Measurement of ammonia in blood

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The measurement of ammonia, now known to be a normal constituent of all body fluids, is fraught with problems. An elevated ammonia level in blood (100 µmol/L or higher) is an indicator of an abnormality in nitrogen homeostasis. The collection, handling, storage, and analysis of blood samples, their limitations, and potential sources of error are discussed. New techniques that permit continuous or real-time estimates of systemic ammonia levels over a broad range are also discussed. The aim should always be to minimize the release of ammonia from the collected sample before analysis. Recommendations are made on the collection and processing of blood samples, for it is by standardization and rigid adherence to these techniques that the reliability of the test results will be improved. (J Pediatr 2001; 138:S11-S20)

Ammonia is now considered a normal constituent of all body fluids, resulting from the metabolism of amino acids. However, doubts of the presence of free ammonia in biologic solutions persisted until approximately 1960. Before this time the various methods available for the determination of ammonia content in biologic fluids relied on the separation or release of ammonia from the fluid sample by volatilization after the addition of an alkaline solution. With these methods, increased levels of ammonia had been reported in the blood of patients with severe hepatic failure. Despite such reports, doubts about the presence of free ammonia in blood continued, mainly as a result of the concern that free ammonia measured by these methods resulted from its liberation from labile amides in blood during incubation in alkaline solutions. Definitive proof was provided by studies showing that the enzyme glutamine dehydrogenase specifically reacts with ammonia, α-ketoglutarate, and a coenzyme, reduced nicotinamide-adenine dinucleotide, to form glutamic acid.[1] This reaction has become the basis for the most commonly used blood ammonia assay in clinical chemistry. One of the attractive features of this assay is that ammonia is determined directly at physiological pH without previous treatment of the sample with either an acid or a base.[2,3]

Today, an elevated ammonia blood level is considered a strong indicator of an abnormality in nitrogen homeostasis, the most common related to liver dysfunction. In excess, ammonia is a potent toxin, principally of central nervous system function. In the venous blood of healthy adults and children, blood ammonia levels are approximately 30 µmol/L, and levels exceeding 1 mmol/L occur under conditions of acute hyperammonemia.[4,5] Over the past 100 years, numerous methods have been developed to measure ammonia levels in various body fluids including blood, plasma, erythrocytes, saliva, sweat, and urine.[6] This brief review considers a few of the most common methods currently used to measure ammonia in blood: alkalization-diffusion, enzymatic, ion exchange, and electrode. Sample collection, handling, storage, and some of the limitations and potential sources of errors associated with these methods are discussed. Finally, some promising novel techniques for the continuous monitoring of ammonia levels that may be used clinically in the near future are also described.

GLDH	Glutamine dehydrogenase
NADH	Reduced nicotinamide-adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
SIFT	Selected-ion flow tube

Preanalytical Method For Handling Blood Ammonia Determination Samples

Most methods recommend collecting from patients who have fasted for at least 6 hours with the use of a verified ammonia-free heparin as an anticoagulant. Heparin is the preferred anticoagulant, because it has been shown to reduce red blood cell ammonia produc-

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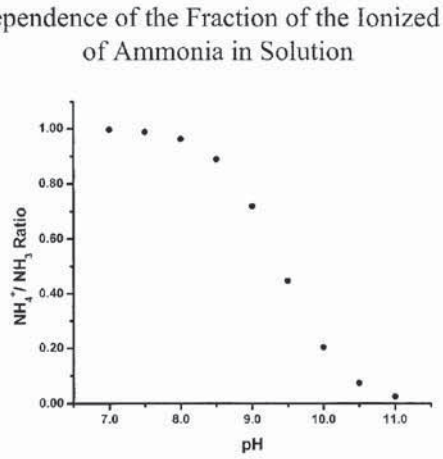

Fig 1. Ratio between ionized and free gas form of ammonia in plasma as function of pH.

