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**Performance of the Du Pont aca Ammonia Method**


We evaluated the performance of the Du Pont aca ammonia procedure with regard to (a) linearity, (b) precision, (c) interferences, and (d) effect of anticoagulants. Linearity extends to 2000 μmol/L. The SD of the method was essentially constant (2 to 3 μmol/L) and independent of the NH₃ concentration. Hemoglobin, bilirubin, and lipemia do not interfere. Either EDTA or heparin is suitable as anticoagulant. Recovery of NH₃ added to plasma samples averaged 102% (range: 97 to 107%). We established normal values by measuring NH₃ in 188 plasma samples from apparently healthy individuals. The 95% confidence range is from 10 to 35 μmol/L. The aca ammonia method compares very well with that of Kingsley and Tager but correlates less strongly with that of Reinhold and Chung. We describe a protein-based solution with stable NH₃ concentration that is suitable as a control material.

Additional Keyphrases: variation, source of normal values, intermethod comparison

An evaluation of the Du Pont aca procedure has been published recently by Jpma et al. (1). The same procedure has been evaluated by our three hospital laboratories. Our studies have been considerably more extensive and include a determination of normal range, effect of anticoagulants, stability of ammonia in blood samples, preparation of a suit-

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Table 1. Ammonia in De-Ionized Water Added to Vacutainer Tubes and Test Tubes (Five Each) as Measured by Two Methods*  

<table>
<thead>
<tr>
<th>Method</th>
<th>EDTA anticoag.</th>
<th>Heparin anticoag.</th>
<th>Test tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>aca</em></td>
<td>0.8</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>Kingsley</td>
<td>0.8</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td><em>aca</em></td>
<td>1.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Kingsley</td>
<td>1.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td><em>aca</em></td>
<td>0.2</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>Kingsley</td>
<td>0.5</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>0</td>
<td>1.8</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>4</td>
<td>1.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* Kingsley-Tager (2) and *aca* methods. Analyses done in duplicate.

Results

Linearity. We confirmed the previously published report (1) and found that linearity extends to at least 2000 μmol/L. We used 13 standard NH₃Cl solutions encompassing a concentration range from 20 to 2000 μmol/L. The linear regression equation, \( y = 1.00\ (0.004)x + 1.2\ (3.7) \) with a correlation coefficient of 0.9999, attests to the excellent linearity of the method (y and x are the found and nominal ammonia values, respectively; figures in parentheses are SD's).

Precision. Within-run precision was evaluated by replicate analyses (n = 20) of two plasma pools. The mean values were 87 and 262 μmol/L with SD's of 2.5 and 3.3 μmol/L, respectively. Aqueous solutions of bovine serum albumin, Fraction V, fortified with ammonium sulfate, were used to evaluate between-day precision. Two such solutions were analyzed 27 times during five weeks. The average ammonia values were 53 and 302 μmol/L with SD's of 2.7 and 3.1 μmol/L, respectively.

Effect of anticoagulant on plasma ammonia. Blood samples were collected concurrently into Vacutainer Tubes containing heparin, EDTA, fluoride-oxalate, or no anticoagulant; 13 individuals were involved in this study. The samples were centrifuged immediately, except those requiring clotting, and analyzed in duplicate in the *aca*. Samples collected with EDTA or heparin provided the following data: EDTA: n = 13; \( \bar{x} = 21.8; SD = 5.7 \); heparin: n = 13; \( \bar{x} = 22.3; SD = 7.6 \). The within-run SD's estimated from the differences among duplicates (SD = \( \sqrt{(SD^2)/n} \) were 1.8 and 2.3 μmol/L for the heparin- and EDTA-containing samples, respectively; thus either EDTA or heparin is suitable as anticoagulant. Samples collected with fluoride-oxalate or without anticoagulant were unsatisfactory because ammonia concentrations were several times higher than those obtained for samples collected with heparin or EDTA as anticoagulant.

Ammonia in Vacutainer Tubes. De-ionized water (7.0 mL) was added to 7.0 mL Vacutainer Tubes containing EDTA or heparin and to plain glass test tubes. Five tubes of each kind were used and the water added was analyzed in duplicate by the *aca* and Kingsley-Tager methods. The results (Table 1) indicate that Vacutainer Tubes containing heparin are ammonia-free, while those containing EDTA contain traces of ammonia.

Comparison of the *aca* method with manual ammonia methods. The *aca* method was compared with manual procedures used by the laboratories conducting the evaluation. Clear specimens, nonjaundiced and nonhemolyzed, were selected for that experiment. The NH₃ concentration in these specimens ranged from 17 to 482 μmol/L. Table 2 shows data obtained with the different methods. Results by the method of Kingsley and Tager, considered a "standard method" because of its thorough evaluation, compare very well with those by the *aca* method; the other two compare less favorably.

Recovery. Plasma samples were analyzed before and after addition of known quantities of ammonia (as ammonium ion). Laboratory "A" used 27 different plasma samples fortified with concentrations of ammonia ranging from 208 to 333 μmol/L. These specimens were analyzed in the *aca* only. The average recovery was 102% (range, 98-107%).

