Measurement of Urinary Ammonium Using a Commercially Available Plasma Ammonium Assay

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Key Points

- Plasma ammonium assay reliably quantifies urine ammonium at physiologic concentrations.
- The enzymatic method compares well with the formalin titration method and is suitable for routine clinical use.

Abstract

Background Determination of urinary ammonium excretion is helpful in evaluating patients with acid-base disorders, chronic kidney disease, and nephrolithiasis. However, urinary ammonium levels are only measured by specialized laboratories in the United States, limiting widespread implementation. We evaluated the performance of a plasma ammonium assay to quantify urinary ammonium excretion and also determined ammonium stability under a variety of conditions.

Methods An enzymatic plasma ammonium assay (Randox) was modified to measure urinary ammonium concentration. Urine samples were diluted 40-fold and then assayed on an Abbott Architect ci8200 analyzer. Assay precision, limit of quantitation, and linearity were determined. The method was compared against the formalin titration method, and stability studies were conducted at different temperatures and pH.

Results After dilution, the assay had total precision of 18% at 2.54 mmol/L, 5% at 15.58 mmol/L, and 2% at 29.49 mmol/L, with a limit of quantitation of 2.92 mmol/L. Assay performance was linear in the range of 0.7–45 mmol/L. Method comparison against the formalin method showed a slope of 0.98 and intercept of –0.37 mmol/L. Urinary ammonium was determined to be stable for 48 hours at room temperature and for 9 days at 4°C and –20°C at pH 5.6–6.3. Ammonium was less stable at pH 3.8 and 8.5. When stored at –80°C, urinary ammonium was stable for at least 24 months.

Conclusions The modified enzymatic plasma ammonium assay reliably quantifies urine ammonium at physiologic concentrations. It compares well with the formalin titration method and is suitable for routine clinical use on an automated chemistry analyzer.

Introduction

A principal function of the kidney is to maintain acid-base homeostasis. On average, the kidneys excrete 1 mEq/kg of hydrogen ions (H+) each day. In healthy individuals, approximately 60% of the acid load is eliminated as ammonium (NH4+) and 40% is eliminated as titratable acids (i.e., H2PO4−); free H+ contributes very little to acid excretion. In CKD, kidney acid excretion declines with worsening kidney function, leading to acid retention and metabolic acidosis, which is associated with CKD progression and death (1–5). Urinary ammonium excretion typically declines before titratable acid excretion in CKD, and findings from large observational studies have linked lower urinary ammonium excretion with higher risks of incident metabolic acidosis, CKD progression, and death (6,7). This suggests that urinary ammonium measurements could identify individuals with CKD who are at risk of poor outcomes and could potentially be treated with alkali before metabolic acidosis develops. Quantification of urinary ammonium levels is also helpful in evaluating patients with normal anion gap metabolic acidosis to determine if the etiology is due to diarrhea or renal tubular acidosis (RTA) (8,9). Urinary ammonium levels can also aid in the management of patients with nephrolithiasis by providing an assessment of the daily acid load, provided kidney function is normal and there is no co-existing RTA, or signaling the presence of struvite stones (10).

Unfortunately, few clinical laboratories offer urinary ammonium testing. Therefore, clinicians use surrogates such as the urinary anion gap (UAG) and

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urinary osmolal gap (UOG) to estimate urinary ammonium concentration (8,9,11,12). Although these surrogates are used in patients with normal anion gap metabolic acidosis who also have normal kidney function, they do not approximate urinary ammonium levels in patients with CKD (13,14). Assays that measure plasma ammonium concentration have been modified to measure urinary ammonium concentration, suggesting that this approach could be employed using modern clinical chemistry analyzers (15,16). In this study, we determined and validated the performance of the plasma ammonium assay to quantify urine ammonium concentration. We also compared results from the plasma ammonium assay with formalin titration using patient samples and determined the stability of ammonium in the urine matrix at different pH levels at various storage temperatures.

**Methods**

**Measurement of Urinary Ammonium Using a Commercial Plasma Ammonia Assay**

Urinary ammonium was measured by the ARUP Clinical Laboratory at the University of Utah Hospital using the Abbott Architect ci8200 automated chemistry analyzer. Patient urine samples used in this study were de-identified in accordance with the University of Utah Institutional Review Board Protocol #7275 for samples used in clinical laboratory test development. Two plasma ammonia assays were initially evaluated: Randox and Multigent. The Multigent assay showed up to 15% negative bias at ammonium concentrations >5 mmol/L and was not evaluated further.

