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Development of a sensitive and selective LC–MS/MS method for the determination of urea in human epithelial lining fluid

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ABSTRACT

A sensitive, selective, and quantitative method for the determination of urea has been developed and validated in human epithelial lining fluid (ELF; the supernatant from bronchoalveolar lavage). The method employs a simple derivatization of urea with camphanic chloride to improve the chromatographic retention and separation. The derivatization was performed after drying an aliquot of ELF (20 μL) without prior sample clean-up. Ultra High Performance Liquid Chromatography (UHPLC) on a HSS-T3 stationary phase column with 1.8 μm particle size was used for chromatographic separation coupled to tandem mass spectrometry. The method was validated over the concentration range of 8.78–103.78 μg/mL, however the dynamic range can be further lowered if needed. The results from assay validation show that the method is rugged, precise, accurate, and well-suited to support analysis of urea in ELF samples. In addition, the relatively small sample volume (20 μL) and a run time of 1.5 min facilitate automation and allow for high-throughput analysis. This derivatization method was compared to a commercially available colorimetric assay kit, and it was used in a preclinical non-GLP mouse study where urea measurements were used as marker of bronchoalveolar lavage fluid dilution.

1. Introduction

Bronchoalveolar lavage (BAL) is a common medical technique in which saline is infused through the lower respiratory tract for sampling epithelial lining fluid (ELF) components and to determine the protein composition of the pulmonary airways. This sampling technique has been performed in the medical community for over 20 years [1] and has become a routine practice for diagnosis of infectious and noninfectious lung diseases [2]. One of the disadvantages when using this technique is significant sample dilution [3]. Also, sample mixing in the alveolar space is a challenge and thus the volume retrieved is never equal to the volume instilled. Because of this, it has been difficult to estimate the actual concentration of recovered pharmaceutical entities in the ELF in situ [2]. Therefore, an endogenous biomarker is needed to determine the volume of ELF after BAL procedure. To estimate BAL volume, many researchers have quantified and compared endogenous substrates in serum and BAL, with albumin [4] and urea [3] being most common. The use of albumin has the disadvantage that the permeability of peripheral lung tissue changes with disease state [5].

Baughman et al. [6] developed a technique using methylene blue, diluted in saline, as the lavage fluid. The absorbance of the sample fluid was measured at 680 nm and by comparing the difference in absorption of the fluid injected and retrieved; the dilution of the fluid dye to lung secretions was determined [6]. Bayat et al. [7] developed a method using technetium labeled diethylentriamine pentaacetic acid infused into the blood prior to and during BAL collection. A number of colorimetric approaches for direct urea measurement have been developed with butane-2,3-dione monoxime being the most widely used reagent [8]. Indirect measurement procedures for urea determination include the Dumas method based on thermal decomposition of organic material to form nitrogen (N_2) with a metal oxide in a carbon dioxide atmosphere, and Kjeldahl method utilizing the formation of ammonia during and sulfuric acid digestion catalyzed by HgO at temperatures around 350 °C [9]. These degradative methods are commonly used in the dairy industry [8]. Other methods with detection techniques such chemiluminescence, ultra violet (UV), nuclear magnetic resonance (NMR) and infrared spectrometry (IR) has been reviewed by Francis et al. [8]. The most commonly used method for urea determination in clinical laboratories utilize assay kits such as the QuantiChrom™ urea assay kit, which is designed to measure urea directly in biological matrices without any sample pretreatment.

The use of urea to quantify the volume of ELF recovered after BAL procedure is based on the assumption that urea is freely diffusible through most body compartments including the lungs [3,10]. After determination of the concentration of urea in plasma and ELF, the volume of ELF obtained can be easily estimated based on the assumption that $[Urea]_{ELF} = [Urea]_{plasma}$ and simple dilution

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principles [3]. Research also indicates that urea may have limited use as a biomarker. Marcy et al. suggested that the high concentration gradient of the urea-free lavage caused a rapid diffusion of urea into the residual lavage fluid, which resulted in an inappropriate increase in the urea level in BAL fluid during lavage procedures, thus leading to an overestimation of the ELF volume [11].