Laboratory "B" performed analytical recovery experiments by both the *aca* and Reinhold-Chung methods. Seven plasma pools were used with the *aca* method and two plasma pools with that of Reinhold and Chung. Aliquots from each pool were supplemented with ammonia, in eight different concentrations ranging from 100 to 467 μmol/L. For the *aca* method the average recovery was 101% (range, 97-104%). The same mean-recovery value (101%) was obtained with the Reinhold-Chung method, but the values ranged from 70-148%.

Interferences. The effect of hemoglobin, bilirubin, and lipemia on the *aca* method was studied by evaluating the recovery of known concentrations of NH₃ added to plasma pools containing various concentrations of hemoglobin, bilirubin, and triglycerides. The hemoglobin added to plasma pools was freshly prepared by lysing washed erythrocytes. Patients' samples with high concentrations of bilirubin or triacylglycer-

Table 2. Plasma Ammonia as Determined by Different Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Lab. &quot;A&quot; (n = 133)</th>
<th></th>
<th>Lab. &quot;B&quot; (n = 69)</th>
<th></th>
<th>Lab. &quot;C&quot; (n = 40)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \bar{x} )</td>
<td>SD</td>
<td>Range</td>
<td>( \bar{x} )</td>
<td>SD</td>
<td>Range</td>
</tr>
<tr>
<td><em>aca</em></td>
<td>101</td>
<td>91</td>
<td>17-482</td>
<td>42</td>
<td>28</td>
<td>0-118</td>
</tr>
<tr>
<td>Kingsley-Tager</td>
<td>100</td>
<td>88</td>
<td>22-458</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Hyland</td>
<td>---</td>
<td>---</td>
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<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Reinhold-Chung</td>
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<td>---</td>
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<td>---</td>
</tr>
</tbody>
</table>

Linear regression analysis

<table>
<thead>
<tr>
<th>Method</th>
<th>Regression equation (SD's in parentheses)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>aca</em> vs. Kingsley-Tager</td>
<td>( y = 1.03\ (0.008)x - 2.0\ (1.0) )</td>
<td>0.996</td>
</tr>
<tr>
<td><em>aca</em> vs. Hyland</td>
<td>( y = 0.931\ (0.039)x + 6.4\ (3.7) )</td>
<td>0.969</td>
</tr>
<tr>
<td><em>aca</em> vs. Reinhold-Chung</td>
<td>( y = 0.915\ (0.056)x + 8.8\ (2.5) )</td>
<td>0.895</td>
</tr>
</tbody>
</table>

*aca = y (dependent variable)
cerols (triglycerides) were used to supplement plasma pools with these constituents. We found no interference from bilirubin and triglycerides in concentrations up to 92 mg/L and 5.1 g/L, respectively. Hemoglobin at a concentration of 3.6 g/L decreased the ammonia values by 6% on the average.

**Stability of NH₃ in plasma.** The stability of NH₃ in plasma has been evaluated by several investigators (4-9) and the reports are quite conflicting. We evaluated the stability of NH₃ in plasma at room temperature and at −20 °C in a few samples, and the results were quite variable. On the basis of our experience and that of other investigators we recommend that NH₃ determinations be performed within 15 min after the blood specimen is centrifuged, because in general the NH₃ concentration tends to change regardless of the storage conditions.

**Normal values.** There is a wide variation in the reported normal values for plasma ammonia (2). This may be due, at least in part, to technical pitfalls described by Gerron et al. (10).

We measured plasma ammonia in 100 ambulatory women (ages 19 to 66 years) and 88 men (ages 19 to 61 years). Blood specimens, collected with EDTA as anticoagulant, were placed on ice, centrifuged, and analyzed immediately by the aca method. The composite distribution was not significantly different from the distribution for either sex separately (p >0.1 by the t-test for means). In addition, no significant deviation from a gaussian distribution was found (p >0.3 by the chi-square test). Thus, the normal values were established by parametric analysis on the composite population. The mean ammonia value for the 188 samples was 22.1 μmol/L, the SD 6.3 μmol/L. The 95% confidence range for the normal population is from 10 to 35 μmol/L and corresponds exactly to that found graphically from the probability plot. This range is almost identical to those reported by other investigators (5, 10), who used resin-adsorption methods and the Berthelot reaction. A frequency histogram of the 188 samples is shown in Figure 1.

**Effect of formaldehyde in the Berthelot reaction.** We have found that formaldehyde strongly inhibits the Berthelot reaction. Formaldehyde is sometimes used to disinfect certain components for preparation of de-ionized water (filters and tanks). If such components are not thoroughly rinsed, serious interference with some laboratory tests may be encountered owing to the presence of traces of formaldehyde in the water. For example, we observed strong interference with the triglyceride procedure of Moses et al. (11), the Technicon SMA 6/60 version of the glucose oxidase method, and the ammonia procedure based on the Berthelot reaction (2).

