Vol. 8, No. 3, 2017
ISSN 2233-4203/ e-ISSN 2093-8950
INVITED ARTICLE www.msletters.org | Mass Spectrometry Letters

Investigation on the Stability of Uric Acid and its Isotope (1,3-$^{15}N_2$) in Ammonium Hydroxide for the Absolute Quantification of Uric Acid in Human Serum

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Received August 04, 2017; Revised September 11, 2017; Accepted September 12, 2017
First published on the web September 30, 2017; DOI: 10.5478/MSL.2017.8.3.59

Abstract : In clinical diagnosis, it's well known that the abnormal level of uric acid (UA) in human body is implicated in diverse human diseases, for instance, chronic heart failure, gouty arthritis, diabetes, and so on. As a primary method, an isotope dilution mass spectrometry (IDMS) has been used to obtain the accurate quantity of UA in blood or serum and also develop the certificated reference material (CRM) so as to provide a SI-traceability to clinical laboratories. Due to the low solubility of UA in water, an ammonium hydroxide (NH_4OH) has been considered as a promising solvent to increase the solubility of UA that enables the preparation of both UA and its isotope standard solution for next IDMS-based absolute quantification. But, because of using this NH_4OH solvent, it gives rise to the unwanted degradation of UA. In this study, we sought to optimize condition for the stability of UA in NH_4OH solution by varying the mole ratios of UA to NH_4OH, followed by ID-LC-MRM analysis. In addition, we also inspected minutely the effect of the storage temperatures. Additionally, we also performed the quantitative analysis of UA in the KRISS serum certificated reference material (CRM, 111-01-02A) with diverse mixing ratios of UA to NH_4OH and then compared those values to its certification value. Based on our experiments, adjusting the mole ratio of 1/2 (UA/NH_4OH) with the storage temperature of -20°C is an effective way to secure both the solubility and stability of UA in NH_4OH solution for next IDMS-based quantification of UA in serum.

Keywords : uric acid, isotope dilution mass spectrometry, certificated reference material (CRM)

Introduction

Measuring the level of uric acid (UA) in human blood (or serum) is very important, since its either high or low concentration induces to increase the chance of diverse human diseases, for instance, chronic heart failure, gouty arthritis, diabetes, chronic renal failure, and kidney stones.[1,2] It's well known that uric acid is formed by the oxidation of purine nucleotides and eliminated through flowing out of urine from the human body.[3] Viewed in clinical diagnosis, the normal ranges of UA in blood represent to be 3.4 ~ 7.2 mg/dL and 2.4 ~ 6.1 mg/dL in blood for men and women, respectively.[4] Regarding to the diagnostic reliability, the development of analytical method for the absolute quantification of uric acid is inevitably concomitant to accurately diagnose a variety of diseases caused by being out of the normal range of UA in blood, called as hyperuricemia for high levels of uric acid and hypouricemia for low ones as well.[5,6]

Differed from conventional methods [*e.g.*, the enzyme-linked immunosorbent assay (ELISA),[7] liquid chromatography-mass spectrometry (LC-MS),[8,9] and LC-UV/VIS[10,11]] in clinical laboratory, isotope-dilution mass spectrometry (IDMS)[12] has been considered as a primary method for the absolute determination of targeted analyte (or biomarker) in biological samples, such as blood, serum, urine, and so on. Because both spiking and equilibrating a biological sample with an isotope-labeled counterpart of targeted analyte are completed at the first stage, prior to the following sample treatments and tandem mass spectrometry (MS/MS)-based MRM analysis, the

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significant bias caused by the variation of sample recovery and degradation during complicated purification steps can be reduced, thereby leading to precise and accurate quantification of analyte in a sample. In ID-MS, it's straightforward that the absolute quantity of analyte in a sample can be determined by means of applying both the value of a response factor (RF) — the observed area ratio of SI-traceable pure standard of analyte to its isotope-labeled counterpart obtained by LC-MRM (or SIM) analysis — and the measured area ratio of analyte in the sample to its counterpart with the known amount of isotope into the equation as below.

