Determination of Derivatized Urea in Exhaled Breath Condensate by LC–MS

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Abstract

Elevation in one or more compounds in exhaled breath condensate (EBC) has been reported to be related to one or another lung disease. The increased concentration might be caused by increased chemicals in the airway surface liquid. However, it might also be due to an increased delivery of liquid samples into the airstream. Being evenly distributed throughout the body, urea is a likely candidate for a marker of such dilution. A liquid chromatography–tandem mass spectrometry method was developed for determination of EBC urea. Urea in EBC samples was converted to 2-hydroxypyrimidine (2-HPM) through a one step reaction, along with $^{15}N_2$-urea added as an internal standard. The product ion *m/z* 97/42 was selected for quantification with *m/z* 99/43 from $^{15}N_2$-2-HPM as a standard. Concentrations of urea in EBC from five lung cancer patients were found to be 35.1, 2.2, 103.5, 19.3, and 3.6 µM, respectively. The highest values were in patients dying of respiratory distress, whose lungs were filled with fluid. Lower values were seen in patients whose conditions were improving. Lately, one of the low EBC urea values was observed in a patient whose airway status did not contribute to his poor clinical condition.

Introduction

Water condensed from the exhaled breath contains measurable quantities of protein, DNA, and lipids. This is surprising because the breath is generally considered to be a simple gas phase, consisting of atmospheric gasses plus products of metabolism generated by the individual and his/her gut microorganisms (1,2). Instead, the exhaled breath condensate (EBC) contains a small sample of the fluid lining the airways, albeit at a dilution of approximately 1:10,000 (3,4). This observation has been seized by pulmonary researchers eager for a means to sample regions of the lung not easily accessed without invasive procedures such as bronchoscopy.

Many report a correlation of an elevation of some compound in EBC to lung disease, but an impediment to understanding the meaning of an elevated concentration of a chemical marker for a disease is that it might have two causes. On the one hand, the observed increase in EBC concentration might be due to an increased concentration of the chemical in the airway surface liquid (ASL); alternatively, it might be due to an increased delivery of the liquid sample into the airstream. A marker of dilution would allow us to discriminate between these two possibilities, and urea is a likely candidate. Being uncharged and having a molecular weight of 60.06, urea is evenly distributed throughout the body, and it is a molecule that has a long history of considerable clinical utility. For example, it is regularly measured as blood urea nitrogen (BUN). Because urea is so widely distributed, the concentration in the tissue fluids is the same as in the blood. Therefore, with the EBC urea concentration being 1/8,600 that of plasma urea in healthy individuals (3), only about 0.12 part per thousand of EBC is derived from their ASL.

Chromatographic methods have been developed for determination of urea; most of them utilized gas chromatography–mass spectrometry (GC–MS) for the assay after urea derivatization. Bjorkhem et al. converted urea into 5,5-diallyl barbituric acid with diallyl malonic acid diethyl ester (5). Patterson and coworkers changed urea into *tert*-butyldimethylsilyl derivatives (6). In the work by Beylot and colleagues (7), dimethylaminomethylene derivative of urea was prepared for GC–MS

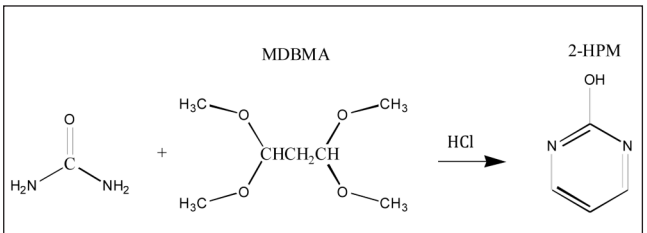

Figure 1. Urea reacts with malonaldehyde bis(dimethyl acetal) (MDBMA) to yield 2-hydroxypyrimidine (2-HPM) in the presence of HCl.

