 20% of B for 6 to 8 min, a linear gradient from 20 to 30% of B for 8 to 8.01 min, 30% of B for 8.01 to 10 min, a linear gradient from 30 to 2% of B for 10 to 10.01 min, and then 2% of B for 10.01 to 15 min. The flow rate was 0.5 mL/min until 11 min, 0.6 mL/min from 11.01 to 14 min, and 0.5 mL/min from 14.01 to 15 min. The total run time was 15 min. Column temperature was set at 50°C, and injection volume was 5 µL. The LC system was controlled by MassLynx software (version 4.1., Waters). The LC eluate was directed to the waste at the beginning of the analytical run, which was controlled by Dual High-pressure Six-way Switching Valve (Shiseido, Tokyo, Japan). The LC eluate was introduced directly into the mass spectrometer from 3 to 10 min.

2.6 Mass spectrometry

Mass spectrometric detection was carried out using an API5000, benchtop quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) with electrospray ionization (ESI) in positive-ion, selected reaction monitoring (SRM) mode. The settings of the mass spectrometer were as follows: ionspray voltage 5,500 V; heated gas temperature 600°C; nebulizer gas, air at 60 psi; heated gas, air at 80 psi; curtain gas, nitrogen at 40 psi. The SRM *m/z* transitions monitored were 526.2 to 481.4 (collision energy (CE): 47 V) for DES, 526.4 to 397.4 (CE: 41 V) for IDES, and 227.1 to 106.1 (CE: 23 V) for I.S.. The mass spectrometer was controlled by Analyst software (version 1.4.2., Applied Biosystems/MDS Sciex).

2.7 Method validation

2.7.1 Selectivity

Using urine collected from six individual humans, six sets of the blank, STD.0, and selectivity samples (20 pmol/mL) were pretreated and measured. Selectivity was evaluated based on the chromatograms obtained.

2.7.2 Linearity of calibration curve

Each calibration standard sample was pretreated in monoplicate (*n* = 1) per concentration point of the calibration curve and was measured. The peak area ratio was calculated from the chromatograms, and the regression equation and correlation coefficient (*r*) with weighting factor of $1/Y^2$ were calculated in order to evaluate the linearity of calibration curves and the quantification range.

The peak area ratio of the analyte to I.S. for each calibration standard is the total content of endogenous substances and spiked concentration in calibration standard sample. The intercept was left out of consideration, to correct the content of endogenous substances.

2.7.3 Accuracy and precision

The accuracy and precision of the assay were determined by analyzing QC samples in quintuplicate (*n* = 5) on three different days. Accuracy was calculated at each concentration by: (Mean determined concentration - Nominal concentration) / Nominal concentration × 100%. Precision was calculated as: Standard deviation of determined concentration / Mean determined concentration × 100%.

The accuracy for intra-day assay was calculated as the percent variation between the mean determined concentration per analytical run and nominal concentration. The accuracy for inter-day assay was calculated as the percent variation between the mean determined concentration after three analytical runs and nominal concentration. The coefficient of variation provided the measure of intra- and inter-day assay precision.

2.7.4 Recovery

The recovery of DES, IDES, and I.S. after solid-phase extraction was evaluated by comparing the response of the samples before and after the extraction, to which the same amount of DES, IDES, and I.S. had been added. The evaluation of recovery for DES and IDES was performed at the concentrations of QC-M and ULOQ in triplicate (*n* = 3).

2.7.5 Validity of urine samples with added elastin

DES and IDES in urine exist as elastin-bound and unbound. To indicate the level of degradation of elastin, resulted from the destruction of the pulmonary alveolus, a quantification of bound DES and IDES besides unbound is necessary in COPD research. To be measured, elastin must be hydrolyzed into DES and IDES with hydrochloric acid. DES and IDES were measured in urine samples with added elastin to evaluate the acid hydrolysis. The urine samples with added elastin were pretreated in triplicate (*n* = 3) at four concentrations and measured in an identical environment (intra-day assay). They were also pretreated in triplicate (*n* = 3) at two concentrations and measured on three different days (inter-day assay). The DES and IDES concentrations derived from elastin were calculated from the calibration curve, and the precision and correlation between DES/IDES and elastin concentrations were obtained. The correlation was calculated at each concentration by: {Mean determined concentration except for 100 pmol/mL sample - (Mean determined concentration of 100 pmol/mL sample × Increasing rate of elastin concentration)} / (Mean determined concentration of 100 pmol/mL sample × Increasing rate of elastin concentration) × 100%.

