Ammonia in human fluids methodology and clinical applications
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SUMMARY

This thesis contains four sections: overview, methodology in vivo, physiology and pathology. The first section gives overviews of the contribution of various organs to the arterial ammonia and of several methods for ammonia determination with their limitations. The second section deals with ammonia methodology in vivo in blood and/or plasma, in cerebrospinal fluid and in saliva. The third section deals with the physiology of lactate and ammonia in blood and sweat during exercise. The fourth section deals with ammonia results in liver disease and during liver transplantation.

A. Overview.

In chapter 1 of the overview, a survey of generation and breakdown of ammonia is given and estimations are made of the ammonia contributed by various organs to the arterial blood as well as the removal of ammonia from the arterial blood by the same or other organs. Contributing organs are - in order of quantitative importance - exercising muscle, intestines, kidneys and pancreas, and ammonia removing organs are liver, resting muscle, gut lumen, kidneys, brain, lung and spleen. In the failing liver the metabolism of ammonia may become impaired and in patients with portal hypertension causing portal-systemic shunting ammonia delivered by the portal vein may by-pass the liver and arrive in the systemic circulation.

In chapter 2 of the overview, the various methods for determination of ammonia in blood and other fluids are discussed and their limitations dealt with. The oldest method - 1859 - makes use of indophenol in a colorimetric reaction. It is performed on protein-free supernatant after adjustment to pH 4.0. It has been in use in our laboratory for more than 30 years. This so-called indophenol reaction has been shown to be interfered with a number of amino acids, also those found in excess in the blood of patients with severe hepatic failure. Ammonia in serum or plasma can be determined with an enzymatic method, based on the conversion of 2-oxoglutarate and NH₄⁺ to L-glutamate. Other methods depend on the liberation of ammonia gas and subsequent quantification as NH₃⁻N. This principle is used in new hand-held devices such as the Blood Ammonia Checker for whole blood and in the Kodak Ektachem for plasma. The arterial reference values using various methods all show a range of about 15-40 μmol/l. Owing to liberation of ammonia resulting from deamination processes, it is senseless to preserve blood or plasma...
at room temperature or at 4 °C (refrigerator). This phenomenon does not take place in the first 2 weeks when samples are stored deepfrozen at - 20 °C. The anticoagulated (heparin or EDTA) blood samples should immediately be placed in an ice bath and brought to the laboratory for analysis. Due to the gaseous nature of ammonia, the determination must be performed in an ammonia-free surrounding.

B. Methodology in vivo.

Blood and plasma.

Chapters 3-7 deal with the determination of ammonia in blood and in all chapters (one of) the methods used was the Blood Ammonia Checker in its earlier (I) or later (II) form.

In chapter 3 the BAC I was compared to an enzymatic method. BAC I was easier, needed only 20 μl blood and was faster - 16 minutes at the bedside - than the enzymatic method. The values were slightly lower than those simultaneously performed in plasma with the enzymatic method. Lack of linearity in the low normal range was a draw-back. In chapter 4 BAC I was compared with both the indophenol reaction (blood) and an enzymatic method (plasma). When the ammonia content was high, precision was excellent to satisfactory with any method. In the normal range, only with the BAC I precision was unsatisfactory, while recovery of added ammonia was unsatisfactory with the indophenol method. Simultaneous determination of the three methods on arterial blood samples of a large reference group showed the reference ammonia values to be decreasing in the following order: indophenol, enzymatic, BAC I. BAC I was the most convenient method in the clinical setting, but the values had to be regarded as semi-quantitative - and as described in chapter 3 - not linear in the low normal range. A further development was the BAC II and in chapter 5, the BAC II was tested against an enzymatic method. The new device had undergone technical improvements and the procedure was speeded up to 4 minutes only, while still 20 μl of blood was needed. There are lower and upper limits to the method: 7 and 280 μmol/l. The precision of BAC II was excellent and equal to the enzymatic method, and the recovery of added ammonia likewise was excellent as well as the linearity. The arterial reference range was 7-28 μmol/l. BAC II combines excellent methodological results with speed and ease.

As a finger prick delivers enough blood for ammonia determination with the BAC II, comparisons can be made between capillary and arterial blood ammonia. Capillary blood is easier to obtain than arterial blood and for investigational purposes there can be medical reasons for using the capillary approach. If, however, capillary blood might not be sufficient, forearm venous blood should be used and the results were found to be identical to arterial ammonia. In a group of healthy adults and in patients with disease, however, there might be a difference in ammonia concentrations. This was a simultaneous determination of ammonia in health and disease and the capillary method did not seem to be indicated for systematic use in healthy populations. The advantages of the capillary method were considered the easy and comfortable sampling technique, which might be more acceptable to patients.

Cerebral ammonia metabolism.

