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An Automated Microassay for Blood Ammonia

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Summary: A microassay for blood ammonia has been developed. After dilution, blood is dialyzed by a continuous flow procedure, and the ammonium ions in the dialysate are assayed colorimetrically by the hypochlorite-phenol *Berthelot* reaction. Concentrations from 1 to 400 μmol/l NH_4^+ can be measured in a 50 μl whole blood sample. Using slight modifications, concentrations up to 800 μmol/l NH_4^+ can be determined. The precision, accuracy, sensitivity, and specificity of the method have been studied. In man, the usual values (mean ± 2 SD) found are 27.4 ± 10.2 μmol/l NH_4^+ in arterial blood, 20.9 ± 9.8 μmol/l in venous blood. The usual values in rats and mice are very close to those given for man.

Blutammoniak-Bestimmung mit einer automatischen Mikromethode

Zusammenfassung: Zur Bestimmung des Blutammoniaks wurde eine Mikromethode entwickelt. Das Blut wird nach Verdünnung kontinuierlich fließend dialysiert. Die Ammoniumionen werden im Dialysat kolorimetrisch mittels der Hypochlorit-Phenol Reaktion von *Berthelot* bestimmt. Ein Gehalt von 1 bis 400 μmol/l NH_4^+ kann in 50 μl Vollblut bestimmt werden. Geringe methodologische Abänderungen erlauben es, Konzentrationen bis zu 800 μmol/l NH_4^+ zu messen. Präzision, Genauigkeit, Empfindlichkeit und Spezifizität der Methode werden untersucht. Beim Menschen sind mit dieser Methode die üblichen Befunde (Mittelwert ± 2 SD) 27,4 ± 10,2 μmol/l NH_4^+ im arteriellen Blut und 20,9 ± 9,8 μmol/l im venösen Blut zu erheben. Bei Ratten und Mäusen finden sich ähnliche Werte.

Introduction

After a critical study of the previously published methods (1), we developed in 1972 a flow dialysis technique (2) which enabled us to measure discontinuously or continuously ammonia levels in arterial and/or in venous blood samples of at least 0.23 ml. We now report a miniaturization of the technique, which is suitable for the screening of congenital hyperammonemia in the new born, the measurement of ammonia levels in the blood of small animals like rats and mice, and the assay of amino acids by determining the ammonia liberated after a specific ammonia forming enzymic reaction.

The microassay that we describe here, can be used to determine as little as 50 pmol of ammonium ions in a sample volume of 50 μl. As we developed the technique, we noticed considerable improvement in accuracy, relative to our previous technique. Ammoniagenesis is reduced due to the short dialysis period (one minute) as compared to other methods.

Material and Methods

Principle of the method

In a continuous flow apparatus, a 50 μl blood sample, or standard solution, is mixed with 50 times its volume of diluted sodium tetraborate, in order to lower the spontaneous blood ammoniagenesis. It is then dialyzed at equilibrium against the same borate solution at pH 8.9. The ammonium ions in the dialysate are assayed colorimetrically using the *Berthelot* hypochlorite-phenol reaction.

Reagents

All the reagents used were previously described (2, 3, 4). They are free of ammonium ions. The enzymatic UV assay for plasma ammonia was performed with the reagents ref. 125 857 from Boehringer, Mannheim.

Apparatus

The determinations are done with an Autoanalyser Technicon L. The diagram of the flow stream is shown in figure 1. Some measures are realized with a set up of two parallel flow streams, one for the micromethod and the other for our previous technique, using a sample rate of 230 μl/min. with a colorimetric expansion rate of 2, as detailed previously (2).

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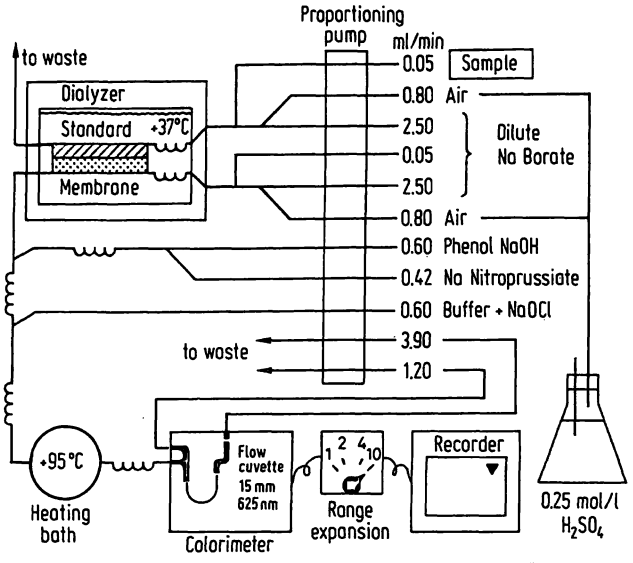

Fig. 1. Diagram of the NH_4^+ microdetermination. Nominal speed of the pump is 25 mm/s; sample to wash ratio is 1/2.

