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Determination of Ammonia in Saliva Using Indophenol, an Ammonium Electrode and an Enzymatic Method: A Comparative Investigation

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Summary: Three methods for determination of ammonia in saliva are reported. The indophenol method on diluted saliva has the best precision (coefficient of variation 0.8%) and the lowest reagent cost. The ammonium electrode method is the quickest, but it requires simultaneous determination of the potassium content of the specimen. The enzymatic method gives the same result as the electrode method, but is more expensive. Deproteinisation proved not to be necessary. In one hour 10, 20 or 40 determinations can be performed with the enzymatic, indophenol- or the electrode method, respectively.

Bestimmung von Ammoniak im Speichel mit der Berthelot-Reaktion, einer Ammonium-Elektrode und einer enzymatischen Methode: Eine vergleichende Untersuchung

Zusammenfassung: Drei Methoden zur Bestimmung von Ammoniak im Speichel wurden geprüft. Die Methode nach *Berthelot* für verdünnten Speichel hat die beste Präzision (VK = 0,8%) und die geringsten Reagenzienkosten. Die Messung mit der Ammoniak-Elektrode ist die schnellste, erfordert jedoch die gleichzeitige Kalium-Bestimmung. Die enzymatische Methode ergibt die gleichen Resultate wie die potentiometrische, ist jedoch teurer. Enteiweißung ist nicht erforderlich. In einer Stunde können mit der enzymatischen Methode 10, mit der *Berthelot*-Reaktion 20 und mit der potentiometrischen Methode 40 Bestimmungen durchgeführt werden.

Introduction

In the past few decades, salivary ammonia has received much attention in dentistry as it might have a rôle in prevention of caries (1). Salivary ammonia has been investigated in patients with chronic renal insufficiency, where it was found to be elevated (2).

The ammonia content of saliva is increased by bacterial hydrolysis of urea in salivary glands and oral cavity (3). Saliva must therefore be collected while it is flowing rapidly (e.g. after chewing gum) from a clean mouth.

The determination of ammonia in saliva has been described using aeration (4), ion-exchange chromatography – i.e. the method of *Folin-Bell* – (5–10), microdiffusion (1, 11–13), distillation in vacuo (14) and common distillation (15, 16).

We have tried to develop simple methods for the determination of ammonia in saliva. *Berthelot*'s indophenol reaction (17) was used after manifold dilution of saliva, to eliminate the effect of inhibitors (18, 19). An ammonium electrode method (20) was also tested. Lastly, the use of an enzymatic method (21), based on the conversion of 2-oxoglutarate and NH_4^+ to *L*-glutamate, was investigated. As deproteinisation might have an effect on the ammonia level measured, we tested both uranylacetate and trichloroacetic acid.

The preservation of saliva for ammonia determination has been dealt with elsewhere (22).

Materials and Methods

Twice distilled water was used throughout. Fresh saliva was obtained after chewing gum, followed by thorough rinsing of the mouth with water. The determinations were performed on freshly voided saliva samples. Because of the high ammonia content of saliva, manifold dilution (1:100) was necessary for the indophenol and enzymatic methods. The electrode method needed dilution of the saliva sample to obtain enough volume for measurement.

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Indophenol method

Apparatus

Test tubes (glass, 10 ml); 1 ml volume pipettes; 2 Pipettors® (Oxford Laboratories, models SA, 1000 ml, and R, 500 ml, respectively); dark 37 °C waterbath; colorimeter (Vitatron DCP, 623 nm filter).

Reagents (23)

Solution A: 5 g phenol, 4 g NaOH pellets and 2 ml sodium nitroprusside 10 g/l, made up with water to 400 ml.

Solution B: the contents of a 10 ml ampoule containing 0.2 mol/l sodium hypochlorite is made up with water to 100 ml.

Reagent blank: water. Stock standard: 23.4 mg $(NH_4)_2SO_4$ in 100 ml water.