Urea is primarily produced in the liver and secreted by the kidneys. Urea is the major end product of protein catabolism in animals and is the primary vehicle for removal of toxic ammonia from the body. Urea determination, in addition to volume biomarker, is very useful for the clinician to assess kidney function, renal ischemia, urinary tract obstruction, and certain extra renal diseases (congestive heart failure, liver disease, and diabetes). The development of a method for analysis of urea in biological matrices by LC–MS/MS is a challenge due to its small molecular weight (60 g/mol), lack of retention on reverse phase HPLC columns and endogenous levels. In the present work, a simple derivatization procedure with camphanic chloride for LC–MS/MS detection was evaluated to address some of these challenges. A small volume of ELF sample (20 μL) is dried down under a steady stream of nitrogen followed by derivatization with camphanic chloride (1 mg/mL) in acetonitrile. After incubation at 37 °C for 30 min, the sample is diluted with water to make a solution of acetonitrile/water (1:1) followed by LC–MS/MS analysis.

The use of multiple reaction monitoring (MRM) in MS detection increases the selectivity and signal to noise ratio, which allows the reduction of sample volume and analysis time. In addition, the MS sensitivity and selectivity was greatly improved using the described derivatization procedure. The relatively small ELF volume (20 μL) allows sample extraction in 96-well format, thereby increasing sample throughput analysis. Moreover, the relatively short run time of 1.5 min, afforded by UPLC separation, has the potential for the analysis of about 300 samples per day.

2. Experimental

2.1. Chemicals and reagents

Urea, [$^{13}C_1$,$^{15}N_2$]-urea, camphanic chloride, ammonium formate, acetonitrile, methanol, isopropanol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Formic acid was purchased from Alfa Aesar (Ward Hill, MA, USA). Human BAL was obtained from Bioreclamation Inc. (East Meadow, NY, USA), and then centrifuged and the supernatant (ELF) removed and used for analysis.

2.2. Equipment

An Eppendorf 5810R centrifuge with a rotor capacity for four 96-well plates (Brinkmann Instrument, Westbury, NY, USA) and a Mettler UMX2 balance (Columbus, OH, USA) were used. Arctic White LLC 96-well round 2 mL polypropylene plates, ArctiSeal silicone mats with PTFE film (Bethlehem, PA, USA), VWR Economy Incubator model 1500E (Radnor, PA, USA) and Barnstead Lab Line Titer Plate Shaker (Radnor, PA, USA) were used for the camphanic chloride derivatization in ELF. Waters 1-ml plastic plates (Milford, MA, USA) along with Arctiseal mats (Bethlehem, PA, USA) were used for sample introduction to the LC–MS/MS. An ACQUITY™ UPLC integrated system from Waters (Milford, MA, USA) consisting of a sample manager combined with a sample organizer, capable of holding ten 96-deep well plates, and a binary solvent manager were used. A triple quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS-Sciex, Concord, Ontario, Canada) was used.

2.3. Optimization of the derivatization parameters

The amount of camphanic chloride, solvent choice, derivatization time and temperature were all optimized during method development. The concentration of camphanic chloride was evaluated from 1 to 10 mg/mL in both acetontrile and toluene. Derivatization time was evaluated from 30 to 120 min under ambient condition, at 37 °C and 65 °C.

2.4. Preparation of calibration standards and quality control (QC) samples

Stock solutions of urea and [$^{13}C_1$,$^{15}N_2$]-urea were individually prepared in 50/50 (v/v) acetonitrile/water solution at a concentration of 20.0 mg/mL. All stock solutions were stored at 4 °C. Separate working solutions were used for the preparation of standards (WS) and quality controls (WQ) and prepared fresh on the day of analysis containing urea in 50/50 (v/v) acetonitrile/water. The concentration of urea in these working solutions was 4000, 1000 and 200 μg/mL. The calibration standards and QC samples were prepared using a pooled sample of ELF with relative low endogenous levels of urea. The endogenous level of urea (3.78 μg/mL) in the ELF pooled samples was determined using the standard addition method described by Robison [12] (see Section 3.4).