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determination. In an earlier work by Nissim et al. (8), urea was mixed with trifluoroacetic anhydride to yield a derivative. There was also an enzymatic method in which urease was used to change urea into CO_2 and NH_3 prior to gas chromatography–isotope ratio mass spectrometry (GC–IRMS) analyses (9,10). More frequently, urea was converted to derivatives of 2-hydroxypyrimidine by multi-step derivatizations (11–16). Usually, complicated preparation and derivatization procedures were needed for these methods; in addition, care must be taken to avoid any water if silylation was involved. Tanigawa et al. developed a high-performance liquid chromatographic (HPLC) atmospheric pressure chemical ionization mass spectrometry (APCI-MS) assay for a direct determination of urea without derivatization (17). Molecular ion was used in their work for quantification. Apparently, tandem MS (MS–MS) is more specific in the analysis; unfortunately only the $[M+1-H_2O]^+$ and $[M+1-NH_3]^+$ can be found in the product ions from a urea mass spectrum. These dissociation channels lack specificity for identification. In the present work, urea was converted to 2-hydroxypyrimidine (2-HPM) along with $^{15}N_2$-urea added as an internal standard, and an HPLC–MS–MS method was developed for the determination of the derivative of urea in EBC.

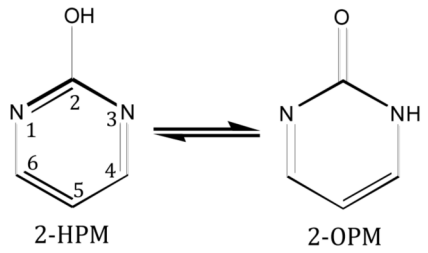

Figure 2. Tautomerism of 2-HPM and 2-OPM.

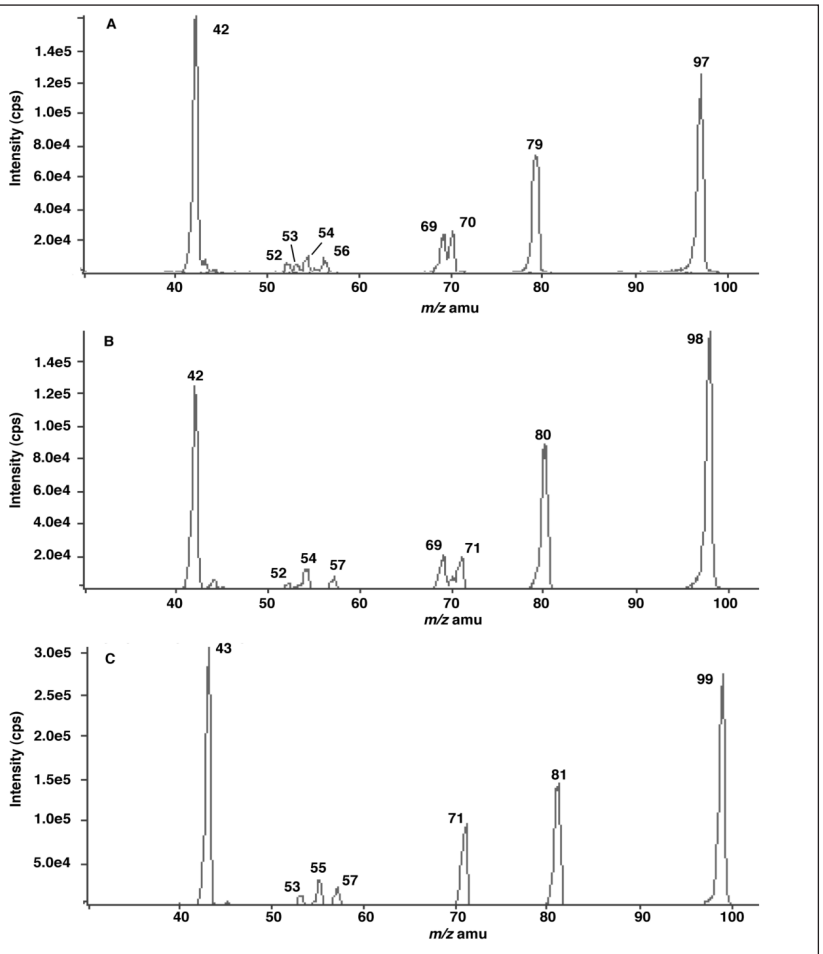

Figure 3. Mass spectra for (A) 2-HPM, (B) ^{13}C-2-HPM, and (C) ^{15}N2-2-HPM.

Experimental

Chemicals

2-Hydroxypyrimidine hydrochloride was provided by Acros Organics (Geel, Belgium). ^{13}C- and $^{15}N_2$-urea were purchased from Cambridge Isotope Laboratories (Andover, MA). Urea, malonaldehyde bis(dimethyl acetal) (MDBMA), and HPLC-grade methanol were obtained from Sigma Aldrich (St. Louis, MO). All other reagents were of analytical-grade, and Milli-Q water was used throughout (Millipore, Billerica, MA).

