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Stabilizing specimens for routine ammonia testing in the clinical laboratory

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ABSTRACT

Background: *In vitro* deamination generates ammonia in freshly collected blood specimens. To prevent this, samples for ammonia testing are usually collected on ice and run rapidly (*e.g.*, within 1 h). We developed a method to stabilize specimens for ammonia analysis.

Methods: Following plasma separation, 500 μmol/l cycloserine or a combination of 2 mmol/l sodium borate with 5 mmol/l L-serine were added to sample pools with normal or increased concentrations of ALT and/or GGT to inhibit deamination; and/or residual platelets were removed *via* centrifugation. Sample pools were then incubated at room temperature or 4 °C. Untreated sample pools were also incubated at − 80 °C. Ammonia was measured at 0, 1, 2, 4, 8, 16, and 24 h.

Results: When incubated at 4 °C without treatment, sample pools with enzymes within their reference limit had an increase of 0.5 μmol/l/h, whereas sample pools with ALT and/or GGT activity above their upper reference limit had an increase of 3.6 μmol/l/h ($p < 0.001$). When sample pools were incubated at 4 °C with sodium borate/L-serine, the rate of ammonia increase was significantly reduced in samples with normal (0.3 μmol/l/h, $p < 0.001$ *vs.* untreated controls) or high enzyme activity (0.1 μmol/l/h, $p < 0.001$ *vs.* untreated controls). Independent of the ALT and/or GGT concentrations, storing the sample at − 80 °C also preserved the specimens for ammonia analysis (0.2 μmol/l/h, $p < 0.001$ *vs.* untreated controls).

Conclusions: By combining sodium borate/L-serine with refrigeration, plasma ammonia specimens can be stabilized for > 12 h.

1. Introduction

Ammonia is formed as a bi-product of protein catabolism or through renal acid/base balance [1]. Blood ammonia concentrations rise when the liver can no longer convert ammonia into urea (*e.g.* in cirrhosis, severe hepatitis or liver failure), or when ammonia production overwhelms hepatic metabolic capacity (*e.g.*, seizures, urinary tract infections, gastrointestinal bacterial overgrowth, and treatment with valproic acid or chemotherapy) [2]. Blood ammonia concentrations are increased in inherited defects in the urea cycle, organic acidurias, disorders of fatty acid oxidation, neonatal illnesses such as Reye's syndrome, and in transient hyperammonemia of the newborn. Ammonia easily crosses the blood-brain barrier and combines with H^+ to form the ammonium cation, which then competes with potassium for transport across neuronal plasma membranes [3,4]. As such, hyperammonemia (≥ 47 μmol/l) is a serious medical condition that can cause encephalopathy.

In addition to these pathological causes of hyperammonemia, the ammonia concentration in blood samples increases over time *in vitro*, after samples are collected. The measurement of ammonia concentration in blood samples is affected by numerous pre-analytical factors including exposure of plasma to red cells and platelets, storage temperature, and plasma aminotransferase activity. As early as the 1990s it was observed that the effect of red cell exposure on plasma ammonia concentrations could be minimized by storing and transporting the sample at 4 °C [5]. However, both storage at 4 °C and even separation of the plasma from the red cells was unable to preserve ammonia specimens > 1 h without experiencing clinically significant elevations in results. In order to avoid falsely-high results, to this day, it has been widely recommended that blood specimens for ammonia testing should be placed promptly on ice after collection and analyzed with an hour [6,7]. Increases over time in ammonia concentrations in both whole blood as well as separated plasma specimens stored at 4 °C is a process now attributed to activity of the enzymes alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) [5,7–9], as well as to platelet contamination [5,7,10–13].

ALT transfers the amino group from alanine to α-ketoglutarate, which creates pyruvate and glutamate, from which ammonia is

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liberated by glutamate dehydrogenase (GLDH). GGT also produces glutamate, leading to ammonia generation *via* GLDH; additionally, GGT may directly deamidate glutamine to produce ammonia [8]. The increase in ammonia associated with platelet contamination is attributed to glutamine metabolism- in particular, a phosphate-dependent glutaminase activity in platelets [14]. In addition, platelets are rich in the unique GGT isoenzyme, GGT-4 [15]. Paradoxically, it has been suggested that volatilization of ammonia gas can lead to a decrease in concentration over time in specimens exposed to air [16]. These requirements have restricted the availability of ammonia testing to large urban centers where specimens are drawn and tested on site.

While the current recommendation to collect ammonia samples on ice and analyze within an hour has led to improved specimen quality in recent years, it remains challenging for laboratories to achieve 1 h from collection to analysis. Additionally, this preanalytical requirement has limited ammonia testing to only centers which could offer the testing on site. In this study, we have sought to significantly improve upon collection practices for ammonia by developing a simple method for preserving specimens for ammonia analysis for many hours after collection, which would improve the lab's ability to offer the testing when sample transportation is required and potentially remove the restriction of only offering the testing on site.

2. Materials and methods

2.1. Reagents and samples

Ammonia was measured using the Randox Enzymatic Manual UV Ammonia reagent (ESBE Scientific) on an open channel of a Roche Cobas 8000 c702 using calibrators from Roche Diagnostics (Laval, QC) [17]. Enzyme activities for ALT and GGT were measured using Roche assays. Platelet counts were assessed using a Sysmex XN 9000 Hematology Analyzer. The enzyme inhibitors cycloserine, sodium borate, and L-serine were purchased from Sigma Aldrich and stock solutions were made using laboratory grade water.