In relation to ammonia metabolism in the brain, however, an alternative method of ammonia determination has been developed. First, ammonia was measured by an indophenol reaction in arterialized ear-lobed blood. Second, ammonia was measured by an enzymatic method in plasma. The results were compared to arterial blood ammonia and showed a correlation with the arterial ammonia values, but there was some discrepancy between the capillary and arterial ammonia concentrations. Further investigations showed that the ear-lobed method was a more sensitive method and might be an alternative to the arterial method.

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medical-ethical problems in the use of arterial blood. There is a problem with fingertips however: sweat contains 100 to 200 times as much ammonia as blood and cleansing of the fingertip grooves has to be done extremely conscientiously in order not to contaminate the capillary blood. In chapter 6, capillary fingertip BAC II blood ammonia was compared to simultaneously collected arterial blood BAC II and plasma enzymatic ammonia. There was a nice correlation between the capillary and arterial ammonia and the capillary ammonia was higher than the arterial. The arterial BAC II and enzymatic ammonia levels did not differ. Reproducibility of the fingertip ammonia, tested during 3 consecutive days in healthy controls, turned out to be poor and despite all the precautions taken, sweat was considered to be the offender. The difference between capillary and arterial BAC II levels might have been eliminated when holding the hand in a warm water bath beforehand. This would have arterIALIZED the capillary blood. Ear-lobe blood might not have the disadvantages encountered with fingertip blood and in chapter 7 capillary blood samples from these two sources were tested against each other and against simultaneously drawn arterial blood using BAC II in a healthy reference group (no arterial determinations), in a reference group of patients without liver disease and in patients with liver disease. The ear-lobe levels were not different between the two reference groups and when compared to the arterial levels showed a small but significant difference in the patients without liver disease. Both the ear-lobe capillary and arterial blood ammonia ranged within the arterial reference values published in literature. The fingertip ammonia was uniformly higher than the ear-lobe ammonia. The overall correlation between ear-lobe and arterial BAC II ammonia was very good and the conclusion was that ear-lobe capillary blood ammonia is an alternative for arterial blood ammonia determination.

Cerebrospinal fluid.

In recent years intracranial pressure measurements in some specialized centers has become a routine procedure in patients with severe hepatic encephalopathy. This allows an ammonia determination in cerebrospinal fluid. In chapter 8 is described how the indophenol reaction was used after pH adjustment. Ammonia was determined in a reference group of patients with neurological disorders, but no liver disease. The recovery of added ammonia was good. Cerebrospinal fluid could not be stored for ammonia determination at room temperature, while it could be kept for 2 days at 4 °C in the refrigerator and for at least 1 month when deepfrozen at - 20 °C. The reference levels
ranged from 8-26 μmol/l.

Saliva.

Saliva is known to have a high ammonia concentration. Until recently, it was not possible to obtain samples from individual salivary glands. In chapter 9, three methods for ammonia determination in total saliva were tested: an enzymatic method and the indophenol reaction on 1:100 diluted specimens and an ammonium electrode. The ammonium electrode was the quickest procedure but it requires correction for the potassium content of saliva (which therefore has to be determined as well). The enzymatic method gave the same result as the electrode but was more expensive. The indophenol method had both the best precision and the lowest reagent cost. The reference values were regardless of any method 1-12 mmol/l. In chapter 10, the preservation of saliva for ammonia determination was studied using the three afore mentioned methods. If immediately diluted and kept at 4 °C ammonia could be reliably measured using any method if determined within 1 hour. When saliva was immediately deepfrozen at -20 °C the specimen could be kept for more than 2 weeks. As it recently became possible to sample saliva from individual salivary glands, more insight into the origin of oral cavity (total saliva) ammonia might be obtained. The results of the ammonia determination with the indophenol method in saliva from parotid and submandibular/sublingual glands and total saliva are described in chapter 11. The median ammonia content of total saliva was 8 to 20 times as high as the content of the salivary glands. One possible reason is bacterial hydrolysis of ureum in the mouth through, e.g. Streptococcus salivarius and Helicobacter pylori.

C. Physiology.

Break-down in the muscle cell during exercise of adenosine 5-monophosphate to inosine 5-monophosphate and ammonia leads to enhanced glycolytic activity with production of lactate, leading to elevated blood levels of both ammonia and lactate. An endurance threshold using blood lactate has been proposed. When during exercise sweat ammonia and lactate would show linear relationships to blood ammonia and lactate, sweat in stead of blood possibly could be used. In chapter 12 is described how 10 healthy subjects cycled a workload of at least 200 watt, while ear-lobe capillary blood as well as sweat samples were analyses on ammonia and lactate. The blood ammonia (BAC II) increased after

workloads with highest values of 2 fold and lactate and lactate and relation.
workloads of 225-300 watt to levels seen in patients with hepatic encephalopathy, the highest value obtained being 227 $\mu$mol NH$_3$-N/l after 325 watt. Blood lactate increased 3-fold and there was a linear relationship between the ammonia and lactate increases. Sweat lactate and ammonia both decreased (with a linear relationship) during exercise. The relationships between blood and sweat ammonia as well as between blood and sweat lactate were non-linear however and the conclusion was that sweat cannot replace blood for lactate and ammonia measurement to determine endurance capacity.

