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Simple and Inexpensive Quantification of Ammonia in Whole Blood

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Abstract

Quantification of ammonia in whole blood has applications in the diagnosis and management of many hepatic diseases, including cirrhosis and rare urea cycle disorders, amounting to more than 5 million patients in the United States. Current techniques for ammonia measurement suffer from limited range, poor resolution, false positives or large, complex sensor set-ups. Here we demonstrate a technique utilizing inexpensive reagents and simple methods for quantifying ammonia in 100 μ l of whole blood. The sensor comprises a modified form of the indophenol reaction, which resists sources of destructive interference in blood, in conjunction with a cation-exchange membrane. The presented sensing scheme is selective against other amine containing molecules such as amino acids and has a shelf life of at least 50 days. Additionally, the resulting system has high sensitivity and allows for the accurate reliable quantification of ammonia in whole human blood samples at a minimum range of 25 to 500 μ M, which is clinically for rare hyperammonemic disorders and liver disease. Furthermore, concentrations of 50 and 100 μ M ammonia could be reliably discerned with $p=0.0001$.

Introduction

Hyperammonemia, a life-threatening condition, is characterized by elevated blood ammonia levels and causes severe neurodevelopmental and neurodegenerative complications. The condition originates from a variety of hepatic diseases. This includes metabolic disturbances in the urea cycle that are caused by several inborn errors of metabolism collectively referred

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Supporting Information. A comparison of the selectivity of phenol and 2-phenylphenol towards as well as the standard curve of the indophenol reaction utilizing low hypochlorite concentrations can be found in the supporting information.

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to as urea cycle disorders, affecting approximately 1 in 35,000 births in the United States,^{1,2} as well as chronic hepatic diseases such as hepatic encephalopathy, carcinomas, cirrhosis and hepatitis. These diseases affect a large number of people, with 5 million in the U.S. alone having cirrhosis.

Current methods for blood ammonia detection lead to prolonged treatment of hyperammonemia due to the requirement of specific sample preparation and access to tandem mass spectroscopy in large central laboratories. Samples must be drawn, placed immediately on ice, separated into plasma, and frozen, as ammonia concentrations will increase in standing whole blood samples. Blood ammonia levels can also increase during the collection process if a tourniquet is used, making it difficult to accurately test for ammonia levels without previous training.^{3,4} Point-of-care (PoC) detection and monitoring of blood ammonia rapidly and in under-equipped environments with limited sample preparation and training would improve the prognosis and disease management for these patients.

A majority of previously reported PoC techniques must first separate the ammonia from blood before analytically determining the concentration. A common approach is to take advantage of ammonia's volatility in alkaline conditions. In solutions with a pH higher than 10, ammonia primarily exists in its gaseous form NH_3 instead of NH_4^+ . The alkalization of ammonia solutions gave way to distillation as a separation mechanism. These techniques have since been consolidated and miniaturized using microdiffusion.^{5,6} An ammonia containing sample is taken up into a reservoir containing an alkaline tablet. The ammonia volatilizes from the solution and passes through a polymer membrane into another reservoir containing a second solution where analysis can be performed. These methods generally suffer from false positives caused by hydrolysis of proteins and amino acids such as glutamine in alkaline conditions which produces ammonia.³ Once separated from blood by alkaline-based distillation, the ammonia must be measured using a quantitative, analytical technique. Titration, a non-specific approach, is frequently investigated for this purpose. The separated alkaline ammonia is added to an acidic solution and the resulting pH change is monitored by a colorimetric indicator such as bromocresol green or by the use of an electrode. The commercial product Blood Ammonia Checker II by Arkray utilizes this technology.⁷ Gas sensing electrodes are also used post distillation, in which ammonia gas is quantified through impedance measurements. These methods are hindered by interference from changes in temperature or humidity.⁸ Such drawbacks are compounded by the aforementioned issues associated with utilizing distillation as the separation mechanism. Other analytic techniques have focused on using the ammonia gas present in a patient's breath, measured by a polyaniline electrode, but these techniques have poor resolution and require fairly complex, large sensor setups.^{9,10}

Enzymatic reactions have also been investigated to measure ammonia, offering specificity as a means to avoid the challenges present in pH or distillation based approaches. Most enzymatic methods described in literature utilize glutamate dehydrogenase, which reductively aminates α -ketoglutarate in the presence of ammonia and the reduced form of nicotinamide adenine dinucleotide (NADH).¹¹ The system can be probed either optically or electrochemically for the decreasing concentration of NADH. This enzymatic technique is

very sensitive; however, measuring a loss in signal can be difficult, especially in consideration of the poor stability of NADH.¹²

