How to Cite this article: Valentinas Gruzdys, Kenneth Cahoon, Lauren Pearson, and Kalani Raphael, Measurement of urinary ammonium using a commercially available plasma ammonium assay, Kidney360, Publish Ahead of Print, 10.34067/KID.0000262022

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Measurement of urinary ammonium using a commercially available plasma ammonium assay

DOI: 10.34067/KID.0000262022

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Key Points:
*Plasma ammonium assay reliably quantifies urine ammonium at physiological concentrations

*Enzymatic method compares well with 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 US 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®, Ireland) was modified to measure urinary ammonium concentration. Urine samples were diluted 40-fold 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 17.7% at 2.54 mmol/L, 5.1% at 15.58 mmol/L, and 2.2% at 29.49 mmol/L with a limit of quantitation of 2.92 mmol/L. Assay performance was linear in the range of 0.7-45.0 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 48h 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.

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

Disclosures: L. Pearson reports the following: Advisory or Leadership Role: College of American Pathologists, Chair of the Instrumentation Committee. K. Raphael reports the following: Consultancy: AstraZeneca. The remaining authors have nothing to disclose.

Funding: US Department of VA Clinical Sciences Research and Development; I01 CX001695

Author Contributions: Valentinas Gruzdys: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing - original draft; Writing - review and editing Kenneth Cahoon: Conceptualization; Data curation; Formal analysis; Methodology; Software; Validation Lauren Pearson: Conceptualization; Data curation; Formal analysis; Funding acquisition; Project administration; Resources; Supervision; Validation Kalani Raphael: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Visualization; Writing - original draft; Writing - review and editing

Data Sharing Statement: All data is included in the manuscript and/or supporting information.

Clinical Trials Registration:
The information on this cover page is based on the most recent submission data from the authors. It may vary from the final published article. Any fields remaining blank are not applicable for this manuscript.
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 physiological concentrations
- Enzymatic method compares well with 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 US 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®, Ireland) was modified to measure urinary ammonium concentration. Urine samples were diluted 40-fold 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 17.7% at 2.54 mmol/L, 5.1% at 15.58 mmol/L, and 2.2% at 29.49 mmol/L with a limit of quantitation of 2.92 mmol/L. Assay performance was linear in the range of 0.7-45.0 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 48h 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.
**Conclusion:** The modified enzymatic plasma ammonium assay reliably quantifies urine ammonium at physiological concentrations. It compares well with 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 (NH\(_4^+\)) and 40% is eliminated as titratable acids (i.e. H\(_2\)PO\(_4^-\)); free H\(^+\) contributes very little to acid excretion. In chronic kidney disease (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 acids 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 and urinary osmolal gap to estimate urinary ammonium concentration\(^8\), \(^9\), \(^11\), \(^12\). While 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 ammonia assay with formalin-titration using patient samples, and determined 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 (IRB) 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. 1:80 and 1:40 dilutions were evaluated. 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

Precision of the enzymatic assay was determined using synthetic urine (Pickering Chemicals, CA, USA) with defined ammonium concentrations (2.54, 15.58, and 29.49 mmol/L)
in accordance with Clinical and Laboratory Standards Institute (CLSI) standards.\textsuperscript{19} Samples were run in duplicate, twice daily, for twenty 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, 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\(^\circ\) 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\textsuperscript{\textregistered} LOQ module, and an acceptable LOQ precision target was pre-defined as having a coefficient of variation (CV) of <20\%, which is the ratio of standard deviation 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) of the enzymatic assay was determined using a high pooled-patient sample (60 mmol/L NH\(_4^+\)) mixed in different percentages (vol:vol) with synthetic urine (2 mmol/L NH\(_4^+\)) to obtain 6 NH\(_4^+\) concentrations (2.00, 2.95, 12.75, 23.50, 34.25, and 45.00 mmol/L). Samples were then assayed in triplicate and the results were evaluated in the EP Evaluator\textsuperscript{\textregistered} 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 to ammonium concentrations obtained by the formalin-titration method in patient samples. The formalin-titration method has been previously described.\textsuperscript{18} Briefly, after measuring pH, titratable acids 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 7.40 using 0.1 M sodium hydroxide. Next, 10 mL of 8\%
formaldehyde is added to the sample, which in the presence of ammonium, forms hexamine and equimolar hydrochloric acid. The sample is subsequently titrated to pH of 7.40 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 stage 2-4 CKD who were participants in a clinical trial testing the effect of oral sodium bicarbonate on urinary transforming growth factor-β1 levels.\textsuperscript{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.