The Randox assay method is based on enzymatic conversion of NH₃ and α-ketoglutarate to glutamate (17). In the process, cofactor NADPH is oxidized to NADP⁺, leading to decreased absorbance at 340 nm. The lower detection limit of the plasma assay is 43.9 μmol/L, and the assay is linear up to 1170 μmol/L. Two dilutions were evaluated: 1:80 and 1:40. The 1:80 dilution showed up to 15% negative bias at concentrations >22 mmol/L, whereas the 1:40 dilution showed minimal negative bias for concentrations >22 mmol/L and was used for all subsequent experiments. The 1:40 dilution also predicted good coverage of ammonium concentration previously observed in patient samples (18).

**Determination of Precision and Limit of Quantitation of the Plasma Ammonia Assay**

The precision of the enzymatic assay was determined using synthetic urine (Pickering Chemicals) with defined ammonium concentrations (2.54, 15.58, and 29.49 mmol/L) in accordance with Clinical and Laboratory Standards Institute standards (19). Samples were run in duplicate, twice daily, for 20 days (n = 80 measurements for each concentration). The precision profile was determined by preparing ten aliquots with ammonium concentrations of 2.92, 3.82, 13.92, 25.35, 36.29, and 46.11 mmol/L using 100 mmol/L ammonium standard solution spiked in pooled patient urine. One aliquot from each concentration was assayed initially; the nine remaining aliquots were frozen at −20°C. On each day of testing, an aliquot from each concentration was thawed, mixed, and tested. Limit of quantitation (LOQ) was determined using the EP Evaluator LOQ module, and an acceptable LOQ precision target was predefined as having a coefficient of variation (CV) of <20%, which is the ratio of SD to the mean value expressed as a percentage. Higher CV values indicate higher imprecision and greater result dispersion.

**Linearity of the Plasma Ammonia Assay**

Linearity (analytical measurement range [AMR]) of the enzymatic assay was determined using a high pooled-patient sample (60 mmol/L NH₄⁺) mixed in different percentages (vol/vol) with synthetic urine (2 mmol/L NH₄⁺) to obtain six NH₄⁺ concentrations (2, 2.95, 12.75, 23.5, 34.25, and 45 mmol/L). Samples were then assayed in triplicate, and the results were evaluated in the EP Evaluator Linearity module. Allowable systematic error was set to 2 mmol/L or 2%.

**Method Comparison between the Plasma Ammonia Assay and Formalin Titration**

Ammonium concentrations from the enzymatic assay were compared with ammonium concentrations obtained by the formalin titration method in patient samples. The formalin titration method has been previously described (18). Briefly, after measuring pH, the titratable acid concentration is determined by adding 10 ml of 0.1-M hydrochloric acid to 10 ml of urine. The sample is then titrated to pH 7.4 using 0.1-M sodium hydroxide. Next, 10 ml of 8% formaldehyde is added to the sample, which in the presence of ammonium forms hexamethylenimine and equimolar hydrochloric acid. The sample is subsequently titrated to pH 7.4 using 0.1-M sodium hydroxide, and the millimolar quantity of sodium hydroxide added to the sample reflects the ammonium concentration (mmol/L) in the sample. Fifty-eight patient samples were used in the comparison analysis. These samples were submitted by 22 US veterans with diabetes and CKD stages 2–4, who were participants in a clinical trial testing the effect of oral sodium bicarbonate on urinary TGF-β1 levels (20). Urine samples were collected under mineral oil over 24 hours. Results of ammonium concentration as determined by the enzymatic assay and formalin titration were compared in the EP Evaluator Alternate Method Comparison module.

**Stability of Urinary Ammonium**

To assess ammonium stability at different pH under different temperatures (room temperature [approximately 23°C], refrigerated [4°C], and frozen [−20°C]), urine samples with pH 3.8, 5.6, 6.3, and 8.5 were generated by adding 6 M hydrochloric acid or 6 M sodium hydroxide into synthetic urine with pH 3.8, 5.6, 6.3, and 8.5. These samples were submitted by 22 US veterans with diabetes and CKD stages 2–4, who were participants in a clinical trial testing the effect of oral sodium bicarbonate on urinary TGF-β1 levels (20). Urine samples were collected under mineral oil over 24 hours. Results of ammonium concentration as determined by the enzymatic assay and formalin titration were compared in the EP Evaluator Alternate Method Comparison module.

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baseline that is distinguishable from difference caused by method imprecision with 95% confidence (21).