A variety of colorimetric reactions exist that respond to ammonia including ninhydrin, Nessler's reagent, and the indophenol reaction. Ninhydrin is a very sensitive chemical, which turns a brilliant purple color in the presence of primary and secondary amines. However, it will react with any and all primary and secondary amines, such as amino acids, making its use in a specific and selective ammonia sensor ineffective.¹³ Nessler's reagent (potassium tetraiodomercurate(II)), is fairly selective and can be found in commercially available ammonia test kits for aquariums and waste water. This reaction generates different products depending on the concentration of ammonia present. The primary product is an opaque red-brown precipitate, which can pose processing issues.¹⁴

The indophenol reaction consists of ammonia, hypochlorite and phenol, which produce a deep blue, water-soluble compound.¹⁵ The largest challenge with this reaction is negative interference from proteins and reducing agents found in whole blood. There have been previous efforts to overcome this challenge and utilize the indophenol reaction to measure blood ammonia. Some approaches utilized cation exchange resins to extract the ammonia from blood, which was then quantified using the indophenol reaction. The reported process was very complex and required multiple washing and extractions steps.^{16–18} Another interesting approach used a variation of the indophenol reaction in a photonic crystal hydrogel matrix.¹⁹ However, it has a long response time and was not demonstrated with human whole blood samples. Due to its inherent sensitivity and selectivity, the indophenol reaction was investigated as a potential means for sensitive blood ammonia detection.

In the following work the indophenol reaction was modified to contain unconventionally large concentrations of hypochlorite to greatly diminish negative interference from small blood-borne reducing agents. The modified reaction was then utilized in tandem with a polyelectrolyte cation-exchange membrane, Nafion. The long range, negatively charged pores provide a means to separate ammonia directly from whole blood without any sample preparation.²⁰ As seen in Scheme 1a, placing the blood in a Nafion bisected well prompts an ion-exchange between ammonia in the blood and sodium ions in the opposing side of the well. To produce Nafion bisected wells, 3D printed, modular, well-halves were fabricated, which allowed for the quick assembly of multiple wells containing different blood samples, bisected with a cation exchange membrane (Scheme 1b). After the ion-exchange has occurred, the extracted ammonia solution can be mixed with the indophenol reagents and the resulting color measured using a plate reader (Scheme 1c). This detection scheme can rapidly quantify ammonia in multiple whole blood samples, allowing for faster diagnosis and management of hyperammonemia.

Experimental Section

Stability Studies

Aqueous solutions of 0.25% hypochlorite, 500 mM sodium hydroxide and a solution of 59 mM 2-phenylphenol in ethanol were stored with limited exposure to light. At intervals of 3, 5, 7, 15, 21, 28, 35, 50, 75 and 100 days the reagents were utilized to develop a standard

curve using ammonium chloride concentrations ranging from 0–500 μM . To a 384 well-plate, 10 μL of 2-phenylphenol, 10 μL of NaOH, 5 μL of hypochlorite and 35 μL of ammonium chloride were mixed. Concentrations ranging from 7 μM to 3 mM of sodium nitroprusside can be used as the coupling agent to initiate the blue color generation. The resulting color was measured using a SpectraMax M5 plate reader at a wavelength of 635 nm after 10 minutes. Significant deviations from the original standard curve indicated the degradation of the stored reagents.

Response to Amino Acids

The selectivity of the indophenol reaction was determined towards ammonia against other primary amines such as amino acids. 1 mM solutions of each of the 21 proteinogenic amino acids were prepared in 1X PBS and tested using the previously described indophenol reaction. 10 minutes after the indophenol reagents and amino acid solution was mixed, absorbance at 635 nm was measured using a plate reader. The response was directly compared to the response measured from a 1 mM solution of ammonium chloride and expressed as a percentage of the ammonium response.

Sensor Design and Construction

A bisected well containing whole human blood in one section and a solution of sodium acetate in the other provide a means for cation-exchange of the whole blood to occur, yielding a high recovery of the ammonium. Modular well-halves were 3D printed from acrylonitrile-butadiene-styrene thermoplastic. The pieces snap together with the 1 cm^2 Nafion 111 membrane in the middle, forming a Nafion bisected well. This design was chosen to provide a uniform platform for all future experiments involving this sensing method. A silicone gasket, at a 1/64 inch thickness, was glued to the inner face of each well-half to ensure a water tight seal.

Extracting Ammonia

Sodium acetate was utilized to extract ammonia through ion-exchange. Concentrations of 0.1, 0.5 and 1 M sodium acetate were prepared using fresh Milli-Q water (18.5 $\text{M}\Omega$), to ensure no ammonia contaminants were present. Bisected wells were prepared, with 100 μL of 500 μM of ammonium chloride in one section and 45 μL of the sodium acetate solution in the other. Ion-exchange took place for 20 minutes before 35 μL of the now ammonia-enriched sodium acetate was tested.

