Measuring Albumin and Calcium in Serum in a Dual Test with the Hitachi 704

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We describe a method for simultaneously determining albumin, by using brom cresol purple, and calcium, by using Arsenazo III, in the same analytical cuvette on the Hitachi 704. Both assays agree well with accepted procedures. The standard curves for the albumin and calcium assays are linear from 0 to 60 g/L and 0 to 5.0 mmol/L, respectively. Calibration is stable for 7 days with use of open reagent in the instrument. Both assays are unaffected by hemoglobin ≤5 g/L and Intralipid ≤4 g/L; calcium is unaffected by bilirubin ≤60 μmol/L.

Additional Keyphrases: colorimetry • multianalyte assay

Recent developments in automated spectrophotometric instrumentation include the ability to simultaneously measure absorbances at several different wavelengths several times a minute. Many of the large analyzers currently available operate two-reagent chemistries with the same throughput as single-reagent chemistries; this has prompted the development of dual assays in which reagent 1 initiates one analysis and reagent 2 another. This approach has three main advantages: increasing the test throughput, broadening the test range on the instrument by making an extra channel available for every assay pairing, and reducing the total sample volume required. Already Boehringer Mannheim (Mannheim, FRG) offers combination assays of urea/glucose, cholesterol/triglycerides, and alanine/aspartate aminotransferases.

Albumin and calcium concentrations are frequently requested clinical analytes. Because ionized calcium is the metabolically active form and albumin is the principal ligand for calcium in plasma, the requests form a natural pair.

Albumin determinations by dye binding are carried out at pH 5.5 for brom cresol purple (BCP) (1) and pH 4.4 for brom cresol green (2). However, the widely used cresolphthalein complexone estimation for calcium is performed at pH 11.0 (3). Recently, Arsenazo III has been used as a complexing agent for measuring plasma calcium (4). This assay is usually carried out around pH 6.0, opening up the possibility of first measuring albumin with BCP at 600 nm, followed by measuring calcium with Arsenazo III at 660 nm. Here we describe conditions whereby both albumin and calcium can be sequentially measured in the same cuvette with the Hitachi 704.

Materials and Methods

Materials

PIPES, Arsenazo III, and bilirubin (from gallstones) were obtained from Sigma Chemical Co. (St. Louis, MO). Intralipid was supplied by Kabivitrum (Cahill May Roberts, Dublin, Ireland). Antiserum to albumin was supplied by Dako (Glostrup, Denmark). All other chemicals were Analar grade from British Drug House Ltd. (Poole, UK).

BCP reagent consisted of 50 mmol/L acetate buffer, pH 5.5, containing BCP, 120 μmol/L, and Brij 35 (30%) surfactant, 1 mL/L.

Arsenazo III reagent consisted of 1,4-piperazinediethanesulfonic acid buffer (50 mmol/L, pH 6.8) containing 500 μmol of Arsenazo III per liter.

Methods

We used a Hitachi (Tokyo, Japan) Model 704 automated analyzer. Given that a sample volume fraction of 0.01 is necessary for good linearity in the BCP assay and given the desirability of using the maximum sam-
ple volume to aid precision in the calcium assay, we decided to use 5 μL of sample and 500 μL of BCP reagent (R 1). Previous investigation (paper submitted) demonstrated that 1,4-piperazinediethanesulfonic acid with Arsenazo III is suitable for estimating serum calcium between pH 6.0 and 7.0. Also, to extend the linearity of the standard curve in the calcium assay to 5.0 mmol/L, the volume ratio of Arsenazo III to calcium in the analytical cuvette should be ~4:1 at the greatest calcium concentration. The maximum cuvette volume for the Hitachi 704 is 700 μL, so we decided to use 200 μL of 500 μmol/L Arsenazo III reagent. This yielded a final Arsenazo III concentration of 142 μmol/L. Because the original sample volume proportion was now reduced to 1/0.0071, the calcium concentration in the analytical cuvette was 5 μmol/L, reflecting a sample concentration of 5.0 mmol/L. The test settings are described in Table 1. Because these are straightforward, they are readily adaptable to other equipment.

Reference method for calcium. Samples were diluted in 1.0 g/L lanthanum chloride and assayed with a Pye Unicam (Cambridge, UK) SP 192 atomic absorption spectrophotometer in accordance with the procedure of Cali et al. (5). The within-run CV for this procedure was 0.85% (mean calcium = 2.28 mmol/L, n = 20).

Comparison procedure for albumin. Samples were assayed with the Baker (Allentown, PA) Encore analyzer by an immunoturbidimetric assay. After prediluting the sample 100-fold in isotonic saline (NaCl 150 mmol/L), we mixed 3 μL of diluted sample with 200 μL of Dako antiseraum that had been diluted 40-fold with polyethylene glycol (M<sub>n</sub> 6000), 40 g/L in isotonic saline. The absorbance change between 6 and 250 s at 290 nm was measured at 30 °C. The mean within-run CV was 2.51% (mean albumin = 37.5 g/L, n = 20). The calibrator used was Behring (Marburg, France) Immuno calibrator.