Standard solutions 50 and 100 μmol NH_4^+/l: 1 and 2 ml of the stock standard are made up with water to 100 ml.

Procedure

1. Saliva, blank and standards are diluted 1:100.
2. One ml of each of the dilutions is pipetted in test tubes.
3. 2 ml of the phenolate-nitroprusside (A) and 1 ml of the sodium hypochlorite solutions (B) are added and the mixture is immediately well shaken, and
4. incubated for 15 minutes in a dark waterbath at 37 °C.
5. Absorbance is read at 623 nm.
6. Calculation:

$$\frac{A_{sample} - A_{blank}}{A_{standard} - A_{blank}} \times \text{standard (mmol/l)} = \ldots \text{ mmol/l } NH_3$$

Ammonium electrode method

Apparatus

10 ml glass beakers; ammonium electrode (Philips IS560-NH_4); reference electrode (Philips R44/2-SD/1); pH electrode (Philips C13-NS); digital voltmeter (Philips PW9414); flame photometer (Corning 450).

Characteristics of the ammonium electrode

Beyond pH 7 the electrode shows a decline in potential (the pH of saliva is 6). Over a 24 hour period the drift of the potential, using a 1 mmol/l NH_4 Cl solution, was 3.5 mV. The electrode stability was better with water than with a buffer solution. The electrode response time (24) was 35 seconds.

The relationship between the electrode potential and the log $[NH_4^+]$ was found to be linear between 1 and 10 mmol/l NH_4^+. The same correlation existed between electrode potential and log $[K^+]$, between 5 and 50 mmol/l K^+ (fig. 1). As the ammonium electrode measures potassium as well as ammonium (while we have not found any interference by other anions), correction for the potassium content has to take place (20). We have constructed (fig. 2) a nomogram by measuring the electrode potentials of series solutions with different ammonium and potassium concentrations. Use of the nomogram can be illustrated by the following example: When the non-corrected electrode potential gives a fictitious ammonium concentration of 7 mmol/l and when the potassium concentration is 20 mmol/l, then the actual ammonium concentration will be 4.3 mmol/l.

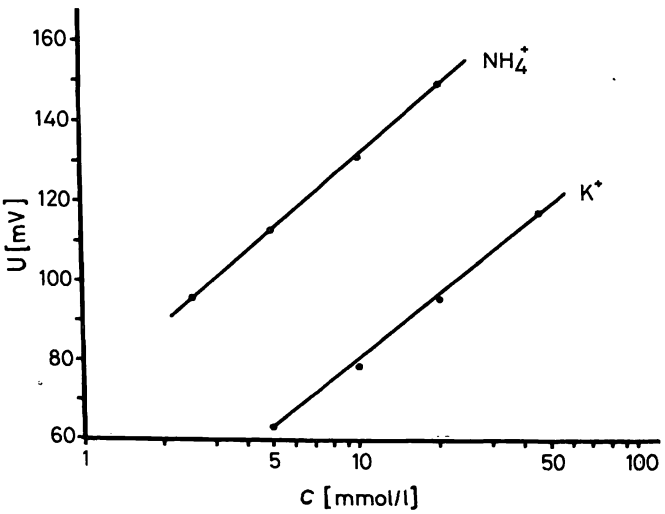

Fig. 1. Electrode response to a series of NH_4^+ and K^+ concentrations.