Reducing Interference from Blood-Borne Small Molecules

Reducing agents in blood such as uric acid can negatively interfere with the indophenol reaction. Increasing concentrations of the hypochlorite were utilized in a modified version of the indophenol reaction to eliminate this negative interference. 500 μM solutions of ammonium chloride were prepared in PBS and in whole human blood. The ammonia was extracted from these samples using previously described ion-exchange protocol. The concentration of hypochlorite was varied between 0.25–2.5% to examine its effectiveness in reducing interference.

Sensor Response to Ammonia in Whole Blood

The 3D printed wells were constructed with 1 cm² pieces of Nafion membrane. Whole human blood was spiked using ammonia chloride to generate concentrations of ammonia of 25, 50, 75, 100, 150, 200, 250, 300, 400 and 500 µM. In one section of the well, 100 µL of the ammonia containing blood sample was added. In the opposing section 1 M sodium acetate was added. Ion-exchange of ammonia was allowed to occur for 20 minutes. To a 384 well plate, 35 µL of ammonia extracted sample, 10 µL of 2-phenylphenol, 10 µL of NaOH, 5 µL of 0.75 % hypochlorite and an appropriate amount of sodium nitroprusside were added. The absorbance of the resulting indophenol reaction was measured at 635 nm after 10 minutes using a microplate reader.

Results and Discussion

One major advantage of using the indophenol reaction for determining ammonia concentrations is that it does not require any biological components such as enzymes, which are high in cost and prone to stability issues. The shelf life of the solutions used for the indophenol reaction was evaluated over the course of 100 days. The response to the range of ammonia chloride concentrations was stable for up to 50 days. As seen in Figure 1, the response to 25, 150 and 500 µM ammonium chloride did not change significantly until day 75. This long-term shelf life is imperative in under-equipped environments.

The indophenol reaction also responds to other primary amine containing compounds. For whole blood applications this is problematic due to the presence of small amine containing molecules such as amino acids, which have the potential to cause positive interference when measuring blood ammonia. Phenol and 2-phenylphenol were examined for selectivity towards ammonia. It was determined that 2-phenylphenol displayed greater selectivity than phenol and was chosen for all future experiments (Supplementary Table S1). This is due to the steric hindrance introduced to the reaction by the phenyl group at the ortho- position of the phenol parent molecule. The selectivity of the reaction utilizing 2-phenylphenol was tested with a large array of the proteinogenic amino acids. A concentration of 1 mM was utilized for both ammonia and the amino acids to ensure selectivity even in cases where amino acid levels are elevated. The absorbance values recorded for the amino acids were normalized with respect to the response from ammonia. The radar graph in Figure 2 shows the response of each amino acid, the highest of which was threonine at just 5% of the ammonia response.

Another potential source of interference for the indophenol reaction is proteins. Small quantities of proteins can completely disable the reaction from proceeding. In order to rapidly separate ammonia from whole blood while excluding any proteins, Nafion, a cation exchange membrane, was utilized. Nafion is a fluorinated ionomer copolymer. When cast into films, usually from solution in a hot press, the sulfonated block aggregates into long-range pores of sulfones surrounded by a matrix of the fluoropolymer. The pores are highly negatively charged due to the sulfonic acid groups and are generally 1–4 nm in size.²⁰ These pores allow for the rapid diffusion of hydroxyl containing molecules and cations while inhibiting the diffusion of anions and macromolecules.

The ion-exchange of ammonia through the use of a Nafion membrane is the mechanism of recovery of the analyte. Sodium salt solutions of different concentrations were tested for their effectiveness in exchanging with the ammonia from a PBS solution. It was expected that higher concentrations of sodium salts would yield larger recoveries of ammonia. Bisected wells were prepared with Nafion membranes. A 500 μM solution of ammonium chloride in PBS was placed on the 'analyte' side of the bisected well and solutions of sodium acetate in the opposing bisection. As seen in Figure 3a, a concentration of 1 M gave the largest recovery in 20 minutes. This recovery was 40% from whole blood and 70% from a 1X PBS solution.

The mechanism at hand is displayed in Scheme 1. At the sample-membrane interface, ammonium will replace a sodium ion. Conversely, at the membrane-sodium salt solution interface, a sodium ion will replace an ammonium ion. This process results in an ion exchange of the sample with the concentrated sodium solution, extracting the ammonia from the blood.

The indophenol reaction is also sensitive to the presence of blood-borne reducing agents such as uric and ascorbic acid. These molecules, amongst others, can react with intermediates of the indophenol reaction as well as the hypochlorite.²¹ This effect was evident when initially testing serial dilutions of ammonia in whole blood (Supplementary Figure S2). The response plateaued at higher concentrations of ammonia due to side reactions with indophenol reagents. To circumvent this issue, larger concentrations of hypochlorite were introduced to the reaction when detecting 500 μM ammonia in both whole blood and PBS. As seen in Figure 3b, with a 0.25% hypochlorite solution, the response to ammonia in whole blood was less than 20% of the response to ammonia in PBS. Increasing the hypochlorite concentration resulted in larger responses to whole blood, with a 50% response as compared to PBS, at an optimum concentration of 0.75%. At larger concentrations the response to both whole blood and PBS began to degrade.

