A RAPID METHOD FOR PLASMA AMMONIA ESTIMATION USING AN INDIGENOUSLY PURIFIED ENZYME

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ABSTRACT

Determination of ammonia level in blood is important, especially in the diagnosis of hepatic disorders. An indigenously purified enzyme was used in the standardisation of the assay. The assay is a two reagent system, requires five minutes for completion and can be performed at temperature between 25-37°C. Performance of the assay was assessed by linearity, imprecision, functional sensitivity and interference studies. Lyophilised reagent I and reagent II were found stable for at least one year. The plasma level of ammonia for the controls was $13.7 \pm 7.3 \mu\text{Mol/L}$, whereas for subjects of hepatic disorders, it was $69.1 \pm 32.4 \mu\text{Mol/L}$ ($P < 0.001$). The functional sensitivity was between 2-1000 $\mu\text{Mol/L}$. Within-run coefficient of variation was between 1.1-2.0% and between-run coefficient of variation was between 1.9-3.7%. The mean recovery after dilution was 99.6%. The present method can estimate ammonia up to 1000 $\mu\text{Mol/L}$ without dilution of sample. Assay time of five minute may be shortened to one minute. This method is suited for routine clinical use in treatment of hepatic disorders.

KEY WORDS : Ammonia, enzymatic assay, glutamate dehydrogenase, deaminase inhibitor.

INTRODUCTION

Determination of blood ammonia concentration is important in the diagnosis and follow up of certain hepatic and renal pathology conditions in adults, children and neonates (1,2). Ammonia analysis, apart from human biology is important in the fields of agriculture and environment (3,4). Several methods are available for the determination of ammonia in various body fluids (5-7). Most of these methods are either tedious, time consuming and insensitive or others, although advanced, are costly. Enzymatic assay is the method of choice in clinical chemistry;

however, various protocols described in literature do not fulfill present day demands such as sensitivity, upper range limit of estimation, assay time and stability of reagents. Commercially available kits fulfill few of the criteria but being costly they are not affordable. It is because of these reasons that ammonia test is not carried out routinely in many ordinary hospitals and pathological laboratories in India.

In the present work we have worked on an enzyme-based assay for ammonia. An indigenously purified bovine glutamate dehydrogenase (GLDH) was used in the standardisation. Emphasis was also given to three other aspects of the assay such as to increase sensitivity and upper detection limit so that the dilution of the samples can be avoided, to improve the stability of the reconstituted reagent I and to find

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out the feasibility of shortening the assay time.

MATERIALS AND METHODS

Clinical specimens

Overnight fasting blood samples either control or of hepatic disorder more specifically inflammation of hepatic cells were collected routinely in EDTA from Hindu Rao Hospital, Delhi and were immediately placed on ice. Subjects were advised not to smoke and consume alcohol 12 hours prior to blood collection. Plasma was separated within an hour and subjected to ammonia estimation. It was subsequently preserved at -20°C for further analysis of ammonia especially after thawing.

GLDH purification

Bovine liver enzyme was purified by a combination of ammonium sulfate precipitation and chromatographic technique. Homogeneity was checked by SDS-gel electrophoresis. Specific activity was determined and the enzyme was lyophilized. In fact, two enzymes were purified by the present method, which would be patented.

Reagents

Reagent I contained 0.40 mMol/L NADPH, 0.10 mMol/L ADP, 0.10 mMol/L 2-oxoglutarate, 0.10 Mol/L KH_2PO_4 and KOH. The reagent was stabilized and lyophilized. It was reconstituted to the desired volume before use. Reagent II contained 0.35 kU/L GLDH, 0.10 mMol/L ADP, 0.05% BSA, 0.10 Mol/L KH_2PO_4 and KOH in 50% glycerol. Reagent II was stored in solution form. A standard at a concentration of 100 $\mu\text{Mol/L}$ was prepared from ammonium sulfate. It was referred as reference standard. The two reagents and the standard were prepared the same day. A fresh standard of 100 $\mu\text{Mol/L}$ was also prepared every month up to a year before performing an assay. It

was used for comparison and shelf life studies and was discarded after use. Distilled water was treated with seralite SRC-120 prior to preparation of reagents and standards. All the reagents were stored at 4°C . They were brought to the desired temperature before use.

Assay

Assay mixture contained 1.0 ml of reagent I and 0.1 ml of either water or sample or standard in separate tubes. Reaction was initiated by the addition of 10 μl of reagent II. Zero time absorbance was recorded immediately, followed by five minutes absorbance at 340 nm. Absorbance difference was obtained by subtracting zero time absorbance from the final absorbance, which was recorded at 5 minutes. Assay was calibrated with standard solutions in the range of 100-1000 μMol ammonium/L. Shimadzu UV-2101PC spectrophotometer was used through out the study. It has all the software for kinetics and calibration studies.