The WS solutions were used to make calibration standards in ELF at 103.78, 83.78, 63.78, 53.78, 33.78, 23.78, 13.78, and 8.78 μg/mL of urea using a serial dilution procedure. The WQ was used to make QC samples in ELF at 503.7 (dilution QC, analyzed after 10-fold dilution), 103.78, 83.78, 43.78, 18.78, and 8.78 μg/mL of urea. QC samples were divided into 0.250 mL aliquots and frozen at −20 °C or extracted immediately. In the first validation run, freshly prepared QC samples were analyzed against freshly prepared calibration standards. For each subsequent validation run, frozen replicate aliquots of the QC samples were thawed at ambient temperature and analyzed against a freshly prepared standard curve.

2.5. Sample preparation

Acetontrile (0.5 mL) was added to each well of the 2 mL Arctic White 96-well polypropylene plate. The plate was sealed with the ArctiSeal mat and vortex-mixed in an inverted position for approximately 3 min. Subsequently, the acetontrile was discarded and the plate was left to dry in a chemical hood. This wash step was used to remove any plastic residue from the plates and plate seals. ELF samples (20 μL) were transferred to the washed 96-well plate. A 200 μL aliquot of internal standard solution (5 μg/mL of [$^{13}C_1$,$^{15}N_2$]-urea in acetonitrile was added to all wells with the exception of the blanks, which instead received 200 μL acetonitrile. The plate was capped and vortex-mixed for approximately 1 min. After vortex-mixing, cap mat was removed and the plate was evaporated at 45 °C with nitrogen for approximately 10 min. It is not recommended to leave the plate in the dryer for an excessive amount of time. Following complete evaporation, 200 μL of 1 mg/mL camphanic chloride (prepared fresh) in acetonitrile was added to all wells. The plate was sealed and vortex-mixed for approximately 1 min followed by incubation at 37 °C under vigorous agitation for 30 min. After incubation, 300 μL of deionized water was added to each well. After mixing, 300 μL was removed and transferred to a Waters 1-mL collection plate. The plate was capped and vortex-mixed for 1 min. The volume of water after derivatization can be reduced depending on the sensitivity of the instrument.

2.6. Chromatographic conditions for derivatized urea

The analytical column used was a BEH, HSS-T3, 2.1 mm × 50 mm with 1.8 μm particle size from Waters Co. The column temperature

was held at 65 °C and the sample compartment was at ambient temperature. Mobile phase A consisted of 10 mM ammonium formate, pH 3 (adjusted with formic acid) and mobile phase B was acetonitrile. Mobile phase B was held at 10% until 0.25 min, followed by a linear gradient from 10% B to 60% B for 0.75 min and held at 60% B until 1.30 min to remove late eluting substances from the column, after which the system was returned to the initial condition. The total run time, including sample loading was approximately 1.5 min and the flow rate was maintained at 1.0 mL/min throughout the run. A typical injection volume of 2 μL in a 10 μL loop (partial loop injection mode) was used.

2.7. Mass spectrometric conditions

A Sciex API 4000 with a TurboIonspray interface (TIS) was operated in the positive ionization mode. The instrument was optimized for the camphanic-derivatives of urea and [$^{13}C_1$,$^{15}N_2$]-urea by infusing a 50 ng/mL solution of purified derivatives in acetonitrile/water (50/50 v/v) at 0.5 mL/min through an Agilent pump 1100 series (Palo Alto, CA, USA) directly connected to the mass spectrometer. The MRM transitions of *m/z* 241–109 and *m/z* 244–109 were chosen for the camphanic-derivatives of urea and [$^{13}C_1$,$^{15}N_2$]-urea, respectively. Dwell times of 100 ms were used for the camphanic derivatives of urea and the internal standards camphanic derivative. The optimized mass spectrometric conditions for the camphanic-derivative of urea included the following MS conditions: TIS source temperature, 650 °C; TIS voltage, 5500 V; curtain gas, 30 psi (nitrogen); nebulizer gas (GS1), 60 psi (zero air); turbo gas (GS2), 60 psi (zero air); collision energy, 32 eV; declustering potential 56 eV.