Long-term stability of urinary ammonium in human samples stored at –80°C was evaluated using 31 urine samples obtained from 17 kidney transplant recipients who participated in a clinical trial testing the effect of sodium bicarbonate on kidney fibrosis markers (NCT 01225796). Overnight urine collections were obtained under mineral oil. Ammonium was measured using the formalin titration method on the day the collection was completed. Samples were subsequently frozen at –80°C, and the ammonium measurements were repeated 24–27 months later using formalin titration.

Results

Precision and LOQ of the Plasma Ammonia Assay in Synthetic Urine Samples

Imprecision (%CV) was higher at lower ammonium concentrations in both the within-run and between-run assessments; yet, it was still acceptable (%CV <20%) for all concentrations tested. The within-run %CV was 5% at 2.54 mmol/L, 0.8% at 15.58 mmol/L, and 0.5% at 29.49 mmol/L ammonium concentrations. Total (between-run) %CV was determined to be 18% at 2.54 mmol/L, 5% at 15.58 mmol/L, and 2% at 29.49 mmol/L ammonium concentrations (Table 1). The results of the LOQ studies showed that the precision limit was below the lowest concentration tested, 2.92 mmol/L (observed %CV of 12%; Figure 1).

Linearity and AMR of the Plasma Ammonia Assay in Urine Samples

The enzymatic assay was determined to be linear in the range of 0.7–45 mmol/L, with a slope of 1.02 and intercept of 0.45 mmol/L (Figure 2). Actual systematic error was calculated to be 1.26 mmol/L or 1%, which did not exceed allowable systematic error limit of 2 mmol/L or 2%. Because the lowest concentration with acceptable precision tested was 2.92 mmol/L, the AMR of the implemented assay would be 2.92–45 mmol/L, even though linearity was demonstrated below the established LOQ.

Comparison of Urinary Ammonium Using the Plasma Ammonia Assay and Formalin Titration

The method comparison using patient samples found that the mean urinary ammonium concentration using formalin titration was on average 5.1 mmol/L higher than values obtained by the enzymatic method. The pH of the 8% formaldehyde was found to be 3.5, indicating that it was contributing acid to the urine samples. However, the $[\text{H}^+]$ concentration at this pH is 0.3 mmol/L and does not itself account for the 5.1 mmol/L difference. Formalin titration was next applied to synthetic urine samples with defined concentrations were run in duplicate, twice daily, for 20 days ($n=80$ measurements for each concentration).

### Table 1. Precision of the plasma ammonia assay

| $[\text{NH}_4^+]$, mmol/L | Within-run SD, mmol/L/%CV | Overall SD, mmol/L/%CV |
|---------------------------|---------------------------|------------------------|
| 2.54                      | 0.14/5.4                  | 0.45/17.7              |
| 15.58                     | 0.12/0.8                  | 0.79/5.1               |
| 29.49                     | 0.14/0.5                  | 0.66/2.2               |

Synthetic urine samples with defined concentrations were run in duplicate, twice daily, for 20 days ($n=80$ measurements for each concentration).
known urinary ammonium concentrations of 0, 2, 16, and 30 mmol/L (n=2 measurements per concentration). Mean (SD) urinary ammonium concentrations obtained from formalin titration were 3.9 (1.9) mmol/L higher than their known quantities. A subsequent titration experiment was performed on the formaldehyde solution, and the measured acid concentration of the solution was 4.9 mmol/L, which closely approximated the difference in ammonium concentration between the formalin titration and enzymatic methods (5.1 mmol/L) in the patient samples.

After accounting for the bias of 4.9 mmol/L in the formalin titration method, the two methods compared favorably, with a slope of 0.98 and intercept of -0.37 (least-squares regression; Figure 3). Plotting pH versus the difference between the two methods detected greater mmol/L differences between methods at higher urinary pH in some but not all instances (Figure 4).

Stability of Urinary Ammonium

At physiologic urine pH tested (pH 5.6 and 6.3), ammonium was determined to be stable at room temperature for at least 48 hours. For samples with urine pH 5.6, ammonium was stable under refrigerated and frozen storage (−20°C) for at least 14 days, whereas samples at pH 6.3 were stable for 9 days at these temperatures (Figure 5, Table 2). Urinary ammonium was less stable at extremes of pH (3.8 and 8.5; Figure 5, Table 2).

Figure 6 shows long-term stability of urinary ammonium from patient samples when stored at −80°C for 24–27 months. Mean (SD) values of ammonium were 19.9 (11.6) mmol/L at the time of collection and 19.4 (11.7) mmol/L after prolonged frozen storage. The mean (SD) difference between the time points were 0.6 (0.9) mmol/L. The range of differences between the measurements at the two time points was narrow (−1.2 to 2.4 mmol/L) and results were generally comparable after long-term frozen storage.