tion. EDTA can also be used. Donors' arms should be as relaxed as possible, because muscle exertion may increase venous ammonia levels.[7] Blood is drawn into a chilled, heparinized vacuum tube that is immediately placed on ice, and plasma is separated within 15 minutes. It is crucial to keep blood samples cold after collection, because the ammonia concentration of standing blood and plasma increases spontaneously. Most of this increase has been attributed to the generation and release of ammonia from red blood cells and the deamination of amino acids, particularly glutamine.[8-11] Plasma ammonia levels of whole blood maintained at 4°C are stable for <1 hour. When promptly separated from blood, plasma ammonia levels are stable at 4°C for 4 hours and for 24 hours if stored frozen at –20°C. To put the problem of rising ammonia levels in perspective, the total nitrogen concentration in venous plasma of healthy adults exceeds 1 mol/L and represents a potential pool of free blood ammonia.[12] In normal healthy adults, homeostasis maintains free ammonia levels at approximately 30 μmol/L.

It is important to note that the criteria for sample stability and the methods for the measurement of ammonia levels in blood were established almost exclusively with blood specimens from healthy subjects.[8] Significantly more difficult but much more constructive would be the establishment of similar criteria for blood ammonia measurements from patients with metabolic or liver pathologic conditions. Standing blood or plasma samples from these patients contain numerous elevated sources of ammonia, resulting in increases in the rate and amount of ammonia formed. Some of these sources include elevated levels of circulating deaminase, γ-glutamyltransferase, an enzyme that may deaminate free amino acids, particularly glutamine in blood and plasma samples, resulting in overestimates of blood ammonia levels. Fast, careful handling and preparation of blood samples is required, especially from patients with metabolic or liver pathologic conditions, to minimize preanalysis increases in ammonia concentration until other assay techniques or methods are developed. Until this is accomplished, measurements in this patient population, in which blood ammonia levels require the closest monitoring, will continue to be the most unreliable. To date, the easiest and most cost-effective method is the stringent and diligent maintenance of blood samples on ice before, during, and after plasma separation.

ANALYTICAL METHODS FOR THE DETERMINATION OF AMMONIA

Many of the procedures for ammonia determination involve 2 general steps: the release of ammonia gas or capture of ammonium ions from the sample and the quantitation of the liberated gas or captured ions. Over the years the most common methods used to volatilize ammonia have been distillation and aeration/microdiffusion, ion-exchange chromatography, and blood or plasma deproteinization.

Properties of Ammonia

In attempting to understand the rationale used to measure ammonia, it is important to review some of the physical properties of the compound. Ammonia (NH_3) is a colorless, acrid-smelling gas at room temperature and pressure. It easily dissolves in water and ionizes to form NH_4^+ as follows:

$$NH_3 + H_2O \rightleftarrows NH_4^+ + OH^-$$

An increase in the pH or temperature of the solution increases the level of the ionized form. Fig 1 shows the ratio that exists in plasma between the ionized or NH_4^+ form versus the gaseous or NH_3 form as a function of pH. Thus in plasma at pH 7.4, the NH_4^+ form represents approximately 98% of the total ammonia. Many of the approaches used to estimate ammonia levels in body fluids involve volatilization of the NH_4^+ form of ammonia into its gaseous form, NH_3 ,by alkalization of the sample to a pH >10.

Distillation

One of the earliest techniques for the measurement of ammonia involves the addition of an alkaline buffer to a sample of blood followed by in vacuo distillation. The released ammonia gas is collected in a trap containing an aliquot of dilute acid, which converts

the gas into the nonvolatile ammonium ion. This approach is rather cumbersome and slow, particularly when many samples require analysis, although initially, speed was the primary advantage of this approach over the early microdiffusion techniques described in the following text. This advantage, however, was temporary, and was lost with the development of smaller microdiffusion vessels. The last reported use of distillation was by Burg and Mook[13] in 1963.