2.8. Quantitative colorimetric urea determination

Urea control stock (QuantiChrom Urea Assay Kit, 50 mg/dL) was used to prepare aqueous solution of urea at concentrations of 12, 6.25, 12.5, 25, and 50 μg/mL. Fifty μL of calibration standards (in ELF, $n = 2$) and QC (validation and Assay Kit QCs, $n = 6$) were aliquoted into the 96-well assay plateQuantiChrom™ Urea Assay Kit (DIUR-500, Bioassay Systems, Hayward CA). Equal volumes of Reagents A and B were combined to create a Working Reagent and mixed well. To all wells, 200 μL of the Working Reagent was added, followed by sealing with adhesive film. The plate was placed on the agitator for 50 min for gentle agitation. Following reaction the plate was read on a Perkin Elmer Envison at 430 nm.

2.9. Data analysis

MS data were acquired and processed (integrated) using the proprietary software application Analyst™ (Version 1.4.2, Applied Biosystems/MDS-Sciex, Canada). Calibration plots of analyte/internal standard peak area ratio versus urea concentrations were constructed and a weighted $1/x^2$ linear regression was used. Concentrations of urea in validation samples were determined from the appropriate calibration line and used to calculate the bias and precision of the method with an in-house LIMS (Study Management System, SMS2000, version 2.3, GlaxoSmithKline).

3. Results and discussion

3.1. Challenges during method development

The objective was to develop a rugged, sensitive, and relatively high-throughput LC–MS/MS method allowing determination of urea in ELF samples with a run time of less than 2 min. Some of the challenges faced during method development include: (1) decrease in internal standard response with increasing urea concentration,

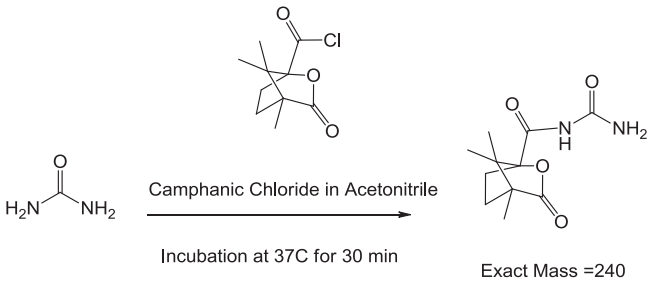

Fig. 1. Structures of urea and derivatized urea following incubation with camphanic chloride.

(2) optimization of derivatization procedures in biological matrices, and (3) dealing with endogenous levels of urea. The approaches used to resolve these challenges are discussed in detail below (see Fig. 1).

3.2. Use of a surrogate matrix and analyte/internal standard response

Due to the high cost and invasive procedure to obtain human BAL, the use of a surrogate matrix, such as de-ionized water, phosphate buffered saline and 0.9% sodium chloride saline, was investigated during method development. A significant difference in internal response was observed (Fig. 2) in different matrices and also with increasing urea concentration (Fig. 3). The internal standard response in ELF was at least 10-fold lower than that for other matrices and it decreased with increasing urea concentration in all matrices for the dynamic range from 5 to 1000 μg/mL. The internal standard response declined significantly as urea concentration exceeded >100 μg/mL. Since the endogenous levels urea in ELF samples is not expected to exceed 100 μg/mL, it was decided to truncate the dynamic range to 5–100 μg/mL.

Saline (0.9% sodium chloride) solution was then evaluated as the surrogate matrix and three full days validation was performed utilizing saline solution for the daily preparation of the standard curve. QC samples prepared in both saline and ELF were analyzed in all three validation runs. The internal standard response for the ELF QC samples was approximately 10-fold lower than that for QC samples prepared in saline. This was originally thought to be related to matrix effects or interferences during the derivatization process (Fig. 2). When comparing the validation data (3 days), both precision and accuracy for both the saline and ELF QC samples, quantified against the freshly prepared calibration standards in saline, were acceptable (≤15% bias and precision). This indicates

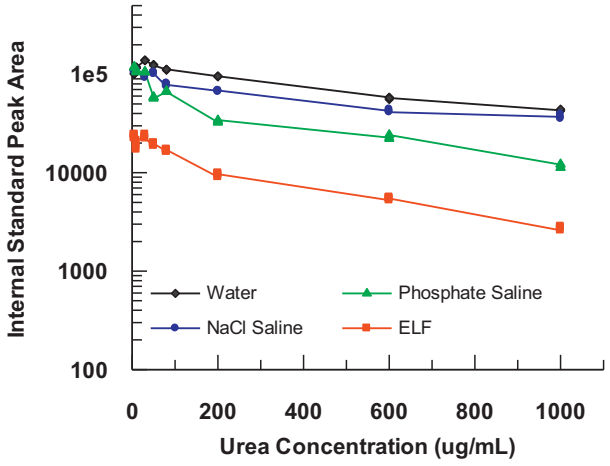

Fig. 2. Internal standard responses in different matrices against urea concentration.