$$C_{\text{sample}} = \frac{M_{\text{is-sol, spiked}} \cdot AR_{\text{sample}} \cdot M_{\text{s-sol, std.mix}} \cdot \text{C}_{\text{s-sol}}}{M_{\text{sample}} \cdot AR_{\text{std, mix}} \cdot M_{\text{is-sol, std.mix}}}$$

Where C_{sample} is the concentration of analytes in the sample, while $C_{s\text{-sol}}$, M_{sample}, $M_{is\text{-sol, spiked}}$, $M_{is\text{-sol, std. mix}}$, and $M_{s\text{-sol, std. mix}}$ signify the concentration of the analyte standard solution, the mass of the sample, the mass of the isotope standard solution added to the sample, the mass of the isotope standard solution added to the isotope ratio standard solution, and the mass of the standard solution added to the isotope ratio standard solution, respectively. Lastly, AR_{sample} represents the area ratio of analyte in the sample to isotope, and $AR_{std. mix}$, the area ratio of standard of analyte to isotope in the standard solution, means precisely the same as RF.

From this concept in IDMS, when preparing the standard solutions (i.e., UA and isotope-labeled UA) for the measurement of the RF value, it's quite important to optimize the confirmative composition of a buffer solution whereby both the standard of analyte and its isotope should be maintain their intact forms during a IDMS-based inter-/intra-day experiments, since the occurrence of a degradation of either the standard of analyte or its counterpart might be directly influenced on the shift of RF value, thereby leading to an inaccurate determination of analyte in a sample. In previous studies, an ammonium hydroxide has been universally considered as one of promising buffer solution to be feasible to prepare the standard solution of UA and also compatible with MS or MS/MS analysis, due to the reason of which the UA has very low solubility in water (i.e, < 0.6 mg/dL at -20°C) or organic solvents.[13-15] While the use of an ammonium hydroxide (NH_4OH) offers the high solubility for UA, it may, however, give rise to the unwanted result wherein NH_4OH is possible to act as the trigger for the decomposition of UA that might be degraded to both urea and allantoin at a certain mole ratio between UA and NH_4OH.

In this study, we investigated on the stability of UA by varying the mole ratios of UA to NH_4OH at five different mole ratios of 1/1, 1/1.5, 1/2, 1/3, and 1/5.6, and followed by ID-LC-MRM analysis. In addition, we also inspected minutely the effect of the storage temperatures [-20°C and room temperature (RT)] of both UA standard solution and its labeled counterpart on the RF value at each mole ratio of UA to NH_4OH. Finally, we performed the quantitative analysis of UA containing the KRISS serum certificated reference material (CRM, 111-01-02A) under diverse mixing ratios of UA and NH_4OH and then compared those values to its certification value.

Materials and Methods

Materials and chemicals

Uric acid (UA, 99.8% ±0.2%) and uric acid-$^{15}N_2$ (ISO-UA, > 98%) were purchased from NIST (Gaithersburg, MD, USA,) and Isotope Cambridge (Andover, MA, USA), respectively. Human serum was used with the certified reference materials [CRM (111-01-02A)] from Korea Research Institute of Standards and Science (KRISS). HPLC-grade acetonitrile (ACN), methanol, and water were obtained from Burdick & Jackson (Ulsan, Korea). Ammonium acetate and ammonium hydroxide (NH_4OH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For purification of a prepared serum sample, 0.2 μm PVDF syringe filter was purchased from PALL Corporation (Port Washington, NY, USA).

Standard solutions of uric acid and its isotope

In order to prepare both UA and ISO-UA standard solutions, UA and its isotope were gravimetrically weighed, and dissolved with 1 mmol/L NH_4OH solution to be 1/1, 1/1.5, 1/2, 1/3 and 1/5.6 at the mole ratios of UA to NH_4OH. The resulting UA and ISO-UA standard solutions were mixed them together to be of the same ratio of UA to ISO-UA, to prepare the internal standard solution.