Discussion

We investigated whether urinary ammonium concentrations could be reliably quantified using a commercially available plasma ammonia assay and modern clinical laboratory equipment. Because plasma ammonia concentrations are measured in micromoles per liter and urinary concentrations are in the millimoles per liter range, urine samples require dilution before analysis. Using this approach, precision of the plasma ammonia assay was found to be greater (lower %CV) with higher urinary ammonium concentrations, but precision met the acceptable threshold %CV (<20%) for all urinary ammonium concentrations tested (Table 1). The results of the LOQ studies showed that the precision limit was below the lowest concentration tested, 2.92 mmol/L (observed imprecision of 12%). The modified assay was found to be linear, with ammonium concentrations in the range of 0.7–45 mmol/L. Because the lowest concentration tested in our precision analyses was 2.92 mmol/L, we conclude that the plasma ammonia assay used here has a broad AMR of 2.92–45 mmol/L after specimen dilution. Moreover, values using this method had excellent agreement with those obtained by formalin titration in patient samples, after accounting for the concentration of acid in formaldehyde.

Analyte stability is also important to demonstrate in clinical laboratory testing. Here, we show that at common urine pH values (5.6 and 6.3), urinary ammonium is stable for at least 48 hours at room temperature. At pH 5.6, ammonium was stable for at least 14 days at 4°C and −20°C, whereas at pH 6.3, ammonium was stable for 9 days at 4°C and −20°C. On the basis of our observations at these common pH values, we conclude that it is best to refrigerate urine samples and complete the measurements within 9 days. Refrigeration is preferable to room temperature storage because this will minimize the effect of urease-producing bacteria, if present, on urinary ammonium levels. Importantly, samples in the stability analyses were...
not stored with a layer of mineral oil, which is commonly added when urinary net acid excretion is measured. Our findings indicate that adding mineral oil is not necessary when measuring urinary ammonium so long as the urine is kept refrigerated and the analyses are performed within 9 days. Lastly, we demonstrate that urinary ammonium levels are stable for at least 2 years when stored at –80°C. Katagawa et al. reported that ammonium was stable for at least 28 days when kept at 4°C and –20°C, which is longer than what we observed. This may be due to a difference in the assays used or equipment. Our results suggest that urine pH may affect stability, and they did not report the pH of their samples. We observed longer stability for samples with pH 5.6 (≥14 days) than those with pH 6.3 (9 days). It is possible that samples studied by Katagawa et al. had pH values toward the 5.6 range, and therefore they had longer stability. We also found that stability was shorter at extremes of pH (3.8 and 8.5). Whether this is due to ammonium degradation, an effect on assay performance, or some combination of factors is unclear and speculative at this time. Alkaline urine with pH values near those tested here (pH 8.5) can be observed in humans, and in such cases ammonium levels could be unreliable. Although urine pH 3.8 does not occur physiologically, this finding cautions against excessively acidifying samples before measuring ammonium. For these reasons, simultaneous measurements of urine pH may increase confidence in the accuracy of the results.

Although urinary ammonium testing is available in a few clinical laboratories in the United States, limited access to this test has led clinicians to rely on the UAG to gauge the robustness of urinary ammonium excretion in patients.

Figure 5. Stability graphs of ammonium in urine at different temperature and pH. The dashed horizontal line indicates the limit of stability, defined as being <2.8×%CV below the baseline value. (A) Room temperature (approximately 23°C). (B) Refrigerated (4°C). (C) Frozen (−20°C).
Ammonium concentration is higher in transplant recipients, where 40% of 70 urine samples had a UOG urinary ammonium concentration in an ambulatory kidney study evaluating performance of the UOG as an estimate of glomerular filtration rate in CKD (13). The UOG has also been used as another estimate of kidney function. However, the validity of the UAG (urinary ammonium gap) that fails to predict ammonium concentration in this setting has been debated (22). Further, the UAG is a qualitative assessment (to determine if the UAG is positive or negative) that fails to predict ammonium concentration in CKD (13), whereas the UOG has been used as another estimate of urinary ammonium concentration.

In a study evaluating performance of the UOG as an estimate of urinary ammonium concentration in ambulatory kidney transplant recipients, 40% of 70 urine samples had a UOG < 0 mOsm/L (14), which would imply that the urinary ammonium concentration is < 0 mOsm/L.