Standard solutions of ammonia prepared with de-ionized water containing traces of formaldehyde gave very little color with the Berthelot reaction. When the same solutions were analyzed in the aca, the nominal ammonia values were obtained, indicating that formaldehyde did not interfere with the enzymatic procedure. Further evidence that formaldehyde was the source of inhibition was obtained by adding known amounts of formaldehyde to a 500 μmol/L ammonia solution. Addition of 0.2 and 2.0 ml of 37% formaldehyde per liter decreased the absorbance of the standard by 30 and 85%, respectively, in the Berthelot reaction. We recommend that, after treatment of a water system with formaldehyde, the water be tested with chromotropic acid (12) to assure that it is formaldehyde-free.

**Discussion**

The precision, both within- and between-run, of the aca method was impressive. The SD was essentially constant (about 3 μmol/L) regardless of the NH₃ concentration. (Under the conditions of the aca assay 3 μmol/L of NH₃ corresponds to a ΔA of 0.00026.) However, one of us (B.T.D.) has heard some complaints from aca users with regard to ammonia packs. Poor precision and lack of linearity are occasionally encountered with a particular pack lot. We suggest that quality control on ammonia packs be improved, to eliminate such complaints and inaccuracies in analyses. The average analytical recovery by the aca method has, in general, slightly exceeded 100%. It is of interest that IJpma et al. (1) had a similar experience; the small bias observed does not affect the medical usefulness of the test.

We found no artifactual suppression or elevation of ammonia values by the aca method in samples containing abnormal concentrations of hemoglobin, bilirubin, or triglyceride.

The aca method gives results that compare very well with those by the method of Kingsley and Tager (Table 2) when the NH₃ concentration varies over a wide range.

We conclude from our study that the aca method provides accurate data.

Either EDTA or heparin is satisfactory as anticoagulant. Although we used tubes containing EDTA for most of the work, we think that heparinized Vacutainer Tubes are preferable because their NH₃ content is practically undetectable.

The advantages of the present method have been pointed out by IJpma et al. (1). We believe that these advantages outweigh the cost of the reagents (pack).

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**References**


New Technique for Solid-Phase Immunoassay: Application to Hepatitis B Surface Antigen

Hydow Park

I describe a new technique for improving the sensitivity of the solid-phase "sandwich" assay, by using the through-passage receptacle in a novel flow-communication device. The technique allows a large volume of serum to flow through the antibody-coated receptacle repeatedly during the incubation and is thus termed the "flow-through large-volume incubation" method. Binding of $^{125}$I-labeled hepatitis B surface antigen to its corresponding antibody on a solid-phase by this method was more rapid and persistent than binding by the conventional method. When the method was applied to the first incubation of the sandwich assay, the test for the antigen was rendered four-, eight-, and 32-fold as sensitive as an accepted third-generation test for the antigen, by incubating 5-ml volumes of serum at (a) room temperature for 18 h, (b) 45°C for 8 h, or (c) room temperature for seven days, respectively.

Additional Keyphrases: "sandwich" assay technique • "flow-through large-volume incubation" method • solid-phase assay

The "sandwich" assay technique, a solid-phase immunoassay, was first used by Ling et al. (1) for detecting HBsAg in serum. In this technique the control or test solution is added to a receptacle containing the anti-HBs bound to a solid phase. During the first incubation, HBsAg, if present in the test solution, binds to the solid-phase anti-HBs. The receptacle is then washed before radio- or enzyme-labeled anti-HBs is added for a second incubation. During the second incubation the labeled anti-HBs combines with the HBsAg bound to the solid-phase anti-HBs, forming an antibody-antigen-labeled antibody "sandwich." The receptacle is then washed, to remove unbound labeled anti-HBs. The remaining (bound) labeled anti-HBs provides a measure of the amount of HBsAg bound to the solid-phase anti-HBs.

Most commercial HBsAg test kits currently used in clinical laboratories are based on this principle, with various modifications in the procedure. In the procedures utilized in these commercial test kits, only a small portion of serum or plasma (0.2 or 0.5 ml of 2- to 6-ml sample) obtained by routine collection methods is used for the actual test. This paper describes a method for using substantially larger volumes of serum to increase the sensitivity of the assay.

Materials and Methods

Antibody to HBsAg (anti-HBs): Chimpanzee anti-HBs purified by the affinity column method was kindly supplied by Ortho Diagnostics, Raritan, NJ 08869. The stock solution had a protein concentration 0.5 g/L.

$^{125}$I-labeled anti-HBs, as supplied in RIAUSURE™; Electro-Nucleonics Laboratories, Bethesda, MD 20014.

Through-passage receptacle (TPR) (2): This funnel-like polystyrene structure (Figure 1A) has a longitudinal through-passage with a large aperture at the upper end and a small opening at the lower tip. The lower portion of the through-passage has a small space (0.1 ml) relative to the area (3.7 cm) of the inner surface because of longitudinally arranged fin-like structures protruding into the central space.

Antibody coating of TPR's. Each TPR was coated with 0.1