Blood Ammonia Stability Revisited

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ABSTRACT

The instability of ammonia in whole blood has challenged clinical laboratory scientists to develop specimen collection and handling methods that produce reliable results for plasma ammonia concentration. Thirty-eight outpatients' plasma ammonia concentrations were measured after heparinized whole blood specimens from each subject were held for 5, 15, and 30 minutes at room temperature and "on-ice". The plasma ammonia concentration from whole blood maintained "on-ice" for 5 minutes was designated the "reference value". Plasma ammonia concentrations from whole blood maintained "on-ice" for 15 or 30 minutes prior to processing were not significantly different from the reference values (15 min., p~0.196; 30 min., p~0.512). Plasma ammonia concentrations from whole blood maintained at room temperature for 5, 15 and 30 minutes prior to processing were also not significantly different from the reference values (p~0.961, 0.610, and 0.948, respectively). These results suggest that reliable plasma ammonia concentrations may be obtained from heparinized whole blood maintained at room temperature for up to 30 minutes.

INDEX TERMS: Ammonia, Blood Specimen Collection, Specimen Stability, Temperature

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INTRODUCTION

Diagnosis and treatment of patients who present with symptoms of congenital urea cycle defects or hepatic encephalopathy depend upon reliable measurement of plasma ammonia concentration. The instability of ammonia in venous blood in vitro has been recognized as a pre-analytical source of error since methods to measure its concentration were established.[1] Numerous studies have shown that plasma ammonia levels increase in vitro over time and may be falsely elevated if the specimen is not chilled "on-ice" prior to processing and analysis.[1-7] The sources of the increase in plasma ammonia concentration in vitro reportedly include deamination reactions in erythrocytes and platelets, and the deamidation of plasma glutamine by γ-glutamyltransferase (γ-GT).[4,6,8-10] While these studies demonstrated the clinical importance of plasma ammonia specimen integrity, many of these studies were performed with blood collection techniques and analytical methods that are no longer in use. In some cases, blood was collected directly into Erlenmeyer flasks, evacuated glass tubes, or via syringe into dipotassium EDTA.[1,6] Other studies utilized microdiffusion or ion-exchange methods to determine ammonia concentration, which are methods not used today.[1,3] Furthermore, previous studies focused on storage of specimens for hours to days prior to ammonia analysis and various temperature conditions for storage.[1-6]

Based upon the aforementioned studies, the established procedure at Parham Doctors' Hospital (Hospital Corporation of America, Richmond, VA) requires that any blood specimen collected for plasma ammonia measurement not received "on ice" be rejected, regardless of how soon after collection the specimen is

received in the laboratory. Rejection of a specimen delays analysis, reporting of results, diagnosis, treatment, and potentially increases the cost of providing healthcare. To examine the relevance of the current specimen rejection policy, an automated enzymatic method was used to investigate the changes in plasma ammonia concentration in blood specimens maintained "on-ice" or at room temperature over 5, 15 and 30 minutes after collection in plastic heparinized blood collection tubes.

METHODS

This study was approved by Virginia Commonwealth University's Institutional Review Board (IRB) as well as Henrico Doctors' Hospital's Research and Review Committee. Blood specimens for this study were collected from 44 outpatients at Parham Doctors' Hospital who voluntarily consented to the collection of two extra tubes of blood in addition to the tubes required for the laboratory tests ordered by their physician. Individuals were excluded from participating in the study if they were incarcerated, under the age of 18 years, or showed evidence of altered mental status.

Blood was collected by routine venipuncture using a 23-gauge butterfly needle and sterile technique. The venipuncture site was cleaned with an alcohol pad, and tourniquet use was minimized at the discretion of the investigator to avoid hemoconcentration. A non-traumatic, non-probing venipuncture was required for an acceptable specimen for this study.[3] In addition to the blood collected for the physician-ordered tests, two 3mL evacuated lithium heparin tubes (BD Vacutainer®) were collected from each individual and labeled with pre-printed study number labels. Immediately after labeling, one of the extra tubes was placed in the side pocket of a 5"x9" biohazard bag filled with approximately one cup of ice, and the second extra tube was maintained at room temperature (23°C) in a 5"x9" biohazard bag. At 5, 15, and 30 minutes post collection, both tubes were mixed by gentle inversion and 1ml of whole blood was transferred into plastic capped pour-off tubes that were labeled with pre-printed study numbers corresponding to the tube of origin and appended with the temperature/time condition. Both tubes were immediately centrifuged at room temperature for 3 minutes at 1220 x *g*. The plasma supernatant from each tube was immediately transferred to sample cups labeled with the study number, time, and temperature conditions. The sample cup was promptly loaded onto a Siemens Vista® 500 chemistry analyzer, the plasma ammonia concentration was measured by an enzymatic method,[7] and the results electronically transferred to the EasyLink® data system for storage.