2.7.6 Stability

The stability is expressed as the percentage variation. The stability of DES and IDES working solutions (2 and 200

pmol/mL of working solutions) was evaluated after storage at -80°C for 3 months. The response was compared with a freshly prepared working solution.

The stability of stock/working I.S. solutions (0.04 and 500 $\mu\text{g/mL}$ of I.S. solutions) was evaluated after storage at -80°C for 1 month and at room temperature for 24 h. The response was compared with freshly prepared stock/working solutions.

The stability of DES and IDES in urine samples with added elastin (100 and 200 pmol/mL of elastin concentrations) was evaluated after storage at -80°C for 3 months. The response was compared with a freshly prepared urine sample with added elastin.

To evaluate the stability of DES and IDES in the pre-treated samples stored in the auto-sampler at 10°C , QC samples (QC-L and -M) were re-injected at 24 and 48 h after the first injection. The response was compared with a first injection.

All stability assays in urine were performed in triplicate ($n=3$).

3 RESULTS and DISCUSSION

3.1 Chromatography and mass sapectrometry

A general approach to enhance separation and throughput is to use LC columns with finer particles. The ultra-performance liquid chromatographic (UPLC) system utilizing high linear velocities with columns packed with porous $1.7\ \mu\text{m}$ particles, coupled to a tandem mass spectrometer, is a another powerful approach that improves peak resolu-

tion, sensitivity, and high-throughput of analysis¹¹⁻¹⁵). In our efforts for the determination of DES and IDES by UPLC system on a column with $1.7\ \mu\text{m}$ particle size, the separa-

Fig. 2 UPLC-MS/MS SRM Chromatograms for DES and IDES of the LLOQ Samples (1 pmol/mL).

Fig. 3 Chromatograms for DES and IDES of Blank, STD.0, and the Selectivity Samples (20 pmol/mL).

tion could be achieved in a much shorter time than ever previously reported (15 min). DES, IDES, and I.S. showed an identical retention time of approximately 6.5, 6.4, and 4.1 min, respectively. The representative chromatograms of the LLOQ samples (1 pmol/mL) are shown in Fig. 2.

3.2. Method validation

3.2.1 Selectivity

For endogenous substances, the analyte peak of DES and IDES appears in the blank and STD.0 samples. However, the content of the endogenous substances is corrected by the calculation of calibration curve. The selectivity of DES and IDES was evaluated by confirming that the peaks had good shape and were clearly separated from the other peaks. The selectivity of the I.S. was evaluated by confirming that there was no interference at the retention time. The representative chromatograms of selectivity (blank, STD.0, and selectivity samples) are shown in Fig. 3.

The chromatograms indicated that there was no interference at the retention time of the I.S. DES and IDES peak shapes were good and showed good separation.

3.2.2 Linearity of calibration curve

The correlation coefficients (r) were 0.9958 to 0.9998 for DES and 0.9966 to 0.9996 for IDES. The accuracy for DES of the back-calculated concentrations of calibration curves was -6.5% to 18.2% at the LLOQ and -14.2% to 10.5% at all other concentrations. The accuracy for IDES of the back-calculated concentrations of calibration curves was -3.2% to 13.3% at the LLOQ and -12.2% to 9.8% at all other concentrations. The representative calibration curves and correlation coefficients (r) of DES and IDES are shown in Fig. 4. These results fall within the acceptable range set by the FDA guidelines on bioanalytical method validation¹⁶⁾, whose deviations up to 20% at the LLOQ and 15% at other concentrations. It was judged that the calibration curves for DES and IDES in human urine had good linearity in the quantification range from 1 to 250 pmol/mL. This result was indicated higher sensitivity than previously reported non-derivatized method.

3.2.3 Accuracy and precision

The results of the accuracy and precision are shown in Table 1. The accuracy for DES was -6.1% to -1.3% at the LLOQ and -6.8% to 14.5% at other concentrations, and the precision for DES was 5.0% to 8.8% at the LLOQ and 2.8% to 11.9% at other concentrations. The accuracy for IDES was -1.5% to 2.9% at the LLOQ and -2.3% to 12.8% at other concentrations, and the precision for IDES was 10.0% to 11.7% at the LLOQ and 3.3% to 13.8% at other concentrations. These results fall within the acceptable range set by the FDA guidelines on bioanalytical method validation. All these results had good reproducibility in both intra- and inter-day assay.

