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A KINETIC DETERMINATION OF AMMONIA IN PLASMA

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Summary

A simple and rapid method is described for the determination of ammonia in plasma without deproteinization, using the enzyme glutamate dehydrogenase and NADPH as coenzyme. ADP is used to stabilize glutamate dehydrogenase.

The measuring principle is a kinetic one, determining the reaction rate on the "Enzyrator". The whole determination takes only 7 min, including the preincubation time.

The plasma sample is only 100 μl, which makes it possible to determine the ammonia values in capillary blood. Comparing the values of venous and capillary blood, we found that capillary values are 2—3 times higher than those in venous blood.

The normal range for venous plasma NH_3 was 6.5—35.0 μmol/l. The mean value of non-fasting persons was 22.0 μmol/l.

Introduction

Many investigators [1—8] have shown that the enzymatic determination of ammonia is the most preferable because it is specific, simple and rapid. It is based on the following reaction:

$$\alpha\text{-ketoglutaric acid} + NH_4^+ + NAD(P)H \xrightleftharpoons{GLDH} NAD(P)^+ + \text{glutamic acid} + H_2O$$

A disadvantage of the use of NADH as coenzyme is the presence in plasma of other NADH consuming systems, which have to be eliminated by a 30 min preincubation. This is not the case with NADPH, so that the preincubation time can be reduced to a few minutes [9]. This not only diminishes the time necessary for one determination, but also reduces the risk of the formation of spurious plasma ammonia. The use of ADP has a favourable influence on the reaction: it stabilizes the GLDH and enhances its activity about 5 times.

This made it possible for us to use the reaction rate as a criterion for the

ammonia concentration, which is preferable to the determination of the total consumption of NAD(P)H, because of the unreliable absorbance readings at the beginning and end of the reaction [6,7].

Principle of the kinetic measuring method

In the presence of an excess of α-ketoglutaric acid and NAD(P)H the reaction will be of a pseudo-first order. This means that the reaction rate is only dependent on the concentration of ammonia and the activity of glutamate dehydrogenase (GLDH). The activity of GLDH originally present in plasma is small compared to the amount added, so the resulting reaction rate is only dependent on the concentration of ammonia.

Materials

Instrument

The reaction rate was measured with an enzyme reaction rate analyzer, developed in our laboratory. Originally the "Enzyrator" was meant for zero order reactions only, but we tried it succesfully for first order reactions as well. This instrument takes 12 absorbance readings every 20 s and from these the ΔA/min is calculated automatically. It is possible to multiply by a factor so that the readout may be International Units. As most reactions have a period of instability right after starting, a delay of one measuring cycle (20 s) is built in as a precaution.

For this determination we used a filter of 340 nm and the thermostat of the instrument was set at 25°.

Reagents

All glassware has to be carefully cleaned, as even small particles may disturb the reaction. Freshly distilled or demineralized NH_3-free water should be used.

(1) EDTA solution. 10% in H_2O.

(2) Phosphate buffer, 66.7 mM, pH 8.3. Dissolve 0.30 g KH_2PO_4 and 11.56 g $Na_2HPO_4 \cdot 2H_2O$ in 1000 ml H_2O. Adjust pH to 8.3 if necessary.

(3) Standard solutions. (a) Stock solution 10 mM NH_3: dissolve 66.08 mg $(NH_4)_2SO_4$ in 100 ml H_2O. (b) Dilute 10 ml stock solution to 100 ml with H_2O to obtain a 1 mM solution. (c) Working standards of 50, 100 and 200 μmol/l NH_3 are made by dilution from the 1 mM solution when needed.

(4) Working solution. Dissolve 2.25 mg NADPH (Boehringer, no. 15501) and 28.0 mg sodium α-ketoglutarate (Merck, no. 12294) in 25 ml phosphate buffer, 66.7 mM, pH 8.3. This solution will be stable for a few days, kept at 4°.

(5) Enzyme solution. Dissolve 4.0 mg ADP (Boehringer, no. 15691) and 300 μl GLDH (10 mg/ml, solution in 50% glycerin, Boehringer no. 15324) in 5.7 ml phosphate buffer, 66.7 mM, pH 8.3. This solution may need filtration before it can be used, because the GLDH solution may not be clear. It should be prepared freshly each day.

Blood sampling

1 ml blood is drawn either from the finger or by venipuncture into a small

tube containing 10 µl of a 10% EDTA solution. Mix well and cool the tube immediately in ice-cold water. All further manipulations should be done as fast as possible.

Centrifuge, decant the plasma in a Beckmann microtube and centrifuge once more for 1.5 min in the Beckmann Microfuge 152.

Procedure

Pipet into a microtube, also cuvet, 100 µl H_2O, standard or plasma. Add 425 µl working solution, mix and allow to stand for about 5 min in a thermostated waterbath at 25° to adjust temperature. Start the reaction by adding 75 µl enzyme solution, start a stopwatch at the same time, then mix well on a Vortex without causing any bubbles. Place the tube in the Enzyrator exactly 15 s after starting the reaction and 40 s later the first readout will follow, which can be printed at the same time. We followed 3 readouts to be able to check the course of the reaction.