Assay procedure

A 50 µl sample is aspirated in exactly one minute by the capillary tube of the analyser, which is washed afterwards for two minutes with freshly bidistilled water. The sample is diluted 50 fold with 5 mmol/l sodium tetraborate and dialyzed for one minute at + 37 °C through a standard membrane against the same borate solution. The pH of the mixture stays at 8.9 during the dialysis. The components of the *Berthelot* reagent (2, 3) are then added in the following order: first the NaOH-phenol solution (80 mmol/l phenol, 1 mmol/l NaOH) mixed with a Na nitroprusside solution (170 mmol/l sodium nitroprusside, 332 mmol/l acetic acid) in a mixing coil, then the phosphate buffer containing the Na hypochlorite (200 mmol/l Na_2HPO_4, 35 mmol/l NaOCl, 150 mmol/l NaOH). The mixture flows for 3 minutes through a + 95 °C heating bath where the blue colour develops; it is read at 625 nm in a 15 mm flow cuvette. The colorimeter response is expanded 10 times (only 4 times if concentrations are higher than 400 µmol/l NH_4^+). The calibration curve is established each day with standard solutions. The result is obtained on the recorder, 7 minutes after sample introduction.

Materials used

In man, 1 ml of arterial or venous blood is drawn in a plastic syringe containing 2.5 mg lithium heparinate. Delay between the blood collection and the beginning of the assay does not exceed 5 minutes. Our reference values have been established in a hospital population of men and women, aged 26 to 75, free of any liver, kidney, heart or metabolic impairment.

In rats, blood is drawn under ether or intravenous or intraperitoneal sodium pentobarbital anesthesia, either by heart puncture with a microneedle of 0.4 mm outer diameter, or after carotidal and/or femoral vein catheterism. Our reference values have been established in male 200 to 350 g Wistar rats, one group having free access to food and the other being fasted for variable periods of time.

In mice, blood is drawn by heart puncture under ether anesthesia.

Results

Quality criteria of the micromethod

Detection limit

By diluting a 3 µmol/l $(NH_4)_2SO_4$ solution with 20 mmol/l H_2SO_4, the lowest concentration giving a detectable signal over the base line is 0.5 µmol/l. The calculated value of three standard deviation (3 SD) of 30 repeated assays with a 0.5 µmol/l NH_4^+ solution is 1.05 µmol/l.

Precision

Repeatability was checked on 32 samples (2 series) of 28 and 111 µmol/l NH_4^+ standard solutions; variation coefficients in the series are respectively 1.00% and 0.24%; variation coefficients between series are 2.04% and 0.58%, over a period of one month.

Reproducibility assayed by the double sample method, with two exactly parallel flow sets, during one month, and 75 pairs with a mean of 77.3 µmol/l, leads to a variation coefficient of 1.6%.

Accuracy

We compared the ammonium ion microassay method with the technique we described earlier (2), taken as a reference. The two analyses were made simultaneously using a double parallel flow set. Thus, the spontaneous ammoniagenesis of the extravasated blood preceeding the analysis was the same, even during dialysis. Analysing 218 pairs of results, we found that the micromethod leads to slightly higher values ($\bar{y}$ = 97 µmol/l) as compared to the reference method ($\bar{x}$ = 94 µmol/l). The high values are particularily better determined with the micromethod. Correlation between the results given by both techniques is very significant ($y = 1.004\ x + 2.658$; $n = 218$; $r = 0.963$).

Correlation between total blood ammonia (14–138 µmol/l NH_4^+) with our micromethod and plasma ammonia with the glutamate dehydrogenase UV method (6) is also significant: $y = 1.021\ x - 2.477$; $n = 30$; $r = 0.946$, the enzymatic method giving slightly higher values; correlation between plasma ammonia with our micromethod and with the enzymatic method is: $y = 0.956\ x - 0.940$; $n = 30$; $r = 0.940$.

In order to check the linearity of the micromethod, we overloaded an arterial blood sample and a pooled serum with increasing amounts of NH_4^+. The recovery of NH_4^+ was between 97 and 102%. These results are better than those hitherto observed with several other methods (1, 2).

The arterial blood and pooled serum were also diluted with 0.15 mol/l NaCl. Experimental values obtained after assays of the dilutions deviated from the theoretical values by less than ±3%.

Contamination study

By subsequently using NH_4^+ solutions of low (20 µmol/l) and of high concentrations (200 µmol/l), we found that the contamination of the low concentration solution by the high concentration solution was 0.7%, and the contamination of the high concentration solution by the low concentration solution was 0.4%.