Assay validation

Linearity : An aqueous stock calibrator of 2.0 mMol ammonium sulfate/L was prepared. This solution was stored at 4°C . Working aqueous standards of 100, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 and 2000 $\mu\text{Mol/L}$ were prepared from the stock calibrator. Assay was performed with each of these standards and linearity was found out.

Shelf-life study : It was carried out in two ways. In one of the studies reagent I was reconstituted every alternate month for a year. Absorbance was noted against water at 340 nm on each reconstitution. The reconstituted reagent I was used the same day for estimation of ammonia in reference standard and a fresh standard of same concentration. In an another study ammonia was estimated in the same standards on alternate days from the day of reconstitution of

reagent I. The absorbance of the reconstituted reagent I were also recorded every alternate day to find out any deterioration.

Reagent II and reference standard prepared in the beginning along with reagent I were routinely incorporated in both the studies. Fresh standard was prepared every month on the day of reconstitution of reagent I and was discarded after use.

Time-course study : Performance of the kinetic reaction was checked in a time-course study. The assay was carried out continuously for five minutes with an interval of one minute by taking known ammonium sulfate concentrations between 100-1000 $\mu\text{Mol/L}$.

Difference in absorbance was calculated for each minute as well as for five minute's reaction time.

Precision study : The precision of ammonia assay was evaluated by using three samples having low, medium and high concentrations within the linear range. The intra-assay imprecision was determined by analyzing each sample ten times in a single analytical run. The inter-assay imprecision was assessed daily over a period of twenty days.

Analytical recovery : Accuracy of the assay was evaluated with reference concentrations of 30, 100 and 208 μMol ammonium sulfate /L. Each reference was supplemented with three known concentrations (volumes) of ammonium sulfate calibrator. The percentage recovery of added ammonium sulfate was calculated.

Specimen Studies : Control and pathological samples were analysed by the present method. A comparison was made with an international method/ kit (Sigma).

Statistical analysis

Data were analyzed by a two-tail, unpaired *t*-test. Significance was set at 0.05. All results are reported as mean $\pm$ SD unless stated otherwise.

RESULTS

Stability study of the reagents was carried out for a period of one year. Lyophilized reagent I was reconstituted every alternate month and it's absorbance was recorded. During this period the absorbance of reconstituted reagent I was 1.32 ± 0.04 with a coefficient of variation (CV) of 2.7% against water. The absorbance differences obtained on estimation of ammonia for 100 $\mu\text{Mol/L}$ of reference and fresh standards were 0.09 ± 0.001 and 0.09 ± 0.002 with CV's of 1.1% and 2.2% respectively. The reconstituted reagent I was studied every alternate day for two weeks. Initial mean absorbance of reagent I against water for this period was 1.33 ± 0.02 with a CV of 1.65%. When used in the estimation of the two standards the absorbance difference obtained during this period was 0.09 ± 0.002 and 0.10 ± 0.002 respectively.

The statistical analysis of precision study is shown in table 1. Within-run CV's ranged from 1.1% to 2.0% and between-run CV's from 1.9 to 3.7% for the three samples. Supplemented ammonia recovered by this method ranged between 97.5-103.8% as can be seen from table 2.

Clinical studies were carried out with control and pathological plasma samples. Ammonia concentration in control subjects was $13.7 \pm 7.3 \mu\text{Mol/L}$ and in pathological samples it was $69.1 \pm 32.4 \mu\text{Mol/L}$, when estimated by the indigenous assay (table 3). The samples were also analysed by an internationally available kit. The results obtained by this kit were identical as can be seen from the same table.

Table 1. Intra and interassay precision study

Precision	Sample 1	Sample 2	Sample 3
Within-Run			
Mean, $\mu\text{mol/L}$	29.9	98.7	208.9
SD, $\mu\text{mol/L}$	0.3	1.1	4.3
CV, %	1.1	1.1	2.0
n ^a	10	10	10
Between-Run			
Mean, $\mu\text{mol/L}$	30.2	97.6	208.6
SD, $\mu\text{mol/L}$	0.6	2.4	7.8
CV, %	1.9	2.5	3.7
n ^b	20	20	20

^{a,b}Number of assays and days respectively.