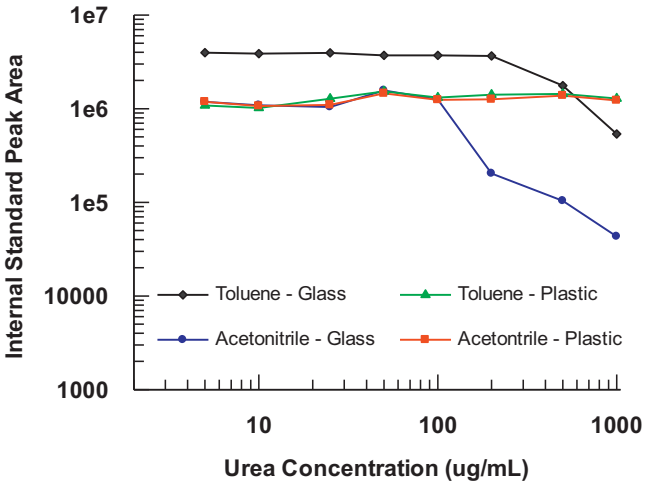

Fig. 3. Internal standard responses in ELF in different solvents and containers with increasing urea concentrations.

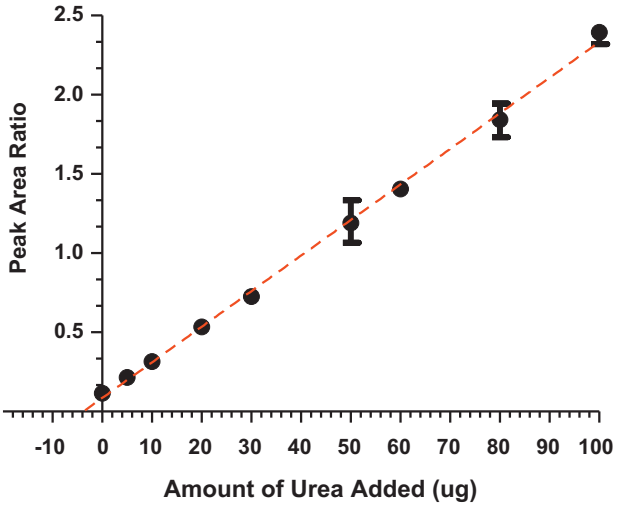

Fig. 4. Determination of the accuracy of the concentration of urea in a pooled ELF sample using the plots of analyte/internal standard area ratios against the additional amount of urea added.

that the decrease in response for the internal standard is most likely to be to the same extent as for the analyte and it seems like the labeled internal standard is correcting for any losses during sample extraction and analysis. However, the method was not considered rugged due to the remarkable difference in internal or analyte standard response for the ELF QC samples as compared to that for saline solution of calibration standards and QC. Other extraction technique such as liquid–liquid and solid phase extraction was evaluated to minimize the discrepancy in response between the internal standard responses in different matrices. Due to these issues, a surrogate matrix was not used and the validation was performed in pooled human ELF.

3.3. Derivatization of urea

A few parameters including reaction time, temperature, solvent composition and concentration of camphanic chloride were optimized for better recovering of urea from ELF samples. Concentrations of camphanic chloride ranging from 1 to 10 mg/mL were evaluated. Even though the recovery was approximately 2-fold higher for the 10 mg/mL solution, the decrease of internal standard response with increasing urea concentration was still prominent for the range 5–1000 μg/mL (data not shown). This indicates that the difference in response is not related to insufficient concentration of camphanic chloride. Therefore, it was decided to select the lower concentration of 1 mg/mL solution for the assay to avoid further sample clean-up and to maintain the instrument as clean as possible. Incubation times ranging from 30 to 120 min and temperatures of 37 °C and 65 °C were also investigated. Even though a 2-fold increase in response was observed for 65 °C as compared to that for 37 °C, the internal standard response decreased with an increase in urea concentration from 5 to 1000 μg/mL. Although a sensitivity gain was noted when increasing both incubation time and temperature, neither modification had any effect on internal standard response decrease with increasing urea concentration. Since sensitivity was not critical for this assay, the incubation and temperature were kept at 30 min and 37 °C, respectively.