The modified indophenol reaction was examined in conjunction with the Nafion based separation technique for its effectiveness in distinguishing blood ammonia concentrations ranging from 25 to 500 μM , representing healthy to diseased levels. In this range, where high resolution measurements are critical for examining treatment effectiveness, the r^2 was 0.9757, seen in Figure 4.

The average relative standard deviation in this range was just 12.23% which falls within the FDA guidelines for validation of a bioanalytical method which require a relative standard deviation of 15% at $n=5$ samples. The lower limit of quantification, 25 μM , is at least 3σ above the mean background reading of 0.0448 ± 0.0012 absorbance, which is the IUPAC standard for determining LLoQ. Additionally, the sensor can reliably differentiate between 50 and 100 μM blood ammonia with a $p=0.0001$, which is highly relevant for disease management, as this is the range presented in treated patients.

Conclusion

The investigated system for evaluating blood ammonia levels demonstrated a high degree of correlation between blood ammonia and sensor response. In the range of 25–150 μM , the most clinically critical concentrations, the relative standard deviation was just 12.23%. The method could discern levels of 50 and 100 μM with a $p=0.0001$, indicating the system is extremely reliable in differentiating these concentrations. The sensor has a 30 minute response time, and the interference from other small molecules and proteins was greatly reduced. The components used are stable at room temperature for up to 50 days and inexpensive. This presented method could lead the way for PoC devices for whole blood ammonia detection. Ultimately, such a device would assist in the rapid diagnosis of urea cycle disorders, which are not included in newborn screening programs. Due to their exclusion from newborn screening, UCDs are often misdiagnosed and treated after severe hyperammonemic episodes. Additionally, measured blood ammonia levels can often result in false positives due to the difficulty in sample processing, which can involve several preparative steps and logistical hurdles. The future development of a point-of-care device would provide an attending physician with the option for rapid diagnosis, especially in the intensive care unit setting. This is particularly imperative as high ammonia levels can be indicative of many disorders outside of UCDs, such as organic acidemias, fatty acid oxidation disorders and other rare inborn errors of metabolism. At home testing is another option for such a point-of-care device in the management of UCDs. Dietary adjustments could be made more readily, however, due to parental expectations and use, this option should be examined carefully and approached strategically to ensure the patient's best interest is taken into account.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlight

- A simple and inexpensive method was developed to determine blood ammonia concentrations in just 30 minutes
- The test utilizes a cation exchange membrane to specifically extract ammonia from whole blood
- The extracted ammonia is then quantified using a colorimetric reaction
- The method could reliably differentiate concentrations of 50 and 100 micromolar ammonia in human blood samples

Scheme 1.

(a) The bisected well contains blood in one section and sodium acetate in the other, initiating a cation-exchange effect. In the blood, ammonium is exchanged for sodium and in the sodium acetate solution, sodium is exchanged for ammonium. (b) The 3D printed wells are modular and will snap together forming multiple, Nafion bisected wells. (c) The extracted ammonia solution is mixed with the reagents for the indophenol reaction producing a concentration dependent gradient of developed blue color in a 384 well plate. This color can be measured at a wavelength of 635 nm.

Figure 1.

The reagents for the indophenol reaction were stored at room temperature and used to generate an ammonia standard curve at regular intervals for 100 days. The response to 500 μ -M ammonia began to degrade at day 75. The reagents of the indophenol reaction are stable at room temperature for up to 50 days before its response to different concentrations of ammonia begins to deteriorate.

Figure 2.

1 mM concentrations of each of the 21 amino were tested using the indophenol reaction. The absorbance measured at 635 nm for each amino acid after the indophenol reaction was calculated as percentage of the response from indophenol reaction with 1 mM ammonia chloride. The radar graph displays the percent response as compared to 1 mM ammonia chloride. The highest response was threonine, which produced an absorbance value at just 5% of ammonia's response.

Figure 3.

(a) It was determined that a 1 M concentration of the sodium salt was necessary for high recovery of ammonia from the analyte bisection of the well. (b) Concentrations of 0.25–2.5% hypochlorite were utilized in the analysis of 500 µ-M ammonia in 1X PBS and whole blood. At concentrations higher than 0.75%, the response to both blood and PBS began to degrade. A concentration of 0.75% hypochlorite was optimal.

Figure 4.

The bisected well sensor was used to extract ammonia in whole human blood. The extracted ammonia solutions were tested with the 0.75% hypochlorite indophenol reaction and the absorbance measured at 635 nm. In the range of 0–500 μ-M the COD was 0.976 with n=5 samples.