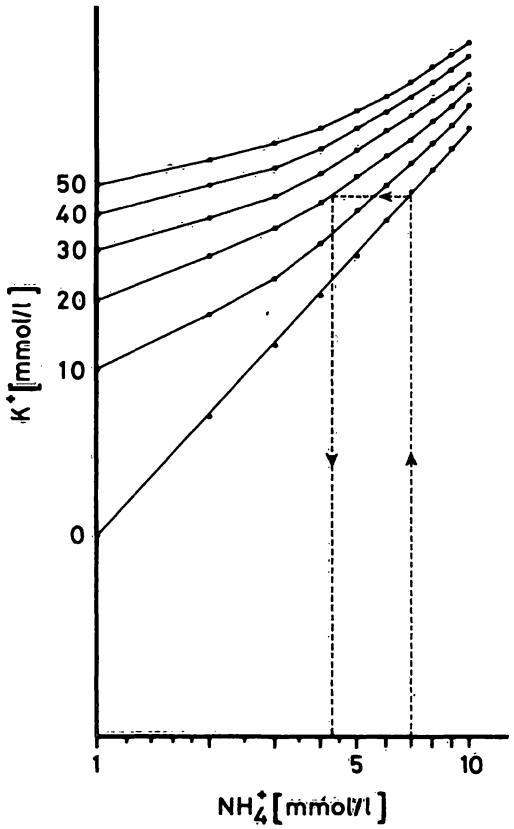

Fig. 2. Nomogram for determination of the actual salivary ammonia with the electrode method. See text for explanation of the example (broken lines).

Procedure

1. Standard solutions 2.5; 5.0; 10.0 and 20.0 mmol/l NH_3 are diluted 1:10 and an electrode standard curve is constructed.
2. Saliva is diluted 1:10 and the electrode potential is measured. This is the fictitious (non-corrected) ammonia concentration.
3. The potassium concentration of the saliva is measured with a flame photometer.
4. The actual ammonia concentration in saliva is determined using the nomogram (fig. 2).

Enzymatic method

Apparatus

Disposable cuvettes (Pharmaseal, cat. no. L 1045 M), 0.5 and 2.5 ml volume pipettes, 20 μl syringe (Hamilton); colorimeter (Vitatron DCP, 340 nm filter).

Solutions

Solution C: 0.15 mol/l triethanolamine buffer pH 8.6.
Solution D: 15 mmol/l 2-oxoglutarate.
Solution E: 1.5 mmol/l ADP.
Solution F: 0.10 mmol/l NADPH.
Solution G: 10 g/l glutamate dehydrogenase suspension.

Reaction mixture: 20 ml C, 1 ml D, 2 ml E, 1 ml F and 6 ml water.
Reagent blank (RB): water.

Procedure

1. 1 ml saliva is diluted with water 1:100.
2. 2.5 ml of the reaction mixture is pipetted into a cuvette and 0.5 ml diluted saliva or 0.5 ml reagent blank is added, mixed and the absorbance A1 is measured after 10 minutes followed by
3. addition of 20 µl of solution G and mixing.
4. After 10 minutes standing the absorbance A2 is read.
5. Calculation:

$A1 - A2 = \Delta A_{sample}$ or ΔA_{RB}

$\Delta A_{sample} - \Delta A_{RB} = \Delta A_{corrected}$

Concentration:

$96 \times \Delta A_{corrected}$ = . . . mmol/l NH_3.

(Factor 96: $\frac{100 \times 6.04}{6.3}$. First dilution 100 times, second dilution 6.04 times.

Molar lineic absorbance of NADH (30 °C) is

$\epsilon_{339\,mm} = 630\ m^2 \cdot mol^{-1}$

Deproteinisation of saliva

Solutions

Trichloroacetic acid 100 g/l (0.61 mol/l).
Uranylacetate 1,6 g/l (3.79 mmol/l).

Procedure

Saliva was deproteinized by trichloroacetic acid or uranylacetate 1 + 1. The sediment was negligible.

After centrifugation the ammonia concentration was measured in the supernatant fluid using the indophenol and electrode methods as described above and the results were compared with those of non-deproteinized specimens.

Statistical analysis

Statistical analysis was performed using the *Wilcoxon* signed rank test (25). A difference was accepted to be significant at the $p < 0.05$ level.

Results

Precision

The ammonia content was determined twenty times in one diluted saliva sample. The results are given in table 1. Precision was best with the indophenol method.