Others have also shown that it is feasible to quantify urinary ammonium using available assays and equipment (15, 16). One of these studies, however, compared urinary ammonium measurements with the UOG rather than direct measurements of ammonium (16). The other study compared ammonium measurements with formalin titration as we did (15), and our comparison with formalin titration reinforces their findings, albeit with a modern chemistry analyzer. We expand on this work by including additional validation studies using synthetic urine samples with known ammonium concentrations to determine precision, linearity, and AMR to meet Clinical and Laboratory Standards Institute standards. The use of synthetic urine for precision studies also provides commercially available quality control material to monitor long-term assay accuracy and precision. Our study also adds important new information regarding the effect of different urine pH values on stability during the first 2 weeks after collection and the long-term stability of ammonium when stored at −80°C. Our finding that formalin titration overestimated urinary ammonium concentration also suggests that enzymatic methods may outperform this technique.

Several studies in CKD provide some insight regarding urinary ammonium values that may be considered clinically meaningful. In the African American Study of Kidney Disease and Hypertension, urinary ammonium excretion rates < 15 mmol/d were associated with a 56% higher risk of CKD progression and death and a 2.56-fold higher risk of incident metabolic acidosis (18). In terms of spot urine values in CKD, a fasting urinary ammonium concentration < 9 mmol/L was associated with a higher risk of ESKD (7). For individuals with normal anion gap metabolic acidosis, one of the original reports of the UAG provides insight as well. In that study, seven of eight individuals with diarrhea-induced metabolic acidosis had urinary ammonium concentrations > 30 mmol/L. On the other hand, all 38 patients with distal RTA had urinary ammonium concentrations well below 30 mmol/L (8). Thus, a urinary ammonium concentration threshold of 30 mmol/L may be useful to differentiate patients with diarrhea from those with RTA. The AMR for this assay after 1:40 dilution is 2.92–45 mmol/L. This range is robust and includes thresholds posited to be of clinical importance in CKD (15 mmol/d or 9 mmol/L on a spot specimen) and those with normal anion gap metabolic acidosis (30 mmol/L). In patients with nephrolithiasis, high urinary ammonium levels could signal the presence of urease-producing bacteria and struvite stones or a high-protein diet (10).

In summary, the enzymatic plasma ammonium assay used here reliably quantified urinary ammonium concentration after specimen dilution. The modified assay we used had a broad AMR (2.92–45 mmol/L in undiluted urine), which is expected to quantify ammonium in most patient samples without additional dilution. Method comparison studies showed excellent agreement with the formalin titration method; however, the formalin-titration method overestimated ammonium excretion, suggesting that the enzymatic assay may outperform this technique. Although findings in this study should generally translate to other commercially available ammonia assays, it is important to check for negative bias with higher sample dilution and other ammonia assays as observed in our preliminary studies. Nevertheless, the enzymatic method used here is fully automated and easy to implement. Our findings provide important information about ammonium stability and a framework for clinical laboratories to implement this test.

Disclosures

L. Pearson reports an advisory or leadership role for the College of American Pathologists and is chair of the Instrumentation

| Table 2. Stability of ammonium in urine at different temperature and pH |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Storage Condition          | pH 3.8                      | pH 5.6                      | pH 6.3                      | pH 8.5                      |
| Room temperature (approximately 23°C) | 17 hr                       | ≥ 48 hr                     | ≥ 48 hr                     | 4 hr                        |
| Refrigerated (4°C)         | 2 d                         | ≥ 14 d                      | 9 d                         | 1 d                         |
| Frozen (−20°C)             | 2 d                         | ≥ 14 d                      | 9 d                         | 1 d                         |

Figure 6. Stability of urinary ammonium when frozen at −80°C. Urinary ammonium was measured by formalin titration from fresh samples on the day of collection and 24–27 months later (n = 31). The mean difference between the initial and repeat measurements was 0.6 mmol/L, corresponding to a 3% difference from the mean initial values of 19.9 mmol/L. The range of the difference between the initial and repeat measurement was low (−1.2 to 2.4 mmol/L).
Committee. K.L. Raphael reports consultancy for AstraZeneca. All remaining authors have nothing to disclose.

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**Author Contributions**

K. Cahoon, V. Gruzdys, and L. Pearson were responsible for validation; K. Cahoon, V. Gruzdys, and K.L. Raphael were responsible for the methodology and software; V. Gruzdys and L. Pearson were responsible for the software; V. Gruzdys, L. Pearson, and K.L. Raphael were responsible for project administration, resources, and supervision; V. Gruzdys and K.L. Raphael were responsible for the formal analysis, and approved the final version of the manuscript; L. Pearson and K.L. Raphael were responsible for funding acquisition; and all authors conceptualized the study, curated the data, conducted the formal analysis, and approved the final version of the manuscript.

**Data Sharing Statement**

All data are included in the manuscript and/or supporting information.

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