Apparatus

Two Perkin ElmerSeries 200 Micro LC pumps supplied mobile phase to a Perkin Elmer Series 200 autosampler installed with a 10 µL loop. Mobile phase Solution A was composed of 10% methanol (v/v, aqueous) containing 0.2% acetic acid (HAc) (v/v). Mobile phase Solution B was composed of 90% methanol (v/v, aqueous) containing 0.2% HAc (v/v). The solutions were degassed with helium. Separation was achieved on a Phenomenex (Torrance, CA) Synergi Polar-RP 80A column (4 µm, 150 × 2.0 mm). The gradient was initially programmed at 100% Solution A for 5 min, then it was linearly ramped to 40% Solution A over 8 min. The flow was held at 40% Solution A for 5 min prior to a linear return to 100% Solution A over 7 min. It was kept at 100% Solution A for 1 min before the run was stopped. The flow rate was 180 µL/min. The gradient was used to resolve 2-HPM from the background that can be observed by UV. The retention time for 2-HPM was less than 4 min. A prolonged time was used in order to clean the column. A TurboIon Spray ion source interfaced the LC system, and a Perkin Elmer SCIEX API 365 triple quadruple mass spectrometer (Foster City, CA) operated in the positive ion mode. The ion spray voltage was 4500 volts, and the temperature was 200ºC. The nebulizer gas was set to 5 (instrument parameter), and collision-induced dissociation spectra were produced at –38 volts collision energy. The *m/z* 97/42 product ion was selected for tandem MS determination, and the *m/z* 99/43 ion from $^{15}N_2$-2-HPM was employed as an internal standard.

Synthesis of 2-hydroxypyrimidine

The method was first reported by Hamburg et al. (18). Urea, or its isotope compound, ^{13}C- or $^{15}N_2$-urea, was dissolved in methanol to make a 0.1 M solution. Fifty µL of the solution was evaporated to dryness under nitrogen flow at 40ºC. Fifty µL of 0.3 M MDBMA was added prior to injection of 80 µL concentrated hydrochloric acid. The mixture was capped and left at room temperature for 1 h. Then, it was evaporated to dryness under nitrogen at 40°C, and was re-dissolved in 50% methanol (v/v) containing 0.2% HAc (v/v). The reaction is illustrated in Figure 1. This quantitative reaction readily proceeded. The mass spectrum of the formed 2-HPM was virtually identical with that of commercially obtained 2-HPM. The synthesized 2-HPMs were diluted with the same solution for MS analyses.

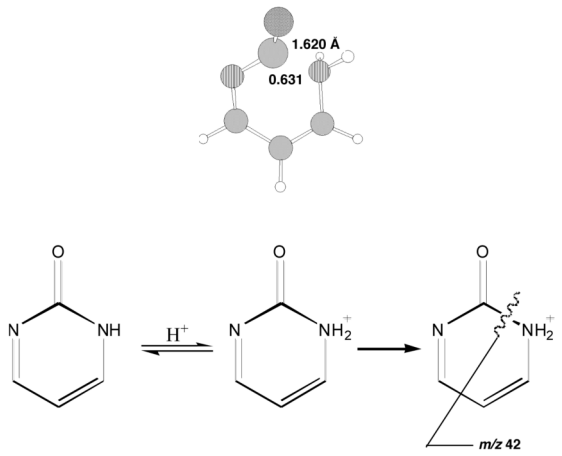

Figure 4. (A) MP2/6-311+G(d,p) predicted bond length and bond order of C2-N3 bond when the N-3 atom of 2-OPM (shown in Figure 2) is protonated. (B) Pathway for formation of the *m/z* 97/42 product ion.

Preparation of EBC samples

EBC samples were collected from five patients. For patients 1–4, the condensate was obtained from the in-line heat and moisture exchange filter (Hygrobac S, Tyco Health Group, Nellcor Puritan Bennett Division, Pleasanton, CA). Fluid from patient 5 was collected from the exhalation limb of the ventilator circuit. In both cases, the samples condensed at ambient temperature, and the fluids were obtained when the respiratory therapists changed out the tubing or filter after 24 h of use, which is the standard maintenance schedule. The samples were stored at –80°C until analyzed. Samples were thawed at 37°C, a fraction was pipetted out, and the internal standard $^{15}N_2$-urea was injected. The sample was vortexed and dried up at 40°C under nitrogen flow. Then, MDBMA and HCl were added, and it was treated in the same way as described previously.

