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Measurement of sputum total desmosines in patients from Al Madinah district using isotope-dilution liquid chromatography-tandem mass spectrometry

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The absolute quantification of total desmosine (DES/IDS) in human sputum was determined utilizing an LC-MS/MS technique employing synthesized internal standard. The procedure includes tandem mass spectrometric analysis (LC-MS/MS), solid phase extraction (SPE), liquid chromatographic separation, and acid hydrolysis. From 0.05 (LLQ) to 20.0ng/mL, the calibration curve displayed acceptable precision and accuracy. The assay's precision and accuracy within and between days were calculated. Precision inter-assay ranged from 2.72 to 20.15%, whereas precision intra-assay ranged from 4.76 to 23.47%. While the inter-assay ranged from -13.33 to 3.33% and the intra-assay ranged from 20.0 to -20.0% according to the accuracy. All of the sputum samples' concentrations fall within the assay's reportable range, which was determined using 40 sputum samples as the reference range for total DES. These findings show that the established method was able to quantitatively measure total desmosine in human sputum samples in a sensitive, repeatable, and accurate manner. In addition, Desmosine has the potential to be a biomarker for elastin degradation in respiratory diseases.

Keywords: Mass Spectrometry, COPD, Biomarkers, Elastin Degradation, Desmosines

INTRODUCTION

The World Health Organization has identified chronic obstructive pulmonary disease (COPD) as the most prevalent chronic disease among children, and it projects that by 2030, COPD will be responsible for one-third of all fatalities globally. Lung elastin degradation was found to be a common factor with respiratory lung diseases. This in turn produces two important metabolites: desmosine (DES) and isodesmosine (IDES), two non-traditional amino acids that both function as crosslinking networks for elastin.(Foster et al. 1974; Brown-Augsburger et al. 1995) Figure 1 depicts the two isomers' chemical structures.

Elastin degradation has emerged as a promising biomarker for both disease progression and patient response to treatment, and it has gained potential as a target for pharmacological intervention in lung diseases(Ma et al. 2003b; Ma, Lin, and Turino 2007; Luisetti et al. 2008; Rennard et al. 2012; Turino et al. 2012; Hu et al. 2007). Therefore, a sensitive and accurate assay is required to increase the clinical validity of elastin degradation as a biomarker for COPD.

Due to the specific characteristics of mature cross-linked human elastin and the fact that elastin degradation reflects/correlates to/with the status of respiratory disease, numerous analytical methods have been developed over the past 30 years to quantify desmosine and isodesmosine as biomarkers of elastin degradation.

Most of the previous studies focused on the measurement of desmosine in urine(Albarbarawi et al. 2010; Stone, Bryanrhadfi, et al. 1991; Viglio et al. 1998; Fill et al. 2006; Sato et al. 2008; Boutin, Ahmad, et al. 2009; Boutin, Berthelette, et al. 2009b; Huang et al. 2012; Laguna et al. 2012; Ongay et al. 2016; Kim et al. 2018; Davies et al. 1983; Janoff 1984; Pelham et al. 1985; Tenholder et al. 1991; Wagner, Accurso, and Laguna 2010; Laguna et al. 2018) due to the low urine matrix complexity and the high concentrations of desmosines predicted in urine compared to plasma or sputum. On the other hand, urinary desmosine represents the whole body elastin turnover(Ma et al. 2003a; Ma, Lin, and Turino 2007) which makes uDES (Urinary Demsosine) less directly associated to the pathological processes of lung disease.

Due to the low complexity of the urine matrix and the higher expected amounts of desmosines in urine compared to plasma or sputum, the majority of earlier studies [9–25] concentrated on focused on measuring desmosine in urine. we are aware that blood and urine desmosines show the body's overall elastin turnover. However, they have a less direct association to the pathogenic mechanisms underlying lung disorders. [4, 26]