3.2.4 Recovery

Generally, the extent of recovery is constant and precise.

Fig. 4 Calibration Curves of DES and IDES in Human Urine in a Dynamic Range from 1 to 250 pmol/mL.

The recovery of DES, IDES, and I.S. from human urine was 79.6% to 84.8%, 81.5% to 82.1%, and 96.1%, respectively. The precision ranged from 0.0% to 5.4%. The results of recovery were almost constant and precise at all concentrations.

3.2.5 Validity of urine samples with added elastin

The results of the validity of urine samples with added elastin are shown in Table 2 (intra-day assay) and Table 3 (inter-day assay).

The precision was 1.4% to 7.6% at all concentrations and DES/IDES concentrations exhibited correlation with the increasing rate of elastin concentrations (-8.4% to 3.1%). This result fall within a 15% variation range. All these results had good reproducibility and correlation between DES/IDES and elastin concentrations. DES and IDES derived from elastin can be measured by hydrolyzing elastin at 110°C for 48 h.

As the result, this UPLC-MS/MS method can be used to measure DES and IDES in both elastin-bound and unbound.

Table 1 Intra-and Inter-day Assay Performance Data for the Analysis of DES and IDES in Human Urine

Compound	QC samples	Run	Nominal conc. (pmol/mL)	Mean determined conc. (pmol/mL)	Precision (% C.V.)	Accuracy (% Bias)
DES	LLOQ	1	4.73	4.46	5.0	-5.6
		2	4.46	4.40	8.8	-1.3
		3	4.97	4.67	6.9	-6.1
		Average	4.72	4.51	7.0	-4.5
	L	1	5.73	5.33	11.9	-6.8
		2	5.46	5.36	6.0	-1.8
		3	5.97	6.17	7.4	3.3
		Average	5.72	5.62	10.8	-1.7
	M	1	53.73	60.27	3.0	12.2
		2	53.46	51.10	2.8	-4.4
		3	53.97	60.26	8.8	11.7
		Average	53.72	57.21	9.5	6.5
	ULOQ	1	253.73	281.80	3.0	11.1
		2	253.46	253.41	3.5	0.0
		3	253.97	290.72	11.8	14.5
		Average	253.72	275.31	9.2	8.5
IDES	LLOQ	1	3.71	3.81	11.7	2.9
		2	3.68	3.63	11.2	-1.5
		3	3.72	3.71	10.0	-0.3
		Average	3.70	3.72	10.4	0.3
	L	1	4.71	4.66	9.7	-0.9
		2	4.68	4.64	5.8	-0.8
		3	4.72	4.89	10.4	3.5
		Average	4.70	4.73	8.6	0.6
	M	1	52.71	57.29	3.7	8.7
		2	52.68	51.49	4.9	-2.3
		3	52.72	56.65	8.4	7.4
		Average	52.70	55.14	7.4	4.6
	ULOQ	1	252.71	265.08	3.3	4.9
		2	252.68	258.57	5.1	2.3
		3	252.72	285.04	13.8	12.8
		Average	252.70	269.56	9.5	6.7

Table 2 Intra-day Assay Performance Data for the Analysis of DES and IDES in Urine Samples with Added Elastin

Compound	Elastin conc. (pmol/mL)	Increasing rate of elastin conc.*	Mean determined conc. (pmol/mL)	Precision (% C.V.)	Correlation (%)
DES	100	1	19.42	1.7	—
	200	2	35.57	7.0	-8.4
	500	5	97.14	1.7	0.0
	1000	10	199.42	6.6	2.7
IDES	100	1	14.32	1.4	—
	200	2	26.92	5.2	0.0
	500	5	71.58	2.8	0.0
	1000	10	147.65	7.2	3.1

* The rate is based on the lowest elastin concentration (100 pmol/mL).