Aeration/Microdiffusion Techniques

Another early technique that is still in use relies on liberation of free ammonia by alkalization by the addition of a strong base to the specimen. The released ammonia diffuses through an air- or nitrogen-filled gap and is trapped in acid within the same apparatus. This approach, introduced by Conway and Berne[14] in 1933, uses a glass container resembling a Petri dish, within which a smaller second chamber is centered. The wall of this inner chamber is approximately half of that of the outside wall. An aliquot of a standard acid solution or ammonia indicator such as bromocresol green is placed into the inner chamber, and the sample is added to the outer chamber (Fig 2). A measured quantity of base is added to the sample, and the "petri" dish is sealed and gently rotated to mix the sample and base in the outer chamber and then left at room temperature for 90 minutes. This same principle is used in the Blood Ammonia Checker[15,16] and in the Kodak Ektachem dry-film method.[17] In these systems the diffusion distance for ammonia is significantly reduced from those in the original diffusion apparatus of Conway and Berne, requiring only 5 minutes to complete (Fig 3).

Distillation and microdiffusion both represent purification procedures that isolate ammonia from the many other constituents of blood and plasma, reducing the possible effects of other constituents and drugs on the ammonia assay. One main drawback of these procedures is that varying amounts of ammonia are liberated from the alkaline hydrolysis of proteins, especially hemoglobin, and labile amides, especially glutamine.[18-20]

Fig 2. Early microdiffusion apparatus for determination of blood ammonia. (Reproduced with permission from Conway E, Byrne A. An absorption apparatus for the micro-determination of ammonia. Biochem J. 1993;27:419-29. © the Biochemical Society.)

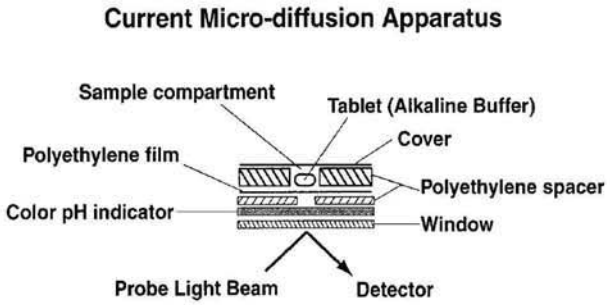

Fig 3. Illustration of microdiffusion technique used in Blood Ammonia Checker. (Reproduced with permission from Tada K, Okuda K, Watanabe K, et al. A new method for screening for hyperammonemia. Eur J Pediatr. 1979;130:105-10.)

Ion-Exchange Chromatography

In this approach ammonia gas is not liberated from the sample. Instead, a strongly acidic cation-exchange resin is used in batch mode to capture ammonium ions, NH_4^+. The resin is added to and subsequently separated from the sample by centrifugation, and the captured ammonium ions are then eluted from the resin by salt solutions or released as ammonia by the addition of dilute alkali,[21] a technique described in detail more recently by Brusilow.[4]

Deproteinization

Whole blood or plasma proteins are precipitated by the addition of trichloroacetic or perchloric acids, and the ammonia is determined directly in the supernatant fluid after alkalization.[22,23]

QUANTITATION OF AMMONIA

After the release or capture of ammonia or ammonium ions, several methods have been described to determine the amount of ammonia present. The general categories for these methods include titration, colorimetric/fluorimetric, electrode-based, and enzymatic.

Titration Method

The ammonia liberated from the sample is trapped in an aliquot of di-

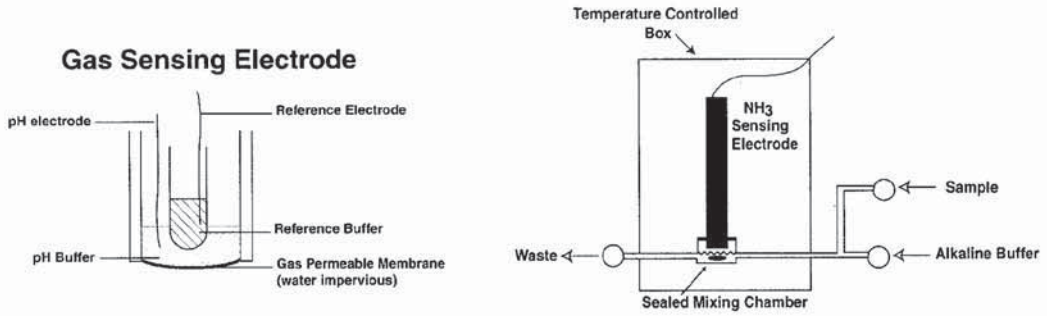

Fig 4. Schematic of ion-selective electrode (*left panel*) and suggested arrangement for continuous-flow analysis of ammonia in plasma samples (*right panel*).

lute acid, and the amount is measured by back-titration of the acid solution with a base while the pH is monitored with an indicator or electrode. The principal advantage of this approach is that it is inexpensive, requiring no specialized equipment. The disadvantages, however, are significant. They include insensitivity, the requirement for large blood samples, and contamination by other volatile bases that may affect the final value. Overall, this procedure is laborious and slow and therefore is not used routinely.