Desmosine concentrations are significantly higher in patients with respiratory conditions like asthma(Huang et al. 2012; Akers et al. 1992; Cataldo et al. 2000; Coultas 1998; Demedts et al. 2005), (CF) cystic fibrosis(Stone, Konstan, et al. 1995; Viglio et al. 2000b; Ferrari et al. 2012; Viglio et al. 2014; Laguna et al. 2009; Wagner, Accurso, and Laguna 2010; Laguna et

al. 2012; Laguna et al. 2018), COPD(Viglio et al. 2000a; Ma, Lin, and Turino 2007; Boschetto et al. 2006; Cocci et al. 2002; Viglio et al. 2000b; Stone, Gottlieb, O'Connor, Ciccolella, Breuer, Bryan- Rhadfi, et al. 1995), alpha-antitrypsin deficiency(Ma, Lin, and Turino 2007; Viglio et al. 2000a; Churg et al. 2001; Churg et al. 2003; Kurucz et al. 2004; Stoller and Aboussouan 2005; Fregonese et al. 2008; Stockley 2014; Hatipoglu and Stoller 2016; Ma et al. 2016; Hazari et al. 2017), and bronchiectasis(Viglio et al. 2000b; Stockley et al. 2013; Gray et al. 2008; Gramegna et al. 2017; Chalmers et al. 2017) than in healthy subjects unless they smoke; elevated desmosine levels were found in healthy smokers(Stone, Gottlieb, O'Connor, Ciccolella, Breuer, Bryan-Rhadfi, et al. 1995; Viglio et al. 2000b, 2000a; Davies et al. 1983; Janoff, Raju, and Dearing 1983; Osman et al. 1985; Churg et al. 2002; Eisner 2002; Jaakkola and Jaakkola 2002; Thomson 2017)

Desmosines were measured using a variety of techniques, including, radioimmunoassay(Starcher, Green, and Scott 1995) immunoassay methods,(Cocci et al. 2002; Luisetti et al. 1996; McClintock et al. 2006; Starcher, Green, and Scott 1995) capillary electrophoresis(Iadarola et al. 2016; Viglio et al. 2014; Guzman, Blanc, and Phillips 2008; Choudhury et al. 2006; Viglio et al. 2000b) electrokinetic chromatography,(Viglio et al. 1998; Viglio et al. 2014; Ferrari et al. 2012; Huang and Kang 2007) nuclear magnetic resonance (NMR)(Dhital et al. 2017; Papaioannou et al. 2015) and high-performance liquid chromatography, HPLC(Stone, Gottlieb, O'Connor, Ciccolella, Breuer, Bryan- Rhadfi, et al. 1995; Stone, Bryanrhadfi, et al. 1991; Stone, Konstan, et al. 1995; Stone, Lucey, et al. 1991), liquid chromatography mass spectrometry (Kaga et al. 2003; Ma, Lin, and Turino 2007; Boutin, Berthelette, et al. 2009a; Albarbarawi et al. 2010; Ma, Turino, and Lin 2011; Ferrari et al. 2012; Lamerz et al. 2013; Ma et al. 2013; Albarbarawi et al. 2013) MALDI ion trap mass spectrometry(Papaioannou et al. 2015; Rathod et al. 2018), ID-LC-MS/MS has the best specificity and sensitivity for measuring desmosines.

Prior to the development of a sensitive nLC-MS/MS approach using heavy desmosine (D4-DES) as an internal standard for absolute quantification of desmosine by Thibault et al. 2009(Boutin, Berthelette, et al. 2009b), methods lacked a more stringent internal standard for matrix effects. The use of nanoflow not only increases ionization efficiency but also, minimizes ionization suppression. However, this method's adoption in ordinary clinical research was rendered difficult by the use of nano-flow liquid chromatography and a derivatization step.

Later, two methods suitable for high throughput clinical applications for the exact determination of total blood and urine desmosines using isotope dilution microflow liquid chromatography tandem mass spectrometry were reported(Albarbarawi et al. 2013; Albarbarawi et al. 2010). to verify the clinical application of these methods as biomarkers of elastin degradation as a result of COPD a combined study published(Huang et al. 2012). The associations between desmosines levels and lung function, illness frequency, and smoking status were investigated further(Huang et al. 2020; Huang et al. 2016).

While numerous methods were employed for sputum desmosines, using an LC-MS/MS(Ma, Lin, and Turino 2007) technique, study showed that in COPD patients with normal alpha anti-trypsin (AAT) levels, total desmosines in sputum (spontaneous and induced) ranged between 0.03-0.58 ng/mL. in other research by capillary electrophoresis Emphysema and COPD patients' total desmosines in forced sputum did not vary from those without emphysema (Boschetto et al. 2006). Recently, Laguna et al reported that using RIA(Laguna et al. 2009), spontaneous sputum desmosine levels in cystic fibrosis ranged from 5 to 128 pmol/mL.