We calibrated the dual test on the Hitachi with Beckman (Beckman Instruments, Brea, CA) Decision III calibrant, using the manufacturer’s quoted values for albumin by BCP and calcium with Arsenazo III because the recovery of this material in the comparison procedures showed close agreement with the quoted values in the package insert.

Assessment of Analytical Variables

Addition of chromophores. Specimens of whole blood from 50 patients were pooled and the plasma was separated. To assess increasing hemolysis, we placed packed blood cells in an ultrasonic bath for 5 min to lyse the erythrocytes. The resulting hemolysate was added to the plasma pool to give various concentrations of hemoglobin. Hemoglobin was quantified as the cyanmethemoglobin derivative by the Technicon H1 (Technicon Instruments, Tarrytown, NY).

To assess the effects of lipemia, we added portions of a 20% Intralipid solution to a plasma pool to mimic lipemia. To assess icterus, we added various volumes of 100 mmol/L bilirubin solution in 100 mmol/L NaOH to the plasma pool. The addition of NaOH did not alter the pH of the pool by more than 0.1 pH units and had no effect on the assay. The dilutional effects of added bilirubin and Intralipid were compensated for by appropriate calculation.

Linearity. To evaluate the range of linearity of the standard curve for the calcium assay, we assayed a calcium chloride-supplemented plasma pool serially diluted with saline. For the albumin assay, we used a plasma pool concentrated with a Minicon (Danvers, MA) B15 concentrator to contain an albumin concentration of 60 g/L and serially diluted this with saline.

Imprecision. Two quality-control materials were measured consecutively 20 times to establish the within-run variation. To quantify the between-run variation, we assayed these again in duplicate each day for a month and calculated the variation from the second result, in accordance with the recommendations of the European Committee for Clinical Laboratory Standards (6).

Results

Correlation studies. For 150 patients’ samples (albumin range 14.0–50.1 g/L)—including samples from patients with renal failure, severe liver disorder, or hyperlipidemia—linear regression of the results by the proposed dual test (y) and by the immunoturbidimetric procedure (x) gave y = -0.36 + 0.974x (r = 0.95, S<sub>y|x</sub> = 2.15). The mean values for the dual test and the immunoturbidimetric procedures were 33.72 and 32.76 g/L, respectively. The mean dual-test albumin value for 20 patients with renal failure was 3.6 g/L lower (range +0.2 to -8.1 g/L) than the immunoturbidimetric mean, consistent with the findings of Maguire and Price (7). The mean dual-test albumin value for 20 patients with hepatic failure was 1.6 g/L lower (range +0.3 to +4.9 g/L), owing to the interference of covalently bound bilirubin (8). The mean value for 10 hyperlipidemic samples was 0.9 g/L higher (—0.5 to +2.7) by the dual test. The dual-test albumin results (y) also correlated well with those of a 10-s bromcresol green method (x) for nonrenal, nonhepatic samples: y = -0.911 + 1.02x (n = 50, r = 0.991).

The same samples assayed for calcium (range 1.04–3.71 mmol/L) by the proposed dual test (y) and by atomic absorption spectrophotometry (x) yielded y = 0.041 + 0.97x (r = 0.988, S<sub>y|x</sub> = 0.045). The mean values for the dual test and the atomic absorption methods were 2.329 and 2.357 mmol/L, respectively.

Analytical variables. The linearity of both assays was
greater absorbance of Intralipid at 600 nm than at 700 nm. No effect was apparent on the calcium assay (Table 2), because true blanking of the sample is part of the three-point protocol in the calcium assay.

Hemolysis demonstrated virtually no effect on the calcium assay. The albumin results were decreased by 0.1 g/L for every 1 g/L concentration of hemoglobin.

As Table 3 shows, within- and between-run variation was acceptable for both analytes.

**Table 3. Imprecision of Dual-Albumin and Dual-Calcium Procedures**

<table>
<thead>
<tr>
<th>Mean</th>
<th>Within-run CV%</th>
<th>Between-run CV%</th>
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</thead>
<tbody>
<tr>
<td>Albumin, g/L</td>
<td>37.2</td>
<td>0.77</td>
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<tr>
<td></td>
<td>44.7</td>
<td>0.69</td>
</tr>
<tr>
<td>Calcium, mmol/L</td>
<td>2.28</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>3.29</td>
<td>0.78</td>
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**Discussion**

Bromcresol green reacts rapidly with albumin but also binds to other proteins, particularly acute-phase proteins (9). Thus, for accurate measurement of albumin, the color change must be monitored within a few seconds of initiating the reaction. Because of the delays inherent in dual assays, we chose instead BCP, which is more specific for human albumin. Also the subsequent addition of Arsenazo III to bromcresol green caused spectral interference in the calcium assay not found with BCP.

Although Arsenazo III had been used to detect calcium transients (10), adaptation of the method for use with plasma samples is quite recent (4). The fact that the binding of calcium to Arsenazo III requires mildly acidic conditions, compared with a pH of 11.0 for the reaction with cresolphthalein complexone, opened up the possibility of combining albumin and calcium assays in the same cuvette.

The method we present gives accurate and precise results for both albumin and calcium. Because ionized calcium is the physiologically active moiety and albumin is the principal ligand