\textit{Stability of urinary ammonium}

To assess ammonium stability at different pH under different temperatures (room temperature [\textasciitilde-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 6M HCl or 6M NaOH into pooled patient urine with final ammonium concentration of 18.6 mmol/L. De-ionized water was added as necessary to match the total volume of each sample. Samples were then stored at the relevant temperature and assayed for pH and ammonium concentration using the enzymatic assay after 0, 4, 17, 24, and 48 hours for room temperature samples, and after 1, 2, 7, 9, and 14 days for refrigerated and frozen samples. The stability limit (Figure 5) for each storage condition was set at -2.8 x %CV from the average baseline value (0 hours) using %CV from the closest quality control (QC) sample concentration. This approach assumes uncertainty in both baseline and subsequent measurements and identifies a statistically significant difference from baseline that is distinguishable from difference caused by method imprecision with 95% confidence.\textsuperscript{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 5.4% 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 17.7% at 2.54 mmol/L, 5.1% 15.58 mmol/L, and 2.2% 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 11.7%, Figure 1).

**Linearity and analytical measurement range of the plasma ammonia assay in urine samples**

The enzymatic assay was determined to be linear in the range of 0.7 – 45.0 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 1.3%, which did not exceed allowable systematic error limit of 2 mmol/L or 2%. Since the lowest concentration with acceptable precision tested was 2.92 mmol/L, the analytical measurement range (AMR) of the implemented assay would be 2.92-45.0 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, $[\text{H}^+]$ 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 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 vs. 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 physiological 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 (Table 2 and Figure 5). Urinary ammonium was less stable at extremes of pH (3.8 and 8.5) (Table 2 and Figure 5).
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 μmol/L and urinary concentrations are in the mmol/L range, urine samples require dilution prior to 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 11.7%). The modified assay was found to be linear with ammonium concentrations in the range of 0.7-45.0 mmol/L. Since 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 analytical measurement range of 2.92-45.0 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. Based on 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 as 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 two 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\textsuperscript{15}, 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 towards the 5.6 range, and therefore 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 of 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.

While urinary ammonium testing is available in a few clinical laboratories in the US, limited access to this test has led clinicians to rely on the urinary anion gap (UAG) to gauge the
robustness of urinary ammonium excretion in patients with normal anion gap metabolic acidosis who have preserved kidney function. However, the validity of the UAG in this setting has been debated. Further, the UAG is a qualitative assessment (to determine if the UAG is positive or negative) that fails to predict ammonium concentration in CKD. The urinary osmolal gap (UOG) has also been used as another estimate of urinary ammonium concentration. However, 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, 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. One of these studies, however, compared urinary ammonium measurements with the urinary osmolal gap rather than direct measurements of ammonium. The other study compared ammonium measurements with formalin-titration as we did, 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 analytical measurement range to meet CLSI 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 two weeks after collection, as well as 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 56% higher
risk of CKD progression and death and 2.56-fold higher risk of incident metabolic acidosis.\textsuperscript{18} In terms of spot urine values in CKD, fasting urinary ammonium concentration <9 mmol/L was associated with a higher risk of end-stage kidney disease.\textsuperscript{7} For individuals with normal anion gap metabolic acidosis, one of the original reports of the UAG provides insight as well. In that study, 7 of 8 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.\textsuperscript{8} Thus, a urinary ammonium concentration threshold of 30 mmol/L may be useful to differentiate patients with diarrhea from those with RTA. The analytical measurement range for this assay after 1:40 dilution is 2.92 – 45.0 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.\textsuperscript{10}

In summary, the enzymatic plasma ammonium assay used here reliably quantified urinary ammonium concentration after specimen dilution. The modified assay we used had broad analytical measurement range (2.92 – 45.0 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. While 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 the following: Advisory or Leadership Role: College of American Pathologists, Chair of the Instrumentation Committee. K. Raphael reports the following: Consultancy: AstraZeneca. The remaining authors have nothing to disclose.

FUNDING
Dr. Raphael is supported by US Department of VA Clinical Sciences Research and Development Service Merit Award I01 CX001695.

ACKNOWLEDGEMENTS
We are thankful for the contributions to this project by Dr. Christopher Lehman during his laboratory directorship at ARUP Clinical Laboratory at the University of Utah Hospital.

AUTHOR CONTRIBUTIONS
Valentinas Gruzdys: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing - original draft; Writing - review and editing. Kenneth Cahoon: Conceptualization; Data curation; Formal analysis; Methodology; Software; Validation. Lauren Pearson: Conceptualization; Data curation; Formal analysis; Funding acquisition; Project administration; Resources; Supervision; Validation. Kalani Raphael: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Visualization; Writing - original draft; Writing - review and editing. All authors approved the final version of the manuscript.