A single instrument and a single lot of ammonia reagent were used to process all specimens in this study. The Siemens Vista® 500 was calibrated with a two-level calibrator, CHEM 3 calibrator (Dade Behring, Inc., Newark, NJ), and quality control was performed on each day with Liquichek™ Ethanol/Ammonia Control (Bio-Rad Laboratories, Irvine, CA). Quality control was acceptable each day, and no corrective action was taken for the ammonia assay at any time during the study. Results for the low control ranged from 29 to 42µmol/L (acceptable range: 16.0-46.0µmol/L) with a mean of 35.5µmol/L and standard deviation of 3.2µmol/L. Results for the high control ranged from 296 to 330µmol/L (acceptable range: 237-359 µmol/L) with a mean of 319.5µmol/L and standard deviation of 7.8µmol/L.

The ammonia result for the 5 minute "on-ice" aliquot was designated the reference value. The differences in ammonia concentration for each of the other aliquots were determined relative to the reference value for that subject. To determine statistical significance, the average difference in ammonia concentration between aliquots and their corresponding reference value were analyzed using a paired t-test (α=0.05).

RESULTS

A total of 44 patients consented to participate over a period of 37 days. One subject verbally consented to participate during blood collection, but failed to sign the required IRB-approved consent form, so the results were excluded from statistical analysis. Samples from three subjects experienced unacceptable delays in analysis due to analyzer process errors and were also excluded from analysis. The reference values for another two subjects were 76 and 60 µmol/L, respectively. All other results for those subjects were < 30 µmol/L, suggesting a random error in the analysis of those two specimens after 5 minutes "on ice". Results from these two subjects were also excluded from statistical analysis.

Figure 1 shows the distribution of changes in plasma

ammonia concentration that occurred while specimens were kept either on ice or at room temperature. Differences in plasma ammonia concentration between 15 minutes "on-ice" and the reference values ranged from -14μmol/L to +10μmol/L with an average difference of -1.13μmol/L (SD=5.30μmol/L). Differences in ammonia concentration between 30 minutes "on-ice" and the reference values ranged from -26μmol/L to +24μmol/L averaging +0.97μmol/L (SD=9.05μmol/L). In the 38 samples maintained on ice, the ammonia concentration increased in 13 samples after 15 minutes, and in 22 samples after 30 minutes. In 30 of the 38 samples, the plasma ammonia concentration changed by no more than ±10 μmol/L after 30 minutes. Only two samples displayed a consistent increase in ammonia concentration from 5 to 30 minutes "on ice", while 6 samples displayed a consistent decrease.

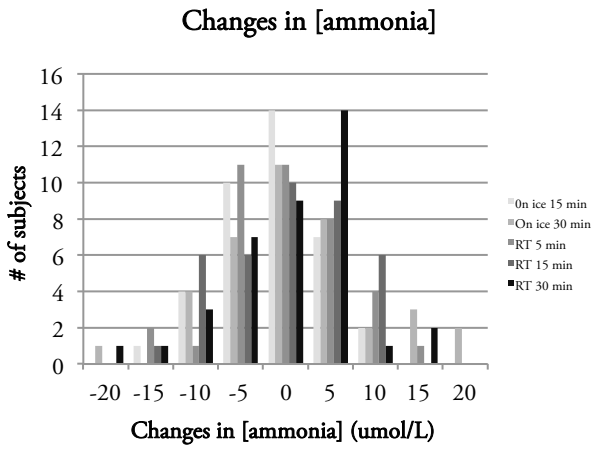

Figure 1. Changes in plasma ammonia concentration. This figure depicts the distribution of changes in plasma ammonia concentration (μmol/L), compared to the "reference value", in specimens on ice and specimens held at room temperature.

The differences between plasma ammonia concentrations observed after 5 minutes at room temperature and the reference values ranged from -15μmol/L to +15μmol/L with an average difference of -0.05μmol/L (SD=6.55μmol/L). The differences between plasma ammonia concentrations observed after 15 minutes at room temperature and the reference values ranged from -17μmol/L to +12μmol/L with an average difference of -0.58μmol/L (SD=6.94). The differences between plasma ammonia concentrations observed after 30 minutes at room temperature and the reference values ranged from -22μmol/L to +17μmol/L averaging +0.08μmol/L (SD=7.35μmol/L). In the 38 samples maintained at room temperature, ammonia levels were higher than the reference values in 21 samples after 5 minutes, in 17 samples after 15 minutes, and in 20 samples after 30 minutes. In 29 of the 38 samples, the plasma ammonia concentration changed by no more than ±10 μmol/L after 30 minutes. Seven samples demonstrated a consistent increase in ammonia concentration from 5 to 30 minutes at room temperature, while 6 samples demonstrated a consistent decrease. A statistical analysis of these results is summarized in Table 1.

Table 1. Changes in plasma ammonia concentration in 38 specimens, relative to the results obtained after 5 minutes on ice (μmol/L).