Sputum desmosines are difficult to accurately quantify due to its low concentrations and technological issues. A reliable, sensitive, and clinically useful method is required due to its clear association with lung diseases. In this study, we do away with the ion-pairing reagents that were mobile phase modifiers in earlier techniques. The ion-pairing reagents significantly reduced the ionization efficiency, which had an impact on the measured analytes' intensities. By overcoming this impact, the method's sensitivity levels have increased, enabling us to identify and precisely measure sputum desmosine in healthy individuals. Consequently, utilizing an in-house synthesised internal standard, an accurate and precise LC-MS/MS technique for the quantification of sputum desmosines was developed (D4-IDES).

MATERIALS AND METHODS

Desmosine and isodesmosine were obtained from the Owensville, Missouri-based Elastin Products Company Inc. Acetonitrile (ACN) of the HPLC gradient grade and formic acid (FA) (analytical reagent grade, 98%) were obtained from Fisher Scientific. The D4-IDES Internal Standard was synthesised in-house. The following items were bought from Sigma-Aldrich: tetrahydrofuran (THF), butanol (Chromasolv grade), heavy water (D2O) (part # 151882-10X1ML), and DBU (1,8-Diazabicyclo[5.4.0]undec-7-ene). 37% HCl and acetic acid came from VWR.

From Agilent Technologies (USA) UHPLC-DAD-ESI-QQQ system, UHPLC(1290 Infinity II), MS/MS(6460Agilent). High recovery certified glass vials (Agilent #5183-2030), and Agilent Polaris C18 HPLC Column (3 μm x 5 cm × 1.0 mm), solid phase extraction C18 columns (C18,40-60μm,120Å,500mg/6mL) from StarLab/China (part#: SLSPE5006C18).

87 human sputum samples were collected from both apparently healthy volunteers and Asthma/COPD patients from King Fahd Hospital in Medina, Saudi Arabia, only 40 of the 87 were used in this pilot study. At -80 °C, all samples were kept frozen until further examination. The study was authorized by the Taibah University Ethical Approval Board, and all study participants provided written Informed-Consent.

Purification and Synthesis of Deuterated Isodesmosine

For the manufacture of desmosine deuterated derivatives, we adhered to a previously reported protocol(Boutin, Ahmad, et al. 2009). Briefly, the reaction was carried out at 70 °C for 36 hours in a closed vessel with 50 µg of IDES reconstituted in 0.1 mL of heavy water (D2O), in addition, 5 µL of DBU was added catalyzing the reaction. The BDU must then be removed using 1 mL of chloroform twice in two subsequent cleanup steps. The remaining heavy water was then eliminated after three cycles of evaporation and reconstitution in 1 mL of water. To inactivate any lingering amounts of DBU and stop the deuterium exchange, the purified Deuterated-IDES was diluted in 1 mL of 0.2% formic acid before being bubbled with nitrogen and stored. To ensure that the synthesized IS was appropriate and to confirm its purity, it was characterized by MS. At least four different Deuterated forms have been found in the resulted mixture of IS. D3, D4, D5, and D6 IDES. Where, D4-IDES was the most common derivative, and mass spectrometry was employed to determine its structure. See the supplemental information figures, figure S1.

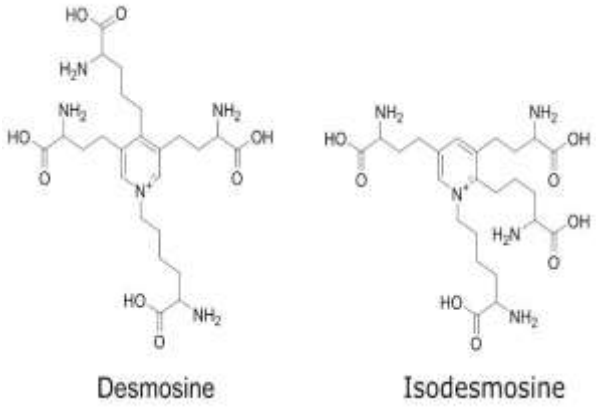

Figure 1: The Chemical Structures of two major metabolites of elastin degradation, Desmosine and Isodesmosine

Desmosines Extraction:

Each 250 µL of the sputum samples had the internal standard D4-IDES added to it to a final concentration of 0.5 µg/mL 250 µL of concentrated HCl were then added. Next the 2 mL Eppendorf safe-lock Sample tubes were placed in a heating block for 18 hours at 110 ºC with lid-lock clips fixed. As the digestion procedure completes, peptide linkages are hydrolyzed liberating bounded desmosines, then mixture had been allowed to cool down for one hour. the C18 SPE filter was used to eliminate impurities and concentrate analytes. In two steps, MilliQ water and the same volume of the mixture (butanol/acetic acid/water=4:1:1) were used to condition C18 SPE columns. Samples were loaded then washed, and lastly eluted using MilliQ water in 1.5 mL Eppendorf safe lock tubes, as described on the manufacturer's manual. Desmosines that had been eluted had been dried and reconstituted in 50 µL of 0.1% FA. Thereafter, vortexed 10sec, sonicated 5min, and lastly centrifuged @ 13900xg for 10 min, the supernatant transferred to MS vials.

Mass Spectrometry and data analysis:

The multi reaction monitoring (MRM) approach was used for the mass spectrometric analysis. The mass spectrometer was placed in positive ion mode with an ion-spray voltage of 4 kV. The precursor ions' MS/MS spectra (m/z = 526>481 for IDES and 530>485 for D4-IDES) were collected with the scan parameters of 500 msec and 0.7 Da. With the exception of the mass shift brought on by deuterium atoms, the fragmentation pattern of D4-IDES and IDES were identical.

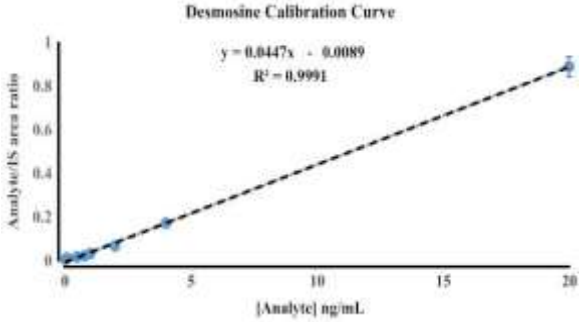

Figure 2: A typical standard curve ranges from 0.05 to 20 ng/mL

10 µL was injected on-column with a flow rate of 250 µl/min, samples were separated and eluted using a linear gradient from 5% to 60% (0.1%FA/Acetonitrile) in 4 minutes, then column washed with 90% mobile phase B then the column recalibrated for 3 minutes with initial conditions.

Chromatogram peak areas for IDES and D4-IDES were measured using Agilent Mass Hunter Qualitative Analysis B.06.00. Over the course of three days, three standard curves were produced, and statistical results for standard deviation (SD), precision %CV, and accuracy (%Bias) were calculated.

Table 1: Intra-Assay Precision and Accuracy of the Desmosines Calibration Curves, Day 1

Calibrator	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Concentration (ng/mL)	0.05	0.1	0.5	0.8	1	2	4	20
Run 1	0.04	0.13	0.3	0.7	1	1.6	4	20.7
Run 2	0.05	0.09	0.5	0.8	0.9	1.9	4	18.8
Run 3	0.04	0.1	0.4	0.7	0.8	1.8	3.9	20.4
Run 4	0.04	0.1	0.4	0.9	0.8	1.8	3.3	21.1
Mean (ng/mL)	0.04	0.10	0.40	0.78	0.88	1.78	3.80	20.25
STD	0.01	0.01	0.08	0.10	0.10	0.13	0.34	1.01
% CV	11.76	5.13	20.41	12.35	10.94	7.09	8.86	4.98
% Bias	-15.00	-2.50	-20.00	-3.13	-12.50	-11.25	-5.00	1.25

Table 2: Intra-Assay Precision and Accuracy of the Desmosines Calibration Curves, Day 2