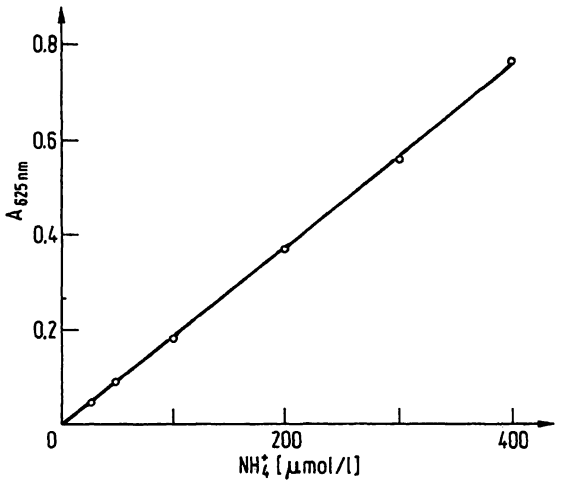

Fig. 2. Standard curve of the micromethod.

Sensitivity

The calibration curve is linear for ammonium ion concentrations from 1 to 400 µmol/l (fig. 2). For higher levels, the colour intensity can be measured by changing the expansion rate from 10 to 4. However, between 400 to 800 µmol NH_4^+/l, the calibration curve is no longer linear, therefore the unknown samples have to be accompanied by the appropriate standard solutions.

Specificity

An ammonium sulfate solution was assayed and then overloaded with 5 different aminoacids, which had previously been shown (2, 5) to disturb the blood ammonia determination, by interfering with the *Berthelot* colorimetric reaction. Ala, Phe, Cys, His, and Trp were added to the ammonium sulfate solution, to give concentrations that are 10 to 100 times greater than the maximal physiological blood concentrations for these aminoacids. The results in table 1 show that the influence of these aminoacids is negligible (less than ± 1.7% deviation) on the results of the ammonium assay using the micromethod.

Determination of reference values

In man

In the hospital population studied, arterial blood ammonia (femoral artery) was ($\bar{x}$ ± 2 SD) 27.4 ± 10.2 µmol/l NH_4^+, and venous blood ammonia (femoral vein) was significantly lower: 20.9 ± 9.8 µmol/l NH_4^+ ($p < 0.001$). These values are lower than those reported in plasma by an enzymatic method (6), which is highly specific for ammonia, but which favours slight ammoniagenesis during plasma separation.

In rats

Blood ammonia values measured in normal rats, reported in table 2, are very close to those observed in man. They are fairly similar whichever way the blood has been drawn or whichever anesthesia was employed. Nevertheless, fed rats have slightly but significantly higher ($p < 0.001$) blood ammonia concentrations than

Tab. 1. Influence of the addition of 5 aminoacids on the results of ammonia microassay.

Solutions assayed	NH_4^+ (µmol/l)
Ammonium sulfate solution T	55.6
Alanine 0.01 mol/l	8.8
Solution T overloaded with 0.01 mol/l alanine	
theoretical value	64.4
experimental value	65.0
Cysteine 0.01 mol/l	40.0
Solution T overloaded with 0.01 mol/l cysteine	
theoretical value	95.6
experimental value	97.0
Phenylalanine 0.01 mol/l	11.6
Solution T overloaded with 0.01 mol/l phenylalanine	
theoretical value	67.2
experimental value	67.4
Histidine 0.01 mol/l	6.6
Solution T overloaded with 0.01 mol/l histidine	
theoretical value	62.2
experimental value	61.4
Tryptophan 0.01 mol/l	7.3
Solution T overloaded with 0.01 mol/l tryptophan	
theoretical value	63.9
experimental value	65.0

Tab. 2. Blood ammonia in healthy rats (NH_4^+ in µmol/l: $\bar{x}$ ± 2 SD).

Anesthesia	Fasting time	Method of blood collection		
		Cardiac puncture	Carotid artery	Femoral vein
Ether	0 h	30.5 ± 13.3 (n = 76)	not determined	not determined
	6 h	23.3 ± 15 (n = 49)		
Pentobarbital intra-venous	6 h	not determined	31.6 ± 13.3 (n = 72)	18.3 ± 8.9 (n = 28)
Pentobarbital intraperitoneal	6 h	28.3 ± 10.5 (n = 14)	not determined	not determined
	24 h	31.7 ± 12.2 (n = 14)		
	48 h	27.2 ± 12.8 (n = 16)		
	96 h	28.3 ± 18.3 (n = 14)		

animals which had been fasted; but the time of fasting does not influence blood ammonia levels.

Otherwise, blood ammonia is significantly lower ($p < 0.001$) in the femoral vein than in the carotid artery, as is the case in man.

In mice

In 21 mice under slight anesthesia, heart puncture blood ammonia was 27.8 ± 16.1 μmol/l ($\bar{x} \pm 2$ SD). This value is of the same order as those found in man and in the rat.

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