Sample preparation of human serum for ID-LC-MRM

KRISS serum CRM (111-01-02A) was equilibrated at RT for about 2 hours prior to sample preparation. 500 μL of serum was taken into a glass amber vial, and mixed with ISO-UA standard solution. The resulting solution was gently mixed for 2 hours, transferred to new vial. In order to remove proteins in human serum, the precipitation step was performed by means of adding 500 μL of ACN into the serum sample, and then the solution was centrifuged at $1{,}400 \times g$ for 10 min. The supernatant of solution was taken to new 1.8 mL tube, evaporated with a speed-vac. Finally, the dried pellets were reconstituted with 1 mmol/L NH_4OH solution following purification using a 0.22 μm membrane filter, and stored at -20°C before a ID-LC-MRM analysis.

UPLC-ESI-MRM

UPLC-ESI-MS/MS was performed by ACQUITY series UPLC system coupled to a Xevo TQ-S MS system from

Waters (Massachusetts, USA) via electrospray ionization (ESI). Chromatographic separation was carried out using Waters Cortecs® UPLC HSS C18 column (100 mm × 2.1 mm, 1.8 μm). For the UPLC runs, the mobile phase was composed of 10/90 (MeOH/water) with 20 mM ammonium acetate. Along with the flow rate of 0.2 mL/min, an isocratic elution was performed for 7 min. The eluted UA and ISO-UA from the analytical column were directly introduced into a triple-quadrupole mass spectrometer equipped with a turbospray source in multiple reaction monitoring (MRM) mode. For MRM analysis, the parameters of mass spectrometer were set as 2.7 kV for capillary voltage, 400°C for desolvation temperature, and 20 eV for collision energy. The transition *m/z* values for MRM analysis were set as 168.94 → 141.02 for UA and 170.94 → 143.02 for ISO-UA, respectively.

Method validation

The litmit of detection (LOD) and the limit of quantification (LOQ) that were MRM-analyzed in triplicate using a SI-traceable UA standard (purity of > 99.8%) were estimated to be about 0.14 mg/g and 0.46 mg/g, respectively. Along with the two different storage temperature (-20°C and RT), the inter-day precision of the assays were performed on 0-, 1-, 3-, and 7-day in triplicate at given mole ratio of UA to NH_4OH and assessed to be 1.16% (RSD). The accuracy in measuring the absolute quantity of UA in serum (KRISS serum CRM, 111-01-02A) was examined by means of comparing with the key comparison reference value (KCRV) from the International Comparison for "Determination of Urea and Uric Acid in Human Serum (CCQM-K109)". The measured quantities of UA in serum that were MRM-analyzed at each mole ratio of UA/ NH_4OH were statistically estimated via a gravimetry-based exact matching IDMS with the expanded uncertainty that is $U = ku_c$ (*i.e.*, $k = 2$). Where u_c is a coverage factor.

Results and Discussion

For the purpose of IDMS-based absolute quantification of uric acid in serum, it's a routine process as follows: i) both UA and ISO-UA standard solutions whose their concentrations are known accurately in NH_4OH solution are prepared and then mixed them together to be of the same ratio of UA to ISO-UA, so as to prepare the internal standard solution, hereafter referred to as "IS solution", ii) For the following the quantitative measurement of UA in serum, the known amount of the ISO-UA standard solution is spiked into the serum sample, equilibrated for at least 12 hours, and followed by the sample purification step prior to LC-ESI-MRM analysis, and lastly, iii) the comparison between the RF value−obtained by MRM analysis of IS solution− and the measured area ratio of UA in serum to ISO-UA allows to figure out the exact quantity of UA in serum as shown in Figure 1. In pursuance of IDMS-based quantification of UA in serum, it's intensively require of modifying the mole ratio of UA (or ISO-UA) to NH_4OH, since the degradation rates of UA might be boosted up when used the low moles of UA against the given moles of NH_4OH. To do this validation for the stability of UA in NH_4OH solution, we performed the LC-ESI-MRM analyses of the five cases of IS solutions, having the different mole ratios of 1/1, 1/1.5, 1/2, 1/3, and 1/5.6 (UA/NH_4OH) in triplicate, along with inter-day analysis at four different time points of 0, 1, 3, and 7 days. In addition, the condition for the storage of IS solution for next IDMS experiment was also tested for the degradation of UA and ISO-UA at two different temperatures (-20°C and RT).