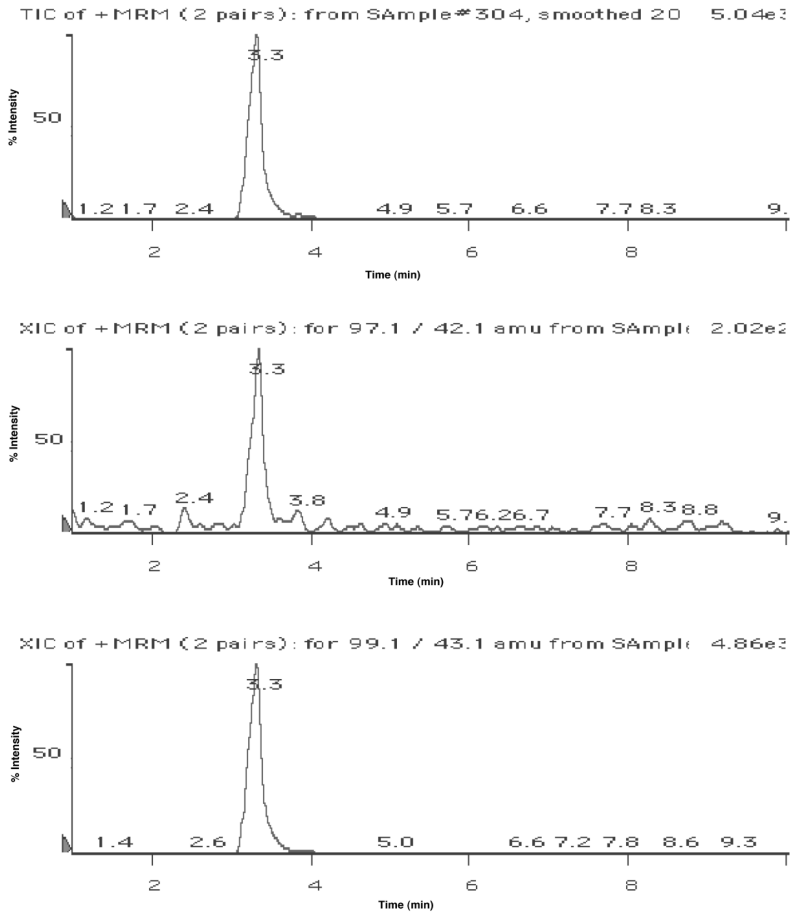

Figure 5. LC–MS–MS chromatograms for determination of 2-HPM derivatized from urea in exhaled breath condensate (EBC): (A) total ion, (B) *m/z* 97/42 for 2-HPM from sample urea, and (C) *m/z* 99/43 from the internal standard $^{15}N_2$-urea.

Recovery and stability

To EBC solutions, the internal standard $^{15}N_2$-urea was injected prior to addition of authentic urea, and the mixtures were derivatized and determined as done previously. The concentration of derivatized 2-HPM from added urea was adjusted at levels of 5 µM or 20 µM, respectively. For stability analyses, samples were thawed, and a fraction was run with LC; the remaining was frozen at –80ºC for two months, then the samples were again thawed and run before results were compared. All LC–MS–MS determinations were duplicated.

Calculation method

The geometries of the studied species were optimized applying the second order Møller-Plesset perturbation theory (MP2) (19). No symmetry constraints were imposed during the optimization process. The location of true minima was confirmed by calculation of frequencies. The 6-311+G(d,p) basis set was applied in most cases. A further expansion of the basis set has less of an impact on the quality of the predicted molecular parameters. All calculations were carried out using the Gaussian 03 program (20). The NBO (localized Natural Bonding Orbitals) analyses were performed with the NBO 3.1 standard program from the Gaussian 03 package (21).

Table I. Urea in Exhaled Breath Condensate*

Sample	Concentration (μM)
1	35.1 ± 0.4
2	2.2 ± 0.0
3	103.5 ± 2.1
4	19.3 ± 0.1
5	3.6 ± 0.2

* Data shown are the result of the average ± SD from duplicated determinations.