Calibrator	1	2	3	4	5	6	7	8
Concentration (ng/mL)	0.05	0.1	0.5	0.8	1	2	4	20
Run 1	0.04	0.09	0.7	0.8	1.2	2.2	4.0	18.2
Run 2	0.05	0.1	0.5	0.7	1.1	1.8	3.6	20.2
Run 3	0.05	0.1	0.4	1.1	0.8	2	3.6	20.1
Run 4	0.04	0.09	0.6	0.8	0.8	1.9	3.8	19.8
Mean (ng/mL)	0.05	0.10	0.55	0.85	0.98	1.98	3.75	19.58
STD	0.01	0.01	0.13	0.17	0.21	0.17	0.19	0.93
% CV	12.83	6.08	23.47	20.38	21.14	8.65	5.11	4.76
% Bias	-10.00	-5.00	10.00	6.25	-2.50	-1.25	-6.25	-2.13

Mean measured Desmosines concentrations from separate analytical runs Day 2

Table 3: Intra-Assay Precision and Accuracy of the Desmosines Calibration Curves, Day 3

Calibrator	1	2	3	4	5	6	7	8
Concentration (ng/mL)	0.05	0.1	0.5	0.8	1	2	4	20
Run 1	0.04	0.12	0.6	0.7	1.1	1.9	4.1	20.5
Run 2	0.05	0.13	0.7	0.8	0.8	1.6	3.5	19.4
Run 3	0.04	0.1	0.6	0.6	0.9	2	4.1	17.8
Run 4	0.04	0.1	0.5	0.9	1	1.4	4.1	18.9
Mean (ng/mL)	0.04	0.11	0.60	0.75	0.95	1.73	3.95	19.15
STD	0.01	0.02	0.08	0.13	0.13	0.28	0.30	1.12
% CV	11.76	13.33	13.61	17.21	13.59	15.96	7.59	5.85
% Bias	-15.00	12.50	20.00	-6.25	-5.00	-13.75	-1.25	-4.25

Mean measured Desmosines concentrations from separate analytical runs Day 3

Table 4: Inter-Assay Precision and Accuracy of the Desmosine Calibration Curves

Calibrator	1	2	3	4	5	6	7	8
Concentration (ng/mL)	0.05	0.1	0.5	0.8	1	2	4	20
Day 1	0.04	0.1	0.4	0.78	0.88	1.78	3.8	20.25
Day 2	0.05	0.1	0.55	0.85	0.98	1.98	3.75	19.58
Day 3	0.04	0.11	0.6	0.75	0.95	1.73	3.95	19.15
Mean (ng/mL)	0.04	0.10	0.52	0.79	0.94	1.83	3.833	19.66
STD	0.01	0.01	0.10	0.05	0.05	0.13	0.104	0.55
% CV	13.32	5.59	20.15	6.47	5.48	7.23	2.715	2.82
% Bias	-13.33	3.33	3.33	-0.83	-6.33	-8.50	-4.170	-1.70

In addition to the calibration curve displayed in Figure 2, all data are presented in the supplemental information tables S1–S3 and the inter-assay table S4.

RESULTS AND DISCUSSION

Improvement of assay sensitivity

We applied two approaches to increase the assay's sensitivity: (1) reducing the LC column diameter from 2.0 mm to 1.0 mm; and (2) changing the mobile phase's compositions without using the previously employed ion-pairing modifiers.(Albarbarawi et al. 2010; Albarbarawi et al. 2013)In brief, sputum samples are spiked with the internal standard, and then subsequently acid hydrolysed at 108 ºC for 18 hours. Desmosines are then extracted using SPE columns and reconstituted in 0.1% FA before analysis. Desmosine and isodesmosine were made to co-elute using a steep gradient (see figure S3 in the supplemental information).

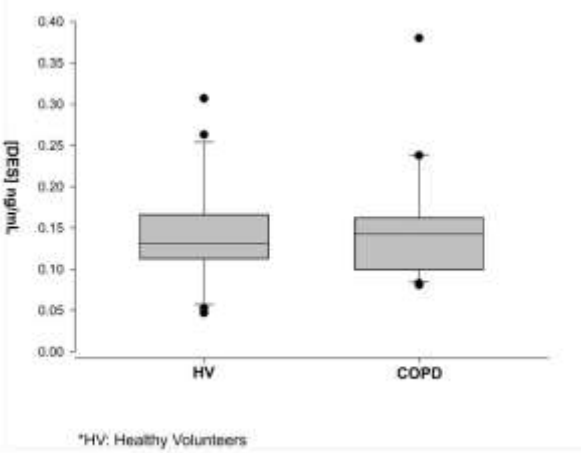

Figure 3: Box and whisker plot showing Sputum total DES/IDS levels in healthy individuals and COPD patients.