Table 2. Enzymatic rate assay of ammonia in ammonia-supplemented sera*

Original	Added	Measured	Measured-original	Recovery, %
30	10.5	40.5	10.5	99.5
30	15.4	45.8	15.8	102.3
30	20.8	50.5	20.5	98.7
100	30.0	130.0	30.0	100.0
100	40.5	140.0	40.0	98.8
100	60.9	159.4	59.4	97.5
208	100.0	311.8	103.8	103.8
208	200.0	404.0	196.0	98.0
208	300.0	501.7	293.7	97.9
			Mean	99.6
			SD	2.1
			CV	2.1

*Concentration of ammonia in $\mu\text{Mol/L}$

The figure represents the results of time-course study with respect to time versus absorbance difference per minute. Assay was performed with each standard; absorbance was noted every minute starting from zero time, which is taken immediately on addition of reagent II.

DISCUSSION

The assay developed by us has two reagents and a standard. The reagents were tested for a period of one year and were found stable during this period. Reagent I was stabilized by lyophilization and reagent II in solution form. Reagent I was found stable for two

Table 3. Analysis of ammonia in control and pathological samples

	Ammonia concentration, $\mu\text{Mol/L}^a$			
	Indigenous assay		Commercial Kit	
	Control	Pathological	Control	Pathological
Mean	13.7	69.1	14.3	68.7
SD	7.3	32.4	7.4	31.6
n ^b	20	20	20	20
p ^c		< 0.001		<0.001

^a Ammonia was analysed as soon as plasma was separated from blood. Assay was performed in duplicate.

^b Number of samples.

^c Significance of difference between control and pathological samples.

Fig. Time course study of the assay. Standard concentrations used for this study were 0.0 (Δ , blank), 100 ($\cdot$), 200 ($+$), 400 ($\cdot$), 600 ($\square$), 800 ($\times$), 1000 ($\diamond$) μmol ammonium sulfate/L. Assay was performed by mixing 100 μL of water or standard with 1.0 ml of reagent I and 10 μL of reagent II. Zero time absorbance was taken immediately followed by every minutes' absorbance up to 5 minutes. Absorbance difference was obtained for the standards. Curve was drawn against the five absorbancies obtained for each respective standard.

weeks on reconstitution. This shift in stability is primarily due to labile nature of NADPH. The concentration of NADPH and other constituents has been brought to a level where it could help estimate more than 1000 μMol ammonium sulfate/L and provide consistent stability over a period of two weeks when in solution form. NADPH may be replaced by NADH which is comparatively more stable in solution, however, non-specificity makes it unsuitable for ammonia estimation (7,8). Rate of reaction was also influenced by the final concentration of GLDH. In the present assay, the enzyme activity was brought to a level where it could convert more than 1000 μMol ammonium sulfate /L in the presence of NADPH to glutamate. The enzyme was found stable in solution form for more than a year. Purification of diagnostic enzymes is one of our objectives; however, the isolation of GLDH has not been discussed as we intend to patent it.

Sensitivity of the assay was tested at various concentrations of ammonium as described in results. It was observed that the reaction was linear up to a concentration of 1000 μMol ammonium sulfate/L. Taking into consideration the importance of the upper limit of ammonia in severe pathological conditions, the present limit of 1000 $\mu\text{Mol/L}$ seemed satisfactory.

In fact, the assay was found linear up to 1200 μMol ammonium sulfate/L under the conditions developed by us. Such a high limit of estimation has not been cited in the literature. Linearity of the assay at high concentrations also avoids dilution of samples as had been advised in an earlier report (6). The assay was equally sensitive to estimate as little as 2.0 μMol ammonium sulfate/L as was observed with a fresh ammonium sulfate solution of 1.0-5.0 $\mu\text{Mol/L}$. However, it is the higher range, which is clinically significant.

In a time-course study, the reaction was observed continuously for a period of 5 minutes as shown in the figure. The absorbance was taken every minute starting from the first absorbance at zero time. The oxidation of NADPH was fast in the first minute of reaction, it slowed to half during the next minute and this pattern was followed with every passing minute. This study suggests that assay time may be shortened to one minute, which would comply with autoanalyzers.

The intra- and interassay reproducibility tests carried out with the three samples were within the acceptable limits; the maximum CV's being 2.0% and 3.7% respectively. Similarly the recoveries were more than 99%. In an interference study the addition of bilirubin, 0.2 g/L; creatinine, 0.1 g/L; hemoglobin, 1.3 g/L; lipids (HDL/LDL/VLDL), 2.0 g/L; urea, 0.4 g/L, and uric acid, 0.15 g/L to a normal sample did not cause any interference in ammonia levels.

During the course of this study, controls and pathological samples of clinical manifestation of jaundice were studied. The ammonia concentration in control subjects ranged between 5.0 to 28.3 $\mu\text{Mol/L}$ (13.7 ± 7.3 $\mu\text{Mol/L}$). The mean plasma ammonia concentration for pathological samples was significantly high ($P < 0.001$). A comparative study of these samples with an international kit provided identical results suggesting that the reagents and the assay system developed by us is of global

competence. Although our results of the control subjects were in agreement with the reported ones, the maximum level of ammonia observed as upper range limit for the controls however, was 28.0 $\mu\text{Mol/L}$. Other groups have reported up to and above 50 $\mu\text{Mol/L}$ (2,10). The variation in normal range could be due to technical pitfalls in methods, and the time taken in sample processing and analysis.

Initiation was also taken to study pediatric samples. However, only five samples could be analyzed for ammonia. Out of these, three children in the age group of 10-11 years showed significantly high concentration, which was above 150 $\mu\text{Mol/L}$ ($P < 0.001$). Of the three children, two were diagnosed of nephrotic syndrome and the third was of yellow fever.

Preserving a sample of clinical significance at subzero temperatures is a routine practice, however, in case of ammonia it seems that the samples be assayed without delay. As evidenced in literature (4,11) and by our own studies the plasma ammonia level increased on storage. Few of the plasma samples when re-estimated within 2-8 weeks of storage showed an increase of 102.9 ± 47.4 $\mu\text{Mol/L}$ ($P < 0.001$) as against 42.2 ± 38.7 $\mu\text{Mol/L}$ which was obtained for the same fresh samples. The possible cause for elevation of ammonia could be deamination of amino acids by deaminases present in blood (4,9,12). Addition of the appropriate inhibitors of these enzymes would check deamination and false positive results.

In conclusion, the present method developed by us using an indigenous enzyme is globally competitive. It is simple and rapid. Assay time may further be reduced which would lend itself to automation. Samples can be used without dilution. The experimental approach detailed here should allow clinical laboratories to develop a cost-effective assay system to meet local demands.

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REFERENCES

1. Ballistreri, W.F. and Rej, R. (1994) Liver Function. In : Tietz Textbook of Clinical Chemistry. Eds. Burtis, C.A. and Ashwood, E.R. WB Saunders. Philadelphia, USA. 2nd edition. p 1449-1513.
2. Diaz, J., Tornel, P.L. and Martinez, P. (1974) Reference intervals for blood ammonia in healthy subjects, determined by microdiffusion. Clin. Chem. 41, 1048.
3. Cabral Joao, P.S. (1974) Comparison of methods to assay ammonia in bacterial suspensions. J. Microbiol. Methods. 19, 207-213.
4. Huizenga, J.R., Tangerman, A. and Gips, C.H. (1994) Determination of ammonia in biological fluids. Ann. Clin. Biochem. 31, 529-543.
5. Ratcliff, C.R. and Hall, F.F. (1982) Ammonia in plasma, enzymatic procedure. Sel. Methods. Clin. Chem. 9, 85-90.
6. Van Anken, H.C. and Schiphorst, M.E. (1974) Kinetic determination of ammonia in plasma. Clin. Chim. Acta. 56, 151-157.
7. Da Fonseca-Wollheim, F. and Heinze, K.G. (1992) Which is the appropriate coenzyme for measurement of ammonia with glutamate dehydrogenase? Eur. J. Clin. Chem. Clin. Biochem. 30, 537-540.
8. Pesh-Imam, M., Kumar, S. and Willis, C.E. (1978) Enzymatic determination of plasma ammonia: evaluation of Sigma and BMC kits. Clin. Chem. 24, 2044-2046.
9. Kurahasi, K., Shihara, A. and Uehara, H. (1972) Determination of ammonia in blood plasma by ion exchange method. Clin. Chim. Acta. 42, 141-146.
10. Solberg, H.E. (1994) Establishment and use of reference values. In : Tietz Textbook of Clinical Chemistry. Eds. Burtis, C.A. and Ashwood, E.R. WB Saunders. Philadelphia, USA. 2nd edition. p 454-484.
11. Howanowitz, J.H., Howanowitz, P.J., Skrodzki, C.A. and Iwanski, J.A. (1984) Influence of specimen processing and storage conditions on results of plasma ammonia. Clin. Chem. 30, 906-908.
12. Linder, A. and Bauer, S. (1993) Effect of temperature, duration of storage and sampling procedure on ammonia concentration in equine blood plasma. Eur. J. Clin. Chem. Clin. Biochem. 31, 473-476.