Calculation

To calculate we used the second readout:

$$\frac{100}{R_{St\ 100} - R_{Blank}} \times (R_{Sample} - R_{Blank}) = x\ \mu mol\ NH_3/l$$

Experiments

Choice of the buffer and concentration of different substances

Originally we started with the method described by Ishahara et al. [6], but several changes were made. Ishahara et al. use a diaethanolamine buffer 0.05 M pH 8.4. The different reagents are added separately to the plasma sample. We simplified the method by dissolving NAD(P)H and sodium α-ketoglutarate in the buffer. The enzyme solution was dissolved in a different buffer, namely a phosphate buffer. We found that mixing the two buffers from working solution and enzyme solution caused an instability of the reaction during the first minute. For this reason we replaced the diaethanolamine buffer with a phosphate buffer 66.7 mM pH 8.3 and used this for both solutions.

Starting from the concentrations mentioned by Ishahara et al. [6] we changed the amounts of NAD(P)H and sodium α-ketoglutarate to find the optimal concentrations. We found that within the standard deviation of the method the reaction rate of the standard solutions 50, 100 and 200 µmol NH_3/l did not change if we kept the concentration of NAD(P)H between $4{,}8.10^{-5}$ and $12{,}0.10^{-5}$ M and of sodium α-ketoglutarate between $2.7 \cdot 10^{-3}$ and $8.25 \cdot 10^{-3}$ M. To get a suitable initial absorbance, considering the absorbance of plasma itself at 340 nm, we decided on final concentrations of $6.0 \cdot 10^{-5}$ M NAD(P)H and $4{,}2 \cdot 10^{-3}$ M sodium α-ketoglutarate.

The activity of GLDH is a determining factor for the reaction rate, as mentioned above, and therefore the concentration has to be exact. Our own experiments (confirmed by other investigators [5, 8, 9]) showed that ADP has a favourable influence on the reaction. Da Fonseca-Wollheim uses ADP to

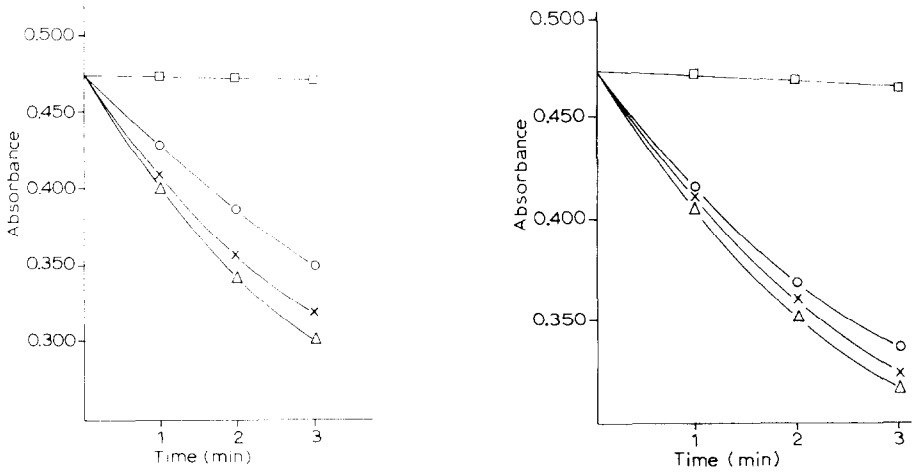

Fig. 1. Effect of the amount of GLDH on the course of the reaction, using standard 200 μmol NH_3/1. Reagent blank (□———□); respectively 100 μl (○———○), 150 μl (x———x) and 200 μl (△———△) GLDH with 2.0 mg ADP and phosphate buffer to 3 ml.

Fig. 2. Effect of the amount of ADP on the course of the reaction, using standard 200 μmol NH_3/1. Reagent blank (□———□); respectively 0.5 mg (○———○), 1.5 mg (x———x), 2.0 and 3.0 mg (△———△) ADP with 150 μl GLDH and phosphate buffer to 3 ml.

stabilize the GLDH, and we found that another advantage of the addition of ADP is that it enhances the GLDH activity in such a way that one-fifth of the amount otherwise needed is sufficient to give approximately the same reaction rate. A final concentration in the reaction mixture of 62.5 mg protein/l for GLDH and 0.18 μmol ADP/l gives a reasonable decrease in absorbance, whereas the velocity of the reaction is not too high (see Fig. 1).

The amount of ADP added to the enzyme solution has to be at least 2.0 mg/150 μl undiluted GLDH and should not be more than 3.0 mg/150 μl, because it will not influence the activity any more and some charges of ADP contain a rather large amount of ammonia, which will make the reagent blank too high (see Fig. 2).

pH of the phosphate buffer, 66.7 mM

To find the kinetically most suitable pH for this system, buffer solutions were made with pH ranging from 7.1 to 8.3. The reaction, using standard 100 μmol/NH_3/1, was followed on the Beckman DB: the reaction rate increased with increasing pH up to pH 8.3. Though the optimal pH lies at 8.6 [9], we had to decide on pH 8.3 because this is the limit for this buffer system.