Acetonitrile, toluene and pyridine were investigated as possible solvents for the camphanic chloride derivatization procedure. The derivatization of camphanic chloride with urea in pyridine resulted in poor recovery and therefore it was then excluded. Derivatization in either toluene or acetonitrile, in glass or plastic vials, was deemed feasible upon initial investigations. The variability (within replicates) of the internal standard response was greater in saline (data not shown) than in ELF samples and therefore a pooled sample of ELF with relatively low endogenous level of urea was selected for assay validation. The internal standard response in ELF samples after derivatization in toluene and acetonitrile in either glass vials (insert into the 2 mL well plate) or individual polypropylene tubes (1.0 mL) is presented in Fig. 3. The variability of the internal standard response was greater when the derivatization was performed in conical glass vials (inserts). This variability was thought to be associated with poor mixing in the conical glass inserts. In plastic polypropylene tubes, both acetonitrile and toluene gave better and similar reproducible internal standard response over 40 replicates with different concentrations of urea. The positive results were associated with better mixing of samples in these sample tubes. For better mixing and less variability, it was decided to perform the derivatization in the 2 mL 96 well plate with larger surface area. For easier sample handling in the laboratory, acetonitrile was chosen as the solvent to be used for the camphanic chloride derivatization.

3.4. Determination of accuracy using standard addition method

The use of surrogate matrix is well accepted in the bioanalytical field; however it is desired to demonstrate parallelism with the actual matrix. Since there was significant difference in the response for the internal standard and analyte between the investigated surrogate matrices and ELF, there was a concern about the accuracy of the result in incurred samples since the calibration standards and QC samples may not adequately mimic the incurred samples. Therefore, it was decided to use a pooled ELF sample with relatively low endogenous concentration of urea for assay validation.

The endogenous level of urea in the ELF pooled samples was determined using the standard addition method described by Robison [12]. Then known quantities of urea (100, 80, 60, 50, 30, 20, 10, and 5 μg) were added to one mL of the pooled sample to create a "calibration line". These spiked "standards" were extracted and analyzed using the validated method. The analyte/internal standard peak area ratios were plotted against the added concentrations of urea using GraphPad Prism 5 software and linear regression was performed with $1/x^2$ weighting (see Fig. 4). The standard addition lines then were extrapolated to the x-intercept, with the concentration of urea in the pooled incurred sample being equal to the absolute value of the x-intercept. The x-intercept for urea was −3.78 indicating that the extrapolated urea concentration of this pooled