Table 3 Inter-day Assay Performance Data for the Analysis of DES and IDES in Urine Samples with Added Elastin

Compound	Elastin conc. (pmol/mL)	Increasing rate of elastin conc.	Mean determined conc. (pmol/mL)	Precision (% C.V.)	Correlation (%)
DES	100	1	20.59	7.3	–
	200	2	37.98	6.9	-7.8
IDES	100	1	15.30	7.6	–
	200	2	28.31	5.8	-7.5

Table 4 Stability Data of DES, IDES and I.S. under Various Conditions

Compound	Storage condition	Concentration	Variation* (%)	Precision (% C.V.)
I.S.	I.S. solution			
	Room temperature, 24 hours	0.04 µg/mL	-6.4	0.1
		500 µg/mL	3.2	0.0
	– 80°C, 1 month	0.04 µg/mL	3.4	0.0
		500 µg/mL	3.3	0.0
DES	Working solution			
	– 80°C, 3 months	2pmol/mL	-10.6	6.7
		200pmol/mL	2.4	0.8
	Urine samples with added elastin			
	– 80°C, 3 months	100 pmol/mL	5.9	6.2
		200 pmol/mL	12.6	5.2
	Pretreated sample			
	10°C, 24 hours	5.76 pmol/mL	4.1	12.9
	(in the auto-sampler)	53.76 pmol/mL	4.2	3.6
	10°C, 48 hours	5.76 pmol/mL	-5.2	13.9
	(in the auto-sampler)	53.76 pmol/mL	1.4	2.1
IDES	Working solution			
	– 80°C, 3 months	2 pmol/mL	-7.7	16.6
		200 pmol/mL	3.8	0.7
	Urine samples with added elastin			
	– 80°C, 3 months	100 pmol/mL	8.1	3.9
		200 pmol/mL	9.7	11.8
	Pretreated sample			
	10°C, 24 hours	4.68 pmol/mL	1.7	6.0
	(in the auto-sampler)	52.68 pmol/mL	6.9	4.7
	10°C, 48 hours	4.68 pmol/mL	-4.7	7.1
	(in the auto-sampler)	52.68 pmol/mL	7.3	2.1

* Variation (%) = (Mean peak area ratio of stability samples-Mean peak area ratio of control samples)/Mean peak area ratio of control samples ×100

3.2.6 Stability

The stability data are summarized in **Table 4**.

3.2.6.1 The stability of working and I.S. solutions:

The variation of DES and IDES in working solutions was –10.6% to 3.8% after storage at –80°C for 3 months. This result suggested that these solutions are stable at –80°C for 3 months. The variation of I.S. in stock/working solutions was –6.4% to 3.4% after storage at room temperature for 24 h and at –80°C for 1 month. This result fall within a 15% variation range and suggested that these solutions are stable at room temperature for 24 h and at

–80°C for 1 month.

3.2.6.2 The stability of urine samples with added elastin:

The variation of DES and IDES in urine samples with added elastin was 5.9% to 12.6% after storage at –80°C for 3 months. This result fall within a 15% variation range and suggested that they are stable at –80°C for 3 months.

3.2.6.3 The stability of pretreated samples stored in the auto-sampler:

The variation of DES and IDES in the pretreated samples stored in the auto-sampler at 10°C was –5.2% to 7.3% after storage for 24 and 48 h. This result fall within a 15%

Fig. 5 Chromatograms for DES and IDES of Clinical Sample.

variation range and suggested that the analytical run can be re-injected after up to 48 h of storage in the auto-sampler at 10°C.

The precision ranged from 0.0% to 16.6%. All the measurements of stability showed good precision.

3.3 Application

The method was applied to quantify of DES and IDES in human urine obtained from 24 patients with lymphangioleiomyomatosis (LAM) that is known to be a destructive lung disease like COPD. DES + IDES in LAM patients was 39.5 ± 2.9 pmol/mg creatinine (mean $\pm$ SD) and significantly increased as compared to that of age- and sex-matched

control (26.0 ± 1.4 , $n = 19$; $p < 0.05$, unpaired t -test). The representative chromatograms of clinical sample are shown in Fig. 5. The samples were pretreated and measured under the guaranteed stability conditions, and DES and IDES were quantified.

4 CONCLUSION

This study was conducted to newly establish a robust analytical method for the quantification by UPLC-MS/MS of DES and IDES in human urine. The method was validated in the quantification range from 1 to 250 pmol/mL with robustness and high-reproducibility, requiring only 100 μ L urine per sample. This robust analytical method will be a valuable tool in measuring DES and IDES in urine and characterizing patients with COPD.

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