K_2-EDTA whole blood specimens were used for this study [18,19]. Specimens stored for up to 6 h at 4 °C in a tertiary care hospital were selected for ammonia testing based on whether a paired lithium heparin plasma sample had normal or increased plasma activity of the enzymes ALT and/or GGT, and was free from interfering concentrations of hemolysis, icterus, or lipemia [17]. Whole blood samples were centrifuged at 4 °C for 10 min at 1200 × *g* to isolate plasma, which was then combined to make pools with normal ALT and/or GGT activity ($N = 68$) or high ALT and/or GGT activity ($N = 67$). Enzyme activity, ammonia concentration and platelet count for all pools are listed in Table 1. Use of these samples for the purposes of method evaluation is classified as a quality assurance/program evaluation activity to be used for assessment, management and/or improvement and is granted quality assurance exemption from ethics review requirement by the Conjoint Health Research Ethics Board at the University of Calgary.

Table 1
Chemical and hematological laboratory findings for the sample pools with normal or high ALT and/or GGT activities used in this study.

Test, unit	Mean	Median	Min	Max.
Normal enzyme activity				
ALT (U/l)[a]	19.3	17.3	10.2	40.0
GGT (U/l)[b]	20.0	19.6	9.4	33.7
Initial ammonia concentration (μmol/l)[c]	51.6	52.4	31.0	71.7
Residual platelet count (10^9/l)[d]	50.2	49.0	5.0	136.0
High enzyme activity				
ALT (U/l)[a]	284.3	203.9	44.4	1728.2
GGT (U/l)[b]	481.4	429.8	124.4	1442.7
Initial ammonia concentration (μmol/l)	104.3	105.2	60.5	217.7
Residual platelet count (10^9/l)	46.5	43.0	10.0	138.0

[a] Laboratory reference interval: 1–60 and 1–40 U/l for males or females, respectively.
[b] Laboratory reference interval: 11–63 and 8–35 U/l for males or females, respectively.
[c] Laboratory reference interval: 12–47 μmol/l.
[d] Laboratory reference interval: 150–400 × 10^9/l.

2.2. Sample preservation conditions

Plasma pools were aliquoted and analyzed under varying conditions (Fig. 1). For each trial, pools were either (1) incubated at 4 °C or − 80 °C; (2) re-centrifuged for 15 min at 1500 × *g* to remove platelets remaining in the separated plasma followed by storage at room temperature or 4 °C; (3) treated with enzyme inhibitors [500 μmol/l cycloserine (ALT) [20] or a mixture of 2 mmol/l sodium borate with 5 mmol/l L-serine (GGT) [8]] followed by storage at room temperature or 4 °C; or (4) were incubated at 4 °C after the removal of residual platelets and the addition of enzyme inhibitors. For each trial of a given preservation treatment condition, a room temperature control was run in parallel. The plasma pools that underwent a second centrifuge step to remove residual platelets had a final platelet cell count with a mean = 6.0 × 10^9/l and range = 1–22 × 10^9/l. Sample pools incubated at − 80 °C were thawed precisely for 30 min prior to ammonia measurement. To assess the effect of each preservation condition, ammonia was measured after 0, 1, 2, 4, 8, 16, and 24 h.

2.3. Data analysis

In each trial, the rate of *in vitro* ammonia increase (μmol/l/h) was calculated as the slope of the least squares fit of data over the 24 h study period. For each treatment condition, summary data of the trials were expressed as the mean slope ± 95% confidence interval ($N = 7$–68). Statistical significance between treatment groups was determined using an unpaired, two-tailed Student's *t*-test. The level of significance for differences in the mean rates of *in vitro* ammonia increase was set as 0.05. Calculated changes in ammonia concentration over time were compared with the Royal College of Pathologists of Australasia performance requirements for this assay (± 5 μmol/l ≤ 50 μmol/l; ± 10% > 50 μmol/l). From these values, we consider treatments that produce an *in vitro* rate of ammonia increase of < 1 μmol/l/h to have preserved the specimen. Using this benchmark, a transit time of several h is possible with no analytically significant change in the measured ammonia concentration.

The Pearson's correlation coefficient (r) was used to determine the relationship between ammonia increase and initial sample conditions (residual platelet count, ammonia concentration, and ALT or GGT enzyme activity). Statistical significance was assessed using the *F*-test and the concentration of significance was set as 0.05. All data were analyzed in Microsoft Excel 2010 (Microsoft) or Graphpad Prism 6.0 (GraphPad Software Inc.).

3. Results

3.1. Effect of sample type and temperature

Irrespective of sample pool type, storing the samples at room temperature significantly increased ammonia concentrations (Table 2; Fig. 2A, D). Over 24 h, the mean rate of ammonia increase in samples with normal activity of ALT and/or GGT was 2.6 (2.3–2.9) μmol/l/h. In samples with increased activities of ALT and/or GGT this rate more than doubled to 6.5 (6.0–7.0) μmol/l/h ($p < 0.001$).

Storing sample pools with normal activities of ALT and/or GGT at 4 °C stabilized the rate of *in vitro* ammonia increase to < 1.0 μmol/l/h [mean rate 0.5 (0.4–0.6) μmol/l/h], but was not as effective for samples with high activities of these enzymes (mean rate of 3.6 (2.8–4.4) μmol/l/h, Table 2; Fig. 2B, D). Although storing the samples with high activities of ALT and/or GGT at 4 °C decreased the rate of *in vitro* ammonia increase by almost half ($p < 0.001$), the observed rate of increase exceeded the target rate of 1 μmol/l/h. At this rate, samples were