The use of NADPH instead of NADH

The determination of plasma samples with NADH as coenzyme needs a preincubation of at least 30 min to eliminate other NADH-consuming systems present in native plasma. By using NADPH this incubation time can be reduced to a few minutes [8]. Following the preincubation with NADPH as coenzyme, we found that hardly any change in extinction took place and if so, only in the first minutes. Therefore a preincubation of 5 min is sufficient to eliminate

possible unspecific changes in absorbance and to thermostat tube and reaction mixture at 25°.

The advantages of the short incubation time are mentioned above.

Results

Linearity and reproducibility

With the reagents mentioned above, standards of 50, 100 and 200 μmol NH_3/1 were determined. The reaction rate is linearly proportioned to the ammonia concentration up to a value of 200 μmol NH_3/1 (see Fig. 3).

The day to day variation could only be determined with the standard solutions. Table I shows the results of determinations during 6 days.

From duplicate determinations of 50 plasma samples we calculated the standard deviation of the method using the R_{Sample}–R_{Blank}: S.D. = 4.8.

Recovery

50 μmol NH_3/1 was added to different plasma samples, the recovery is shown in Table II.

Normal values

The ammonia level of native plasma was determined for 50 normal, non-fasting, healthy persons, aged 22—63. The mean value of these determinations was 22 μmol NH_3/1, ranging from 6.5 to 35 μmol NH_3/1.

From another group of 17 policlinical patients we compared the capillary and venous values. The mean value for venous plasma NH_3 was 19.3 μmol/1, ranging from 11 to 36 μmol/1. The capillary determinations showed a mean value of 48.0 μmol NH_3/1 ranging from 22.0 to 84.0 μmol/l. The mean factor capillary/venous value is 2.6, range 1.5—3.9.

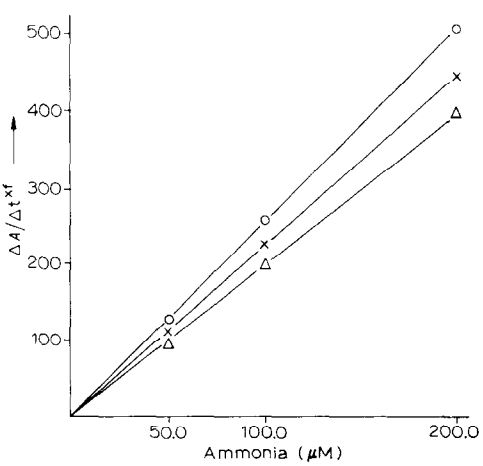

Fig. 3. Linearity of the method. The reagent blank has been subtracted. First readout: ○———○ (35—55 s); second readout: x———x (55—75 s); third readout: △———△ (75—95 s).

TABLE I

	$Rate_{St\ 50}$	$Rate_{St\ 100}$	$Rate_{St\ 200}$
Day 1	116	230	456
Day 2	122	240	460
Day 3	113	238	470
Day 4	113	232	482
Day 5	116	233	468
Day 6	114	235	468
$\bar{x}$	116	235	467
S.D.	3.4	3.8	9.0
C.V.	2.9%	1.6%	1.9%

Discussion

Most of the enzymatic ammonia determinations described before now, start with deproteinization and use NADH as coenzyme [1, 2, 5]. From our experiments and from others [6, 7] it appears that deproteinization is not necessary when performing the method kinetically.

The time necessary for one determination is shortened considerably when using NADPH [8].

Da Fonseca-Wollheim [8] found that the amount of new-formed ammonia in whole blood is negligible until 30 min after drawing the blood. We found however that separating the plasma as soon as possible gives a more realistic ammonia value. Like Da Fonseca-Wollheim we observed a slight increase of the ammonia value after keeping the plasma 30 min either at room temperature or at 4°.

When using heparin as anti-coagulant we found, like Da Fonseca-Wollheim, that it gives unspecific changes in absorbance. Increasing concentrations of heparin in the plasma gave elevated ammonia values, however, not related to the amount of heparin. We did not have this problem when using EDTA as anti-coagulant.

Contrary to Jacobs and Olthuis [7] we did not find it necessary to perform a plasma-blank for pathological cases. No changes in absorbance took place during preincubation of the plasma of two cirrhotic patients and all added ammonia was recovered.

TABLE II

Sample	Plasma NH_3 (μmol/l)	Added 50 (μmol/l)	Recovery (μmol/l)	%
1	11.6	60.3	48.7	97
2	5.6	55.7	50.1	100
3	20.7	68.8	48.1	96
4	54.0	101.3	47.3	95
5	80.3	135.6	55.3	111
6	31.3	86.1	54.8	110

The mean value and range of non-fasting normal persons correspond well with those found by other investigators [3, 7].

Because only a 100 µl sample is needed, it is possible to determine the ammonia values in capillary blood. However we did not have the opportunity to investigate why these values are 2—3 times higher than those in venous blood.

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