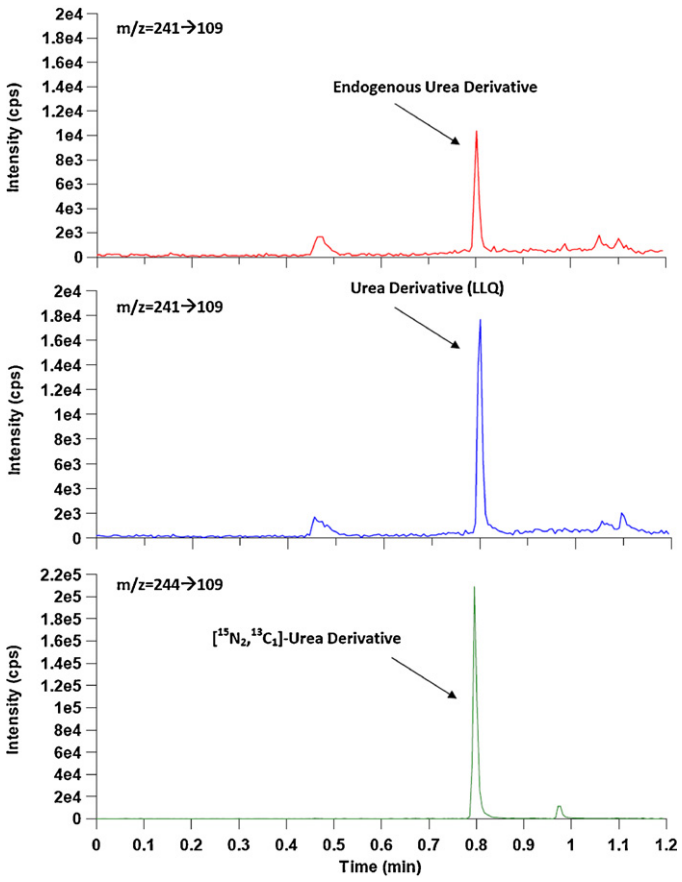

Fig. 5. Representative UHPLC–MS/MS chromatograms of a double blank, sample at the lower limit of quantification, and internal standard.

incurred sample was 3.78 μg/mL. Knowing the endogenous level of urea in the ELF pooled sample, this matrix was used to prepare a calibration curve and QC samples as described in Section 2.4.

3.5. Selectivity and linearity

The characteristic precursor $[M+H]^+$ to product ion transitions, *m/z* 241–109 and 244–109 were consistent with the structures of derivatized urea and the internal standard, respectively. These transitions are used as multiple reaction monitoring transitions to ensure high selectivity. The selectivity of the method was established by the analysis of blank and double blank samples of control human ELF from 6 individual volunteers. The selectivity of the method was also assessed by the inclusion of blank and double blank samples prepared from pooled control human ELF in validation assays. UHPLC–MS/MS chromatograms of the blanks (representing the endogenous level) and validation samples were visually examined and compared for chromatographic integrity and potential interferences. Representative chromatograms of a blank, validation samples at the lower limit of quantification (LLQ), and internal standard are illustrated in Fig. 5. Post-column infusion experiments were performed to investigate potential ion suppression effects from endogenous ELF interferences on the MRM transitions of the camphanic-derivatives. For this purpose, a control human ELF sample was processed as described in Section 2.6. Two microliters of the extracted control ELF was injected into the LC system with a continuous post-column infusion at 20 μL/min of a solution containing 10 ng/mL camphanic-urea derivative. No evidence of ion suppression was observed at the retention times for the investigated camphanic-derivatives of urea and the internal standard.

Table 1
Urea bias, precision and individual validation sample concentrations.

Concentration (ug/mL)	Urea				
	8.78	18.78	43.78	83.78	103.78
Run 1, *n* = 6					
Mean	8.66	20.50	45.63	88.36	108.37
Precision (%CV)	6.3	6.7	5.8	4.0	5.3
Bias %	−1.3	9.2	4.2	5.5	4.4
Run 2, *n* = 6					
Mean	8.95	18.59	46.97	82.77	104.96
Precision (%CV)	13.5	10.1	4.5	14.6	1.7
Bias %	1.9	−1.0	7.3	−1.2	1.1
Run 3, *n* = 6					
Mean	9.06	17.68	47.10	79.29	110.45
Precision (%CV)	5.7	4.6	5.2	5.2	3.6
Bias %	3.2	−5.9	7.6	−5.4	6.4
Overall Totals, *n* = 18					
Mean	8.89	18.92	46.57	83.47	107.93
Precision (%CV)	8.9	9.5	5.1	9.8	4.2
Bias (%)	1.3	0.8	6.4	−0.4	4.0
Between-run precision (%)	Negligible	7.0	Negligible	4.0	2.0

Linear responses in the analyte/internal standard peak area ratio were observed over the range of 5–100 μg/mL (8.78–103.78 μg/mL, after endogenous correction). The correlation coefficients obtained using $1/x^2$ weighted linear regression were better than 0.9942.

3.6. Bias and precision

The maximum bias observed for urea was 9.2% (see Table 1). The maximum within- and between-run precision values observed were 14.6% and 7.0%, respectively. As defined by the lower and upper QC concentrations possessing acceptable accuracy and precision, the validated range of this method based on 20 μL of human ELF is 5–100 μg/mL (8.78–103.78 μg/mL after endogenous correction).