Colorimetric/Fluorimetric Reactions

This method is based on the reaction of ammonia with a reagent to form a colored complex that is measured by spectrometry or fluorometry. Among the first reactions used was the indophenol reaction, described by Berthelot in 1859[23a]: the formation of a bluish color by the reaction of ammonia with phenol and hypochlorite. This method is commonly referred to as the phenol reaction. Another colorimetric reaction is the Nessler reaction, in which a brown-orange color is formed by the reaction of ammonia with mercury or potassium iodide in an alkaline solution. Some other colorimetric reactions include the use of isocyanurate, cyanate, ninhydrin, or thymol hypobromite. Detection of ammonia and primary amines down to the nanogram range is routinely performed with fluorescence derivatization reagents such as fluorescamine and o-phthalaldehyde.[24-29] The principle advantages to this approach are speed, simplicity, specificity when used carefully, and excellent sensitivity. The disadvantage is that other substances in the blood affect some reactions, for example, the Berthelot reaction is inhibited by excess amino acids, creatine, glutamine, and some therapeutic agents.[30]

Gas-sensing Electrode

With the introduction of the gas-sensitive electrode (eg, Orion, Model 951201), a number of reports have appeared describing the methods required and the use of the electrode in measuring ammonia levels in samples of blood, urine, cerebrospinal fluid, and saliva over a broad range from 10 μmol/L to nearly 1 mmol/L.

When immersed in the sample or held closely above it, the dissolved gas of interest diffuses across a gas-permeable membrane into a small volume of buffer. The reaction changes the pH of the buffer, which is sensed by an internal pH electrode or sensing electrode. The change in pH results in a change in the potential between the sensing electrode and a reference electrode immersed in a separate reference buffer, all housed within the same electrode body. The arrangement is illustrated in Fig 4, in which the scale of some of the components is exaggerated for clarity.

The main advantages of electrode-based ammonia assay are its cheapness, ease of use, and because it never comes in contact with the sample, its imperviousness to sample color, turbidity, viscosity, or the presence of drugs or other metabolites in the sample. The electrode is best arranged above the surface of the sample in a sealed environment (Fig 4). This avoids the accumulation of proteins and cell fragments on the membrane surface that would normally occur during immersion into plasma or blood sample and minimizes the loss of ammonia from the sample away from the electrode during the measurement.[22,31,32] The disadvantages of the electrode-based system include the requirement of large sample volumes and slow sample reads, especially at low ammonia levels, requiring 10 to 15 minutes. In addition, major differences in either the osmolarity or temperature of the sample and the sensing electrode buffer must be avoided.

Enzymatic Method

The specificity of most methods for ammonia determination in biologic fluids relies on the physical separation of ammonia from interfering substances by volatilization after alkalization. In contrast, the most common method used in clinical laboratories is an enzymatic method that measures ammonia directly. Thus sample preparation is relatively simple, because the previous liberation of ammonia from the sample is not required. This assay is based on the reductive amination of 2-oxoglu-