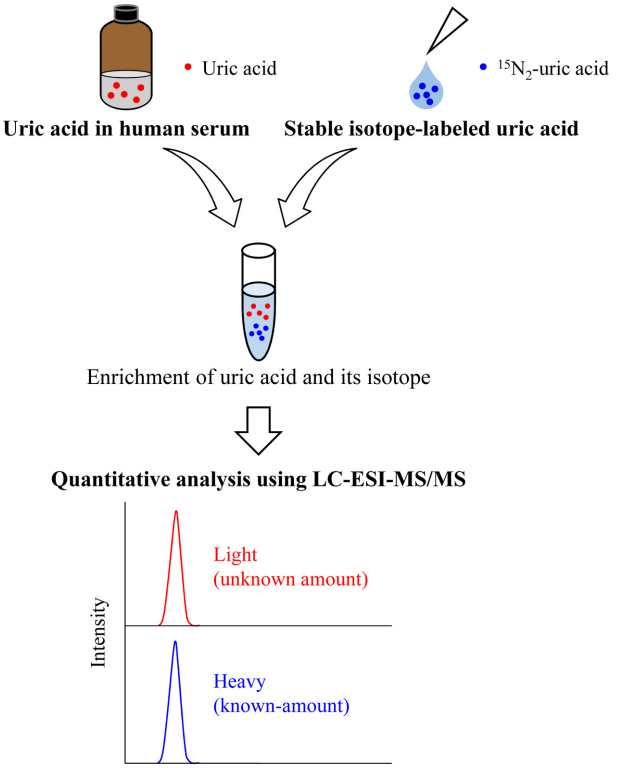

Figure 1. The schematic workflow for the isotope dilution mass spectrometry (IDMS)-based absolute quantification of uric acid in serum.

Figure 2 shows that the trends of RF values, obtained from LC-ESI-MRM experiments, were not significantly changed at certain mole ratios of UA/NH_4OH over time, along with two different storage temperatures of -20°C and RT. At both temperatures of -20°C and RT, two mole ratios of 1/1 and 1/1.5, in particular, were measured to be 1.024 ± 0.004 and 0.987 ± 0.004, respectively, in the first test of inter-day analysis on 0-day, and these results were distinctly different from that of three others (i.e., 0.972 ± 0.003 for 1/2, 0.971 ± 0.007 for 1/3, and 0.969 ± 0.004 for 1/5.6). In previous study, Dai Xinhua[12] has issued in that