Ammonia and potassium in saliva of healthy controls

Ammonia and potassium were determined in saliva samples of 23 healthy controls (tab. 2). The median of ammonium concentration was about 4 and the median of potassium concentration about 18 mmol/l (flame photometry). There was no difference in results between the potentiometric and enzymatic methods ($p = 0.2$), while a higher concentration was measured with the indophenol method ($p \leqslant 0.005$).

Tab. 1. Precision. Ammonia contents determined 20 times with three methods in one saliva sample.

n = 20	Range (mmol/l)	Mean (mmol/l)	S.D. (mmol/l)	CV (%)
Ammonia				
Indophenol	6.1–6.3	6.2	0.05	0.8
Electrode (K^+-corrected)	6.9–7.8	7.4	0.3	3.6
Enzymatic	6.4–7.1	6.7	0.2	2.9

Tab. 2. Ammonia and potassium contents determined with three methods in saliva samples from 23 healthy subjects.

	Mean (mmol/l)	Median (mmol/l)	Range (mmol/l)
Ammonia			
Indophenol	4.8	4.5	1.1–12.3
Electrode (K^+-corrected)	4.0	3.5	1.4–12.1
Enzymatic	4.4	3.9	1.1–12.0
Potassium	18.0	18.0	13 –31

Recovery

Saliva was diluted 1:10 with standard ammonia solutions containing 0.5 and 1.0 mmol/l, which resulted in an addition per sample of 4.5 and 9.0 µmol respectively. The ammonia concentration then was determined with all three methods in samples from the same 23 healthy controls (tab. 3). The recoveries with the indophenol method were higher than with the potentiometric and enzymatic methods (addition of 4.5 µmol $p \leqslant 0.005$ and of 9 µmol $p \leqslant 0.02$). There was no difference in recovery between the potentiometric and enzymatic methods (addition of 4.5 µmol $p = 0.6$ and of 9 µmol $p = 0.9$). A small but significant difference was found between the 4.5 and 9.0 µmol additions when the indophenol method was used ($p < 0.0001$).

Tab. 3. Recoveries of ammonium added to saliva (n = 23) using three methods.

Method	Ammonia addition 4.5 µmol Range (%)	Mean ± S.D. (%)	9 µmol Range (%)	Mean ± S.D. (%)
Indophenol	91–109	100 ± 5.2	87–107	96 ± 5.7
Electrode (K^+-corrected)	67–109	89 ± 12.1	72–106	91 ± 8.9
Enzymatic	76–104	90 ± 7.7	81–108	91 ± 6

Deproteinisation

In saliva samples of two controls, the ammonia content was measured before and after deproteinisation with trichloroacetic acid and uranylacetate respectively (tab. 4). Deproteinisation resulted in a slightly lower ammonia level.

Tab. 4. The effect of deproteinisation of saliva on the ammonia measurement using the indophenol and (K^+-corrected) electrode methods (mmol/l).

Deproteinisation	Electrode method	Indophenol method
No	3.5	3.8
Trichloroacetic acid	3.3	3.7
Uranylacetate	3.2	3.6

Discussion

Determination of salivary ammonia with one of these three methods is far easier than using any of the older procedures. The performance of all three methods is sufficient, but the indophenol method on diluted saliva has a better precision than both potentiometric and enzymatic methods and therefore is the method of choice for research work.

Some economical aspects are summarized in table 5. The electrode method is rapid and cheap when large numbers of determinations have to be performed over a short period (the operational life of the membrane is about 4 weeks). The enzymatic method is the most laborious and expensive of the three. As the larger part of the equipment used will be available in a routine clinical chemistry laboratory, the cost of apparatus has not been included in our considerations.

Tab. 5. Economic aspects of the three ammonia determinations.

	Parallel determinations per hour possible	Reagent costs ($)
Indophenol	20	0.02/determination
Electrode	40	33 per membrane
Enzymatic	10	2.50/determination

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