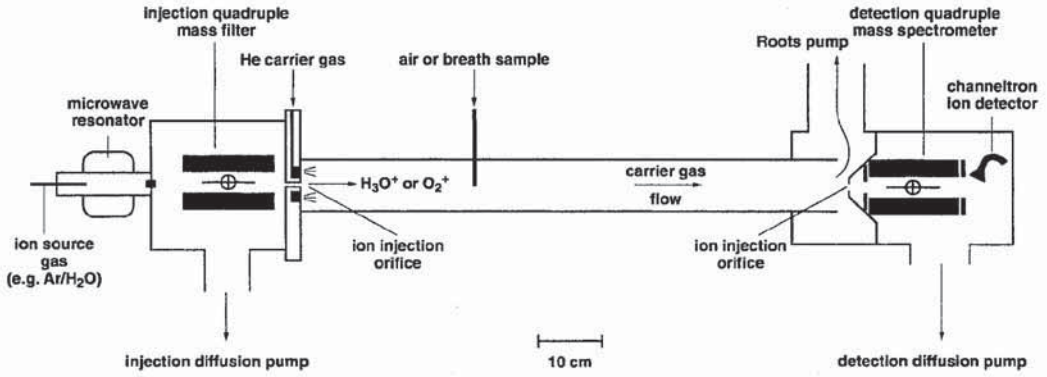

Fig 5. Simplified schematic of selected-ion flow tube (SIFT).

tarate with glutamate dehydrogenase and reduced nicotinamide adenine dinucleotide phosphate:

$$\text{2-Oxoglutarate} + NH_3 + \text{NADPH} \xrightleftharpoons{\text{GLDH}} \text{Glutamate} + \text{NADP}$$

The decrease in absorbance at 340 nm caused by the oxidation of NADPH is proportional to plasma ammonia. GLDH is specific for ammonia and does not react with methylated amines. Early studies describing the enzymatic determination of ammonia used NADH as a coenzyme. Because other NADH-consuming systems are present in blood, many of these reports overestimate the level of free ammonia, for example, Muting.[33] The effect of these systems can be minimized, usually by a 30-minute preincubation period. There are considerably fewer NADPH-consuming sources in plasma, so the preincubation time can be reduced to a few minutes.[3]

The assay can be used to measure ammonia levels over a broad range, from as low as 12 μmol/L to as high as 1 mmol/L. The disadvantage of this approach is the length and complexity of the procedure, thereby enhancing the potential for variation in reported blood ammonia levels. If not handled properly, ammonia concentrations rise in standing blood or plasma. Indeed, because standard procedures for preanalysis sample processing do not incorporate any attempts to inhibit the continued liberation of ammonia from the samples, except for lowering the temperature, ammonia levels in the sample will continue to increase during the assay and up to the time of the initial readings. Thus overestimates of ammonia levels are most likely in poorly handled samples containing elevated levels of transaminases and amino acids.

Normal Values for Blood Ammonia Levels

Table I lists selected blood ammonia levels for various blood sample types and assay methods from a number of studies. The average values for arterial blood, plasma, venous blood, and plasma are 18, 23, 28, and 32 μmol/L, respectively. The average value for venous blood and plasma is 30 μmol/L.

FUTURE DEVELOPMENTS

The major limitations of conventional in vitro blood ammonia measurements are the complexity involved in the proper drawing and handling of the sample, the time allowed between drawing and assaying, and finally, the assay itself. A consequence of these limitations is that blood ammonia measurements are performed only a few times each day. Alternative methods are still sought that are noninvasive or require a small catheter in a peripheral vein but provide a continuous monitor of blood ammonia levels. Such methods could alert medical staff to an impending hyperammonemic condition and would more easily permit earlier selection and regulation of therapeutic interventions. Two promising methods that are under development and may eventually be used clinically are the selected-ion flow tube technique, which analyzes trace gases in breath, and a fiber-optic catheter tipped with an ammonia-sensitive indicator.

Selected-ion Flow Tube

Selected-ion Flow Tube is a quantitative method for the rapid, real-time analysis of the trace gas content of atmospheric air. It was originally developed to study ionic reactions in the gas phase and is particularly valuable for providing kinetics data on ion-molecule reactions, contributing to the current understanding of the chemistry of some low-temperature gaseous plasmas, especially interstellar clouds. The same technology is currently being developed to analyze trace gases in breath. Previous methods for measurement of ammonia in breath have required large sampling flow rates[34] or long sampling times[35] and are therefore unsuitable for assessing the ammonia concentration from a single breath. A schematic of the SIFT apparatus is shown in Fig 5 taken from Smith and Spanel.[36] This technology is being further developed and may soon be a sensitive, quantitative method for the continuous real-time analysis of the trace-gas content of human breath and

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