	"On Ice"		Room Temperature		
	15 min.	**30 min.**	**5 min.**	**15 min.**	**30 min.**
Mean	-1.13	0.97	-0.05	-0.58	0.08
S.D.	5.3	9.05	6.55	6.94	7.35
p-value	0.196	0.512	0.961	0.61	0.948

Curiously, 14 out of 38 specimens yielded reference values that were between 34-45 μmol/L, slightly above the laboratory's stated reference range for plasma ammonia (<32 μmol/L). Half of these specimens subsequently yielded at least one result that was <32 μmol/L. Out of the 24 specimens with "normal" reference values, 10 subsequently yielded one or more results that were slightly elevated (33-46 μmol/L). Using the reference value for each subject to initially classify them as normal or abnormal with respect to plasma ammonia concentration, Table 2 shows the proportion of the subjects who would have been misclassified by their results obtained after holding the whole blood on ice or at room temperature for up to 30 minutes.

Table 2. Misclassification of subjects due to changes in plasma ammonia concentration. The proportion of results that would have led to a different interpretation of the subjects' status, compared to the reference values.

	"On Ice"		Room Temperature		
Reference Values	**15 min.**	**30 min.**	**5 min.**	**15 min.**	**30 min.**
Normal	1/24	5/24	5/24	3/24	2/24
Abnormal	3/14	2/14	3/14	2/13	4/14

Collectively, our findings suggest that in our sample of

subjects, and with the method used to measure plasma ammonia concentration, imprecision obscured any in vitro generation of ammonia in the whole blood specimens, either on ice or at room temperature.

DISCUSSION

Current blood specimen handling policies and procedures for plasma ammonia determination are based upon studies that demonstrated in vitro increases in ammonia concentrations. These studies were conducted with enzymatic methods, which are the basis for most methods used today. The rates of increase estimated in these studies ranged from only 2-4 µmol/L per hour at 0-4°C, and 5-6 µmol/L per hour at 20-22°C, although confidence limits on these rates of increase were not given.[4,6] In our study, we observed no statistically significant difference in plasma ammonia concentrations after heparinized whole blood was held for 5, 15, or 30 minutes either "on ice" or at room temperature. These findings were not unexpected, after extrapolating the published hourly rates of increase in ammonia concentration back to the time points that we examined, and given the published analytical standard deviations for current methods for measuring ammonia.[4,6,11] In a recent retrospective study, Hashim and Cuthbert examined the plasma ammonia results for over 1,800 patients, where samples were collected in EDTA and transported on ice. They found no significant relationship between ammonia levels and pre-analytical time up to 30 minutes, and concluded that ammonia results are reliable if the total time that elapses from collection to result is <120 minutes.[11]

There were two findings in our study that were unexpected. One was the variability in the differences in measured ammonia concentration between time points, illustrated in Figure 1. The standard deviation of the differences in ammonia concentration within subjects, between time points, ranged from 5.3 – 9.0 µmol/L. This is considerably larger than the analytical standard deviation for the ammonia method used in our study, which was 3.2 µmol/L at a mean of 35.5 µmol/L. This suggests that there were specimen-specific effects that contributed to the variability in ammonia results between time points. One possible source of this variability is that the only centrifuge available in the lab for our whole blood specimens had a fixed relative centrifugal force of 1220 x *g* for three minutes. This may have led to incomplete and inconsistent removal of platelets between aliquots of the same specimen. Cowley et.al. reported a 62% higher mean plasma ammonia concentration in plasmas that had a mean platelet count = 100 x 10^9/L, than in plasmas with a mean platelet count = 3.5 x 10^9/L, even though the samples were analyzed immediately after preparation.[9] Another possibility is specimen-specific interactions with heparin and the ammonia reagent system. Dowart and Saner observed unstable initial absorbance readings with 5-10% of heparinized plasma samples that were not observed with samples collected in EDTA.[12] This phenomenon has since been shown to be related to the specific buffer used in the ammonia reagents; however, differences of >50% between the same specimens collected in heparin and EDTA have been reported in 45% of samples measured with the Dade Dimension RxL® analyzer.[13,14] The buffer utilized in the Siemens Vista® ammonia reagent is proprietary, but the Dade Dimension RxL® and the Siemens Vista® analyzers share many of the same reagent systems.

The second unexpected finding was that 37% of the specimens yielded slightly elevated reference values for plasma ammonia concentration, despite being kept on ice, processed within 5 minutes of collection, and analyzed immediately. Access to the subjects' medical records was not provided for this study, so it cannot be determined if there were any dietary factors, gastrointestinal conditions, or therapeutic drugs that might explain these findings. In a retrospective study, Maranda et.al. found that 31% of pediatric patients with an ammonia measurement had elevated values. After examining follow-up test results for these patients, they found that 48% had subsequent values within the reference range. In over 47% of these cases, there was no plausible explanation for the initially elevated result. The authors did not, however, examine the effect that the time or temperature for specimen handling and processing may have had on their findings.[15]

CONCLUSION

Regardless of whether whole blood is held on ice or at room temperature, delays of up to 30 minutes prior to centrifugation have no significant effect on plasma ammonia results. Other pre-analytical sources of variance such as the anticoagulant utilized and the centrifugation step, as well as analytical sources of variance such as reagent composition must be identified and controlled to optimize the reliability of those

results.

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