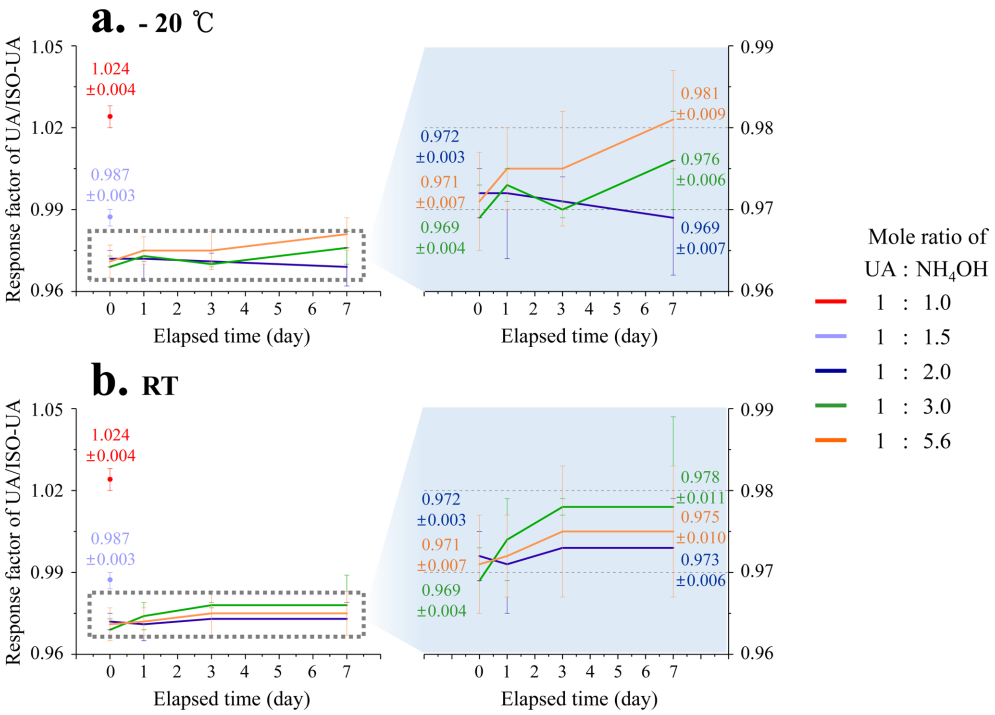

Figure 2. The profiling of RF values were obtained by MRM-analyzing the internal standard solutions that have the five different mole ratios of 1/1, 1/1.5, 1/2, 1/3, and 1/5.6, for UA/NH_4OH with two different storage temperatures of internal standard solution at (a) -20°C and (b) RT.

the use of 1 mmol/L NH_4OH at a mole ratio of 1/1.7 (UA/NH_4OH) is not sufficient to completely dissolve UA after several hours to several days. So, it can be inferred that the initial difference of the RF values might be caused by the limited solubility of UA in 1 mmol/L NH_4OH used in this study. Based on these results, it can be conclude that at least 1/2 or more is suitable to completely dissolve UA and ISO-UA.

To further examine the effect of the temperatures (-20°C and RT) for the storage of UA and ISO-UA standard solutions on the changes of RF values, the IS solutions, having the three different mole ratios of 1/2, 1/3, and 1/5.6, were MRM-analyzed in triplicate, excepting those of both 1/1 and 1/1.5. At -20°C, the inter-day variations estimated over a span of seven days were shown to be about 0.3, 0.7, and 1.0% for 1/2, 1/3, and 1/5.6, respectively, whereas those counterparts of about 0.1, 0.9, and 0.4% at RT. Based on these results, we confirmed that the different temperatures between -20°C and RT did not greatly impinge on the stability of UA for 7 days. However, it's very interesting that the storage temperature of -20°C can be an effective way to maintain the stability of UA in 2 mmol/L NH_4OH in previous study.[15] To further investigate on the effect of storage temperature on the stability of UA, we lengthened the duration from 7 to 14 days. Figure 3 shows that the EICs obtained via MRM experiments of IS solution at the mole ratio of 1/5.6 on 0-, 7-, and 14-day. At RT, the peak areas of UA and the corresponding isotopic counterpart were dramatically decreased over time and eventually disappeared after 14 days, whereas those that were not detectable any change at -20°C during the same time. Viewed in the peak area ratio of UA to ISO-UA in the Figure 3a, it's unusual that the decreasing rate of ISO-UA in the peak area was a little faster than that of natural one, despite of that both UA and its isotope have the same chemical structure. Unfortunately, it has not been proven yet this cause that leads to making a difference in the degradation rates of UA and its corresponding isotope (UA-$^{15}N_2$). Viewed in the recovery of UA over time (i.e., 10.1% at RT after 7 days; 96.0% at -20°C after 14 days), It can be, however, inferred that the lower moles of ISO-UA in 1 mmol/L NH_4OH may be more rapidly decomposed than the natural UA at RT. Alternatively, this unwanted effect could be reduced with the temperature of -20°C, regardless of any mole ratios of 1/2, 1/3, and 1/5.6. However, because all of IDMS experiments are typically completed in a week, the temperature condition for the storage of UA and its isotope is not an indispensable factor for the following IDMS-based absolute quantification of UA in serum.

Finally, we performed the quantitative profiling of UA containing in KRISS serum CRM (111-01-02A) with three different IS solutions (i.e. 1/2, 1/3, and 1/5.6 for UA/NH_4OH) that were stored at RT. As shown in the Figure 4,