3.7. Stability of urea in ELF

The stability of urea in spiked human ELF samples stored at ambient condition was assessed at 18.78 and 83.78 ng/mL (in replicates of 6) by comparing the mean concentrations of samples extracted after storage for 24 h against the nominal concentrations. The bias and precision was less than 15%, and indicates that urea is stable in human ELF stored at ambient temperature for at least 24 h. The long term stability of urea in spiked human ELF samples stored at −20 °C was assessed at 18.78, 43.78 and 83.78 n/mL (in replicates of 6) by comparing the mean concentrations of samples extracted after storage for 24 days against the nominal concentrations. The bias and precision was less than 15%, and indicates that urea is stable in human ELF stored at −20 °C for at least 24 days.

3.8. Stability of urea in ELF during freeze thaw cycles

The stability of urea in spiked human ELF samples after 4 freeze-thaw cycles from −20 °C to ambient temperature was assessed at 18.78 and 83.78 ng/mL (in replicates of 6) by comparing the mean concentrations against the nominal concentrations. The bias and precision was less than 15% and indicates that urea is stable in human ELF after at least 4 freeze-thaw cycles from −20 °C to ambient temperature.

3.9. Matrix dilution and analysis of urea in plasma samples

The ability to dilute samples containing urea at concentrations above the HLQ was demonstrated by performing 6 replicate 10-fold

dilutions of human urea samples diluted with pooled ELF sample spiked at 500 μg/mL. Concentrations of urea in these matrix dilution samples were determined and corrected for the dilution factor. The bias and within-run precision values were less than 15% indicating that a 10-fold dilution of human ELF samples containing urea above the HLQ is valid.

In order to determine the volume of ELF after BAL procedure, it is required the measurement of urea in plasma samples as well. In contrast to ELF plasma samples are not diluted and analysis of urea in plasma would require an analytical method with higher dynamic range. Since the endogenous level of urea is relatively high it is impractical to use plasma as control matrix. Therefore, it was decided to dilute plasma samples with the control pooled ELF sample (10-fold) and analyze the diluted sample using the validated ELF method. This approach was used to measure urea concentrations in BAL and plasma samples, for estimation of BAL dilution volume, in a preclinical non-GLP study as part of the investigation of the penetration of drug candidate into the lungs. In this particular study, the drug candidate degrades in neutral pH and for stabilization an acidic buffer was added to the ELF and plasma samples. The presence of buffer interfered with the performance of the commercially available colorimetric assay kit for urea measurement. The presence of the buffer had no impact on the performance of the LC–MS/MS assay and made it possible to generate urea quantification in both plasma and ELF samples.

3.10. Stability in processed samples

The stability of urea derivative in processed samples derived from 20 μL of human ELF was assessed by re-injecting a validation run after storage at ambient temperature for 120 h. The accuracy, precision and sensitivity of these samples were found to be acceptable on re-injection, indicating that the processed samples were stable when stored at ambient temperature for at least 120 h.

3.11. Comparison to immuno assay data

Calibration standards ($n = 2$) and QC (validation and Assay Kit QC, $n = 6$) samples were analyzed using the QuantiChrom Urea Assay Kit and the response was measured on an Envision spectrophotometer plate reader at 430 nm. Calibration was performed using instrument response with a five parameter – weighted $1/x^2$ regression. Both sets of QC (validation and Assay Kit QC) were quantitated against the standard curve prepared in human ELF from 8.78 to 103.78 μg/mL. All results from QC samples were within 20% of their nominal concentration (data not shown) indicating correlation of the two detection techniques.

4. Conclusion

A semi-automated sample preparation method in 96-well plate format for determination of urea in human ELF was developed and validated over the concentration range of 8.78–103.78 μg/mL. The method employs a simple and rapid (30 min) derivatization of procedure of urea with camphanic chloride to improve the chromatographic retention and peak resolution. The LOQ of 8.78 μg/mL of the assay can be easily lowered if desired by using greater sample volume, decreasing the reconstitution volume and increasing the LC injection volume. The results reported herein suggest this method is rugged, precise, accurate, and well-suited to support pharmacokinetic studies. The relatively small ELF volume (20 μL) allows sample extraction in 96-well format, thereby increasing throughput analysis. Moreover, the relatively short run time of 1.5 min, afforded by UPLC separation, allows for the analysis of up to 300 samples per day. The method can also be adapted for determination of urea in plasma samples after dilution with endogenous ELF, in particular when sample additives interfere with the kit assay.

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