Results and Discussion

Fragmentation

The tautomerism between 2-hydroxypyrimidine (2-HPM) and 2-oxypyrimidone (2-OPM) has been well-known (Figure 2), which was also observed under the conditions in this study. In the mass spectrum shown in Figure 3A, the *m/z* 79 peak was apparently from loss of water from the enol form of the protonated molecular ion. Although neutral loss of water from the enol form of 2-HPM is a major gas-phase dissociation channel, it lacks specificity for identification of 2-HMP. From the same figure, it can also be deduced that some of the product ions were from the keto form of the tautomers. One of them was the *m/z* 97/42 ion, which was selected for quatification of urea in EBC. Comparing the mass spectra of the ^{13}C and $^{15}N_2$ labeled 2-HPMs in Figure 3 and taking into account the computational result (Figure 4), it was concluded that once the keto form of the tautomers was protonated as in Figure 4, the C2-N3 (see Figure 2 for assignment of positions) bond was cleaved, followed by the breakage of the C5-C6 bond to produce the *m/z* 42 ion. Figure 5 shows the chromatograms for an LC–MS–MS run; no interference peak was found to co-elute out.

Calibration, recovery, and stability

Peak area was used for quantification; 100 μM $^{15}N_2$-urea was added as the internal standard, and the peak area for the *m/z* 99/43 dissociation channel served as the reference. A five-point calibration curve was prepared using authentic urea at concentrations ranging from 5 μM to 1000 μM. Linear regression analysis of the result yielded an equation as the following:

$$y = 0.011x + 0.0206$$

where y stands for the ratio of the peak area for 2-HPM (*m/z* 97/42) relative to that of 100 μM $^{15}N_2$-2-HPM (*m/z* 99/43); x represents the concentration of 2-HPM (μM); $R^2 = 0.994$. The detection limit was found to be 0.41 μM when signal-to-noise ratio (S/N) was 3. It could actually be further improved as samples could be concentrated with less volume of solvent when they were re-dissolved following completion of derivatization and evaporation of the original solvent. Satisfatory recoveries of 97 ± 4% and 98 ± 3% (n = 3) were obtained at fortified levels of 5 μM and 20 μM 2-HPM, respectively, in EBC. Sample stability was satisfactory and was evaluated by determining the 2-HPM level before freezing, 13.6 ± 18.0 μM and after freezing for two months, 13.4 ± 18.0 μM; the average ratio of levels before and after freezing was found to be 102 ± 4% (n = 3).

Analysis of urea in EBC

The result for determination of urea concentrations in EBC samples of five lung cancer patients by the present method is summarized in Table I. The individual determinations illustrate the potential importance of EBC urea as a clinical indication of health and disease. The first patient's sample contained 35.1 μM urea and was obtained shortly before death due to cancer. This individual's blood urea nitrogen was measured to be 24 mM or 4–5 times of normal value, and the EBC urea concentration was 68-fold greater than the normal value (0.52 ± 0.12 μM). Thus, knowing the actual value of the blood and presumably the tissue-urea, it can be deduced that the expired breath contained about 15 times the normal quantity of ASL. Clinically, the patient's breath sounds were described as exhibiting bilateral crackles, and these noises are generally interpreted as meaning that an inhalation snaps open innumerable small airways stuck closed by the increased surface fluid. Hence, these sounds could represent the physical basis by which the ASL was aerosolized and expelled with the exhaled air. The third patient had an EBC urea of 103.5 μM, exhibited extensive bilateral crackles, and died shortly after the sample was obtained. In this case, the greatly increased quantity of EBC urea was consistent with the breath sounds and the inferred large quantity of ASL.

Patients who were improving and who were shortly removed from artificial ventilation had smaller values for their EBC urea (patient 2, 2.2 μM; patient 4, 19.3 μM). The one pre-morbid sample that had little urea (patient 5, 3.6 μM) was from a patient who was unlikely to have increased ASL. This individual had normal breath sounds and pulmonary hypertension, a disease where the pulmonary arteries' blood flow was restricted, leading to a very high pressure at the heart but a very low pressure in the lungs distal to the restriction.

Conclusion

A method was developed for determination of urea in EBC. $^{15}N_2$-urea was added as the internal standard. Urea in the sample was converted to 2-HPM through a simple one step reaction. The quantitatively formed 2-HPM was analyzed by HPLC–MS–MS. The method was applied for determination of urea in EBC from five cancer patients. The results illustrated the importance of knowing the quantity of ASL. The highest values were in patients dying of respiratory distress, whose lungs were filled with fluid. Lower values were seen in patients whose condition was improving. And lastly, one of the low values was observed in a patient whose airway status did not contribute to his poor clinical condition.

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