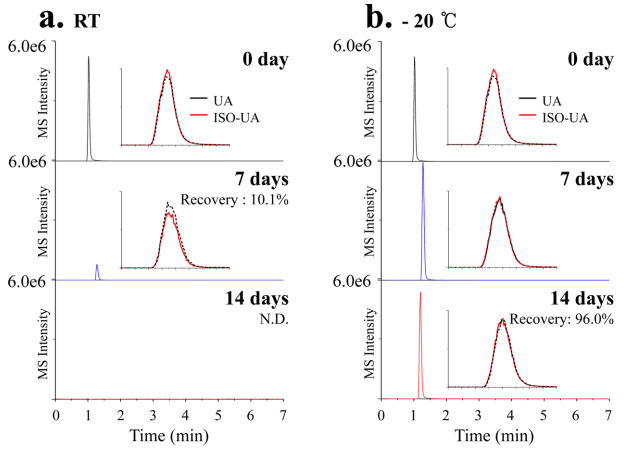

Figure 3. The extracted ion chromatograms (EICs) were obtained via MRM analyses of the internal standard solution at the mole ratio of 1/5.6 on 0-, 7-, and 14-day at (a) RT and (b) -20°C.

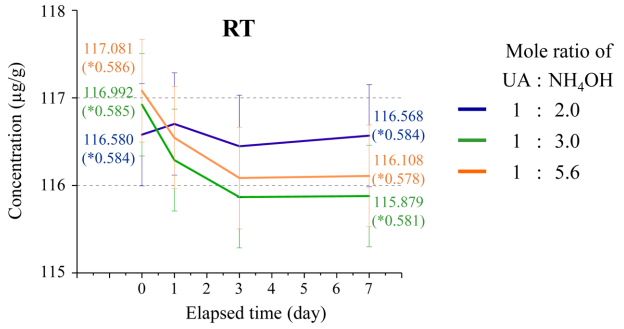

Figure 4. The quantitative profiling of uric acid in KRISS serum CRM (111-01-02A) were determined in compliance with the RF values at three different mole ratios of 1/2, 1/3, and 1/5.6 (UA/NH_4OH) at RT. *the parenthesis refers to as "the expanded uncertainty" that is $U = ku_c$ (*i.e., k=2*). Where u_c is a coverage factor.

the quantities of UA in serum CRM were altered slightly in compliance with the different mole ratios of UA to NH_4OH over time, except that of 1/2. Differed from two cases of 1/3 and 1/5.6, the quantities of UA between 0-day and 7-day, based on the RF values at the mole ratio of 1/2, were measured to be 116.580 ± 0.584 (expanded uncertainty, *U*) and 116.568 ± 0.584 (*U*), respectively, whereas both of 1/3 and 1/5.6 were represented a little change in the measurement of UA between 0-day and 7-day. Especially, all of the measured quantities of UA that were acquired on 0-, 1-, 3-, and 7-day with IS solution at 1/2 for UA/NH_4OH were well matched with its certificated value of 116.60 ± 2.10 (*U*). As a result, using the IS solution at the mole ratio of UA to NH_4OH that to be 1/2 is promising to provide the stability of UA in NH_4OH solution, thereby increasing the inter-day precision and accuracy for the quantification of UA in serum.

Conclusion

In this study, we sought to find out the confirmative method for the preparation of both standard solutions of UA and its isotope (UA-$^{15}N_2$), along with maintaining their stability in 1 mmol/L NH_4OH solution for at least 7 days. To do this, the IS solutions were prepared by varying the mole ratios of UA to NH_4OH at five different mole ratios of 1/1, 1/1.5, 1/2, 1/3, and 1/5.6, and followed by ID-LC-MRM analysis at two different temperatures of - 20°C and RT. Viewed in both of solubility and stability of UA, we found that both 1/2, 1/3, and 1/5.6 at the mole ratios of UA/NH_4OH are suitable to completely dissolve the UA and its isotope, and the low temperature of -20°C for the storage of both UA and its isotope standard solution is much benefit to maintain the their intact structures for 14 days, compared to those that were stored at RT. As a result, it's promising that the mole ratio of 1/2 (UA/NH_4OH) has in common provided a high reproducibility in the measurement of RF values of UA at both temperatures of -20°C and RT. Consequently, we believe that this optimized condition for the stability of UA is of use to determine the accurate quantity of UA in serum and develop a serum CRM with SI-traceability, thereby increasing the reliability in clinical diagnosis.

Acknowledgements

This research was supported by the Basic Research Projects of KRISS, "Nutrient Metrology Program."

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