DATA SHARING STATEMENT
All data is included in the manuscript and/or supporting information.
REFERENCES


Table 1. Precision of the plasma ammonia assay. Synthetic urine samples with defined concentrations were run in duplicate, twice daily, for twenty days ($n = 80$ measurements for each concentration).

<table>
<thead>
<tr>
<th>$[\text{NH}_4^+]$ (mmol/L)</th>
<th>Within-run SD (mmol/L) / CV (%)</th>
<th>Overall SD (mmol/L) / CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.54</td>
<td>0.14 / 5.4</td>
<td>0.45 / 17.7</td>
</tr>
<tr>
<td>15.58</td>
<td>0.12 / 0.8</td>
<td>0.79 / 5.1</td>
</tr>
<tr>
<td>29.49</td>
<td>0.14 / 0.5</td>
<td>0.66 / 2.2</td>
</tr>
</tbody>
</table>

Table 2. Stability of ammonium in urine at different temperature and pH.

<table>
<thead>
<tr>
<th>Storage condition</th>
<th>Stability limit</th>
<th>pH 3.8</th>
<th>pH 5.6</th>
<th>pH 6.3</th>
<th>pH 8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room temp. (~23 °C)</td>
<td>17h</td>
<td>≥ 48h</td>
<td>≥ 48h</td>
<td>4h</td>
<td></td>
</tr>
<tr>
<td>Refrigerated (4 °C)</td>
<td>2 days</td>
<td>≥ 14 days</td>
<td>9 days</td>
<td>1 day</td>
<td></td>
</tr>
<tr>
<td>Frozen (-20 °C)</td>
<td>2 days</td>
<td>≥ 14 days</td>
<td>9 days</td>
<td>1 day</td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Precision profile over a range of ammonium concentrations. Lowest concentration tested is 2.92 mmol/L. The precision profile was determined by preparing ten aliquots with ammonium concentrations of 2.92, 3.82, 13.92, 25.35, 36.29, 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 and tested on subsequent days. The horizontal line at 20% indicates the acceptable %CV threshold.

Figure 2. Linearity plot (N=6). Linearity (analytical measurement range) of the enzymatic assay was determined using a high pooled-patient sample (60 mmol/L NH$_4^+$) mixed in different percentages (vol:vol) with synthetic urine (2 mmol/L NH$_4^+$) to obtain 6 NH$_4^+$ concentrations (2.00, 2.95, 12.75, 23.50, 34.25, and 45.00 mmol/L). Samples were then assayed in triplicate. Slope=0.99. Intercept 0.78 mmol/L. Error 0.30 mmol/L or 0.3%.

Figure 3. Comparison of ammonium concentrations obtained by the enzymatic and formalin-titration methods from patient urine samples (n=58).

Figure 4. Difference between formalin and enzymatic methods by urine pH in patient samples (n=58).

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 less than 2.8 x %CV below the baseline value.
  a. room temperature (~23 °C)
  b. refrigerated (4 °C)
  c. frozen (-20 °C)

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).
Figure 2

The graph shows a linear relationship between measured (mmol/L) and assigned (mmol/L). The data points are plotted on a 1:1 line, indicating a perfect agreement between the measured and assigned values.
Figure 3

Urine NH$_4^+$ (mmol/L)

Enzymatic method

Formalin method

\[ y = 0.98x - 0.37 \]

\[ R^2 = 0.9558 \]

Line of identity

Fitted values
Figure 5 a
Figure 5 b

The graph depicts the change in ammonium concentration (mmol/L) over time (d) at different pH levels. The x-axis represents time in days, ranging from 1 to 14, while the y-axis represents ammonium concentration from 11 to 20 mmol/L.

- **pH 3.8** is represented by black circles connected by a black line.
- **pH 5.6** is represented by light grey diamonds connected by a light grey line.
- **pH 6.3** is represented by grey squares connected by a grey line.
- **pH 8.5** is represented by light grey triangles connected by a light grey line.
- A dotted line indicates the stability limit.

As time progresses, the ammonium concentration decreases at all pH levels, with the concentration at each time point marked by the respective symbols.
Figure 5 c

Graph showing the decrease in ammonium concentration (mmol/L) over time (d) at different pH levels:
- pH 3.8
- pH 5.6
- pH 6.3
- pH 8.5

The stability limit is indicated by the dotted line.

The x-axis represents time in days (1 to 14), and the y-axis represents ammonium concentration (mmol/L).
After frozen storage

Urinary $\text{NH}_4^+$ (mmol/L)

Day of collection

$r=0.9972$