Desmosines' calibration curve was built using eleven calibrators, with values ranging from 0.05 to 20.0ng/mL. All statistical calculations were elaborated based on three analytical runs for each of the eight calibrators (0.05 - 20ng/mL), Table S1, S2, and S3 show the intra-assay precision (%CV) and accuracy (%Bias), with %CV ranging from 4.76 to 23.47% and %Bias from -20.0 to 20.0%. The results of inter-assay precision (%CV) and accuracy (%Bias) are shown in (Table S4). Bias varied from -13.33 to 3.33%, while the %CV ranged from 2.72 to 20.15%.

The effectiveness of this approach was evaluated by evaluating the total sputum desmosines in both COPD patients and healthy volunteers. Twenty samples of sputum from healthy volunteers make up the first cohort, whereas twenty samples from COPD patients make up the second group. Table in supplemental information detailed the complete results (Table S5). All measured sputum total desmosines values, which range from 0.047 to 0.380ng/mL, are higher than the LLOQ. Figure 3 Box and Whisker plot used to summarize results where the COPD patient's average has higher value compared to healthy volunteers as expected.

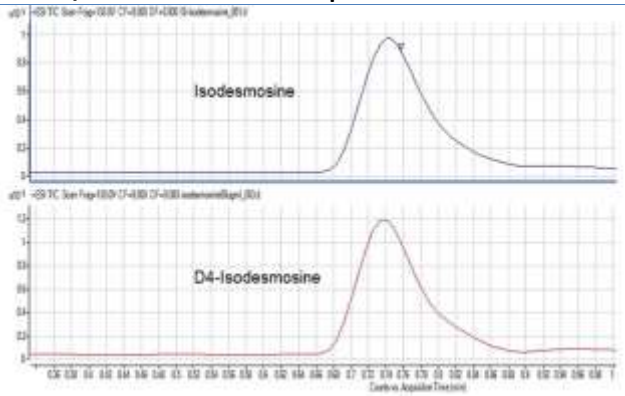

Figure S2: Two separate chromatograms of IDES vs D4-IDES show identical coelution of both compounds under identical conditions

Sputum total DES/IDS levels in healthy individuals and COPD patients.

To assess the utility of this method for measuring total sputum DES/IDS in healthy volunteers and COPD patients, we analysed two clinical cohorts: 20 sputum samples from healthy volunteers and 20 COPD patients (Table 6). All healthy volunteers and COPD patients had sputum total DES/IDS levels above the LLOQ (0.047 to 0.380 ng/mL).

Table 5: Reference Range of Total Desmosine in Sputum Samples

Subject	Final [DES] (ng/mL)	Subject	Final [DES] (ng/mL)
HV 1	0.113	COPD 1	0.122
HV 2	0.113	COPD 2	0.184
HV 3	0.125	COPD 3	0.116
HV 4	0.149	COPD 4	0.102
HV 5	0.106	COPD 5	0.155
HV 6	0.263	COPD 6	0.141
HV 7	0.053	COPD 7	0.081
HV 8	0.172	COPD 8	0.16
HV 9	0.127	COPD 9	0.083
HV 10	0.047	COPD 10	0.097
HV 11	0.17	COPD 11	0.154
HV 12	0.307	COPD 12	0.237
HV 13	0.175	COPD 13	0.097
HV 14	0.128	COPD 14	0.147
HV 15	0.134	COPD 15	0.163
HV 16	0.121	COPD 16	0.099
HV 17	0.097	COPD 17	0.238
HV 18	0.152	COPD 18	0.144
HV 19	0.154	COPD 19	0.111
HV 20	0.152	COPD 20	0.38
Min	0.047	Min	0.081
Max	0.307	Max	0.38

CONCLUSION

In conclusion, a technique for quantifying total sputum desmosines using isotope dilution liquid chromatography mass spectrometry with enhanced sensitivity, repeatability, and quantification accuracy was devised. This method could be used as a biomarker for monitoring elastin degradation in diseases like asthma and COPD

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

OA designed and performleed the experiments and data analysis and also wrote the manuscript. SA performed IS synthesis and sample collection, and. MA (Dept. of Pulmonology, King Fahad General Hospital, Medina, Kingdom of Saudi Arabia) sample collection. All authors read and approved the final version.

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