D. Pathology.

Chapter 13 and 14 deal with the usefulness of determining arterial ammonia in patients with liver disease.

In chapter 13, we investigated three ammonia methods in liver patients without overt encephalopathy and compared them with control patients. The three methods were the enzymatic method in plasma and in whole blood the indophenol method and the Blood Ammonia Checker (BAC) I, all in arterial samples in the fasting patient. There were 106 non-encephalopathic liver patients, 56 with and 50 without cirrhotic and 51 controls (patients without liver disease). Patients with cirrhosis had higher ammonia levels with the enzymatic method and with BAC I than the non-cirrhotic and control group, and in addition the enzymatic method separated the non-cirrhotics from the control groups. The indophenol method could not discriminate liver patients from the control group.

While in chapter 13 liver patients with overt encephalopathy were excluded, in chapter 14 arterial ammonia was determined using the BAC II and indophenol method in liver patients with (70) and without (55) overt hepatic encephalopathy (HE) and compared with the findings in 39 patients without liver disease and 13 patients with neurological encephalopathy, without liver disease. We had observed earlier that in patients with severe hepatic failure the indophenol reaction could become depressed and even totally inhibited and an in vitro study had shown that this interference was due to a number of (aromatic) amino acids, which may be found in excess in blood of liver encephalopathy patients. The difference between BAC II ammonia and indophenol ammonia was regarded to express this interference of the indophenol reaction (IFI) and was expressed in $\mu$mol - N/l. While in the control group differences between BAC II and indophenol ammonia were small or absent, in liver patients indophenol ammonia lagged behind BAC II ammonia, and IFI increased with increasing BAC, which supposedly means an increase of...
indophenol inhibiting amino acids. BAC II was effective in discriminating hepatic encephalopathy from neurological encephalopathy. As in the earlier investigation, indophenol ammonia showed no difference between the control group and liver patients. Using the two methods and IFI there was a difference between patients with and patients without overt hepatic encephalopathy.

In chapter 15, the ammonia concentrations during four episodes of the orthotopic liver transplantation (OLT) operation procedure are presented. Ammonia was determined in 17 OLT patients using the indophenol method during: donor announcement (I), operation until heptectomy completed (II), anhepatic phase (III) and 1 h after implantation with restored blood circulation (IV). For reference a group of 16 patients without liver disease was used. The ammonia concentrations in stage I, II and III were higher and ammonia’s in stage IV did not differ from the reference group. Within 1 h after implantation of the new liver and with restored blood circulation, the arterial blood ammonia returned to normal. In a longitudinal analysis, ammonia concentration in stage III was higher and in stage IV lower than in stages I and II. Conservation of the urea cycle is critical with regard to the vitality of the liver, and ammonia determination before the OLT operation and directly after recirculation can give insight in the eliminative power and function of the new liver.

CONCLUSIONS

Determination of ammonia in biological fluids need different methodology. For whole blood the Blood Ammonia Checker II is an improvement over other methods, also because it can be performed in ear-lobe capillary blood, which accurately reflects the arterial blood ammonia concentration. For other biological fluids - biological and non-biological - the indophenol reaction is both easy and effective, and in arterial plasma or serum the enzymatic method is excellent.

Although hepatic encephalopathy (HE) easily can be discriminated from non-liver diseases and from other encephalopathy, a more precise definition of liver patients without HE, with subclinical HE and with overt HE will enhance the usefulness of the blood ammonia determination. The fact that very high ammonia levels - compatible with severe HE - occur after a heavy workload in healthy persons, will not give rise to confusion as HE patients will not be able to demonstrate the same muscular performance, but it showes the
usefulness of physiological alongside clinico-pathological studies. The interference by a number of amino acids, notably those incriminated in the pathogenesis of HE due to severe hepatic failure, is an interesting by-product of Berthelot's indophenol ammonia method of 1859, which however need quantitative amino acid determination in vivo to establish its true clinical value. The accumulation of ammonia in the liverless state, leading to elevated blood levels, is understandable and well known, but even more interesting is the extremely rapid normalisation of the ammonia levels during liver transplantation by the newly implanted liver, indicating the well-functioning of one of its most vital functions, deposition of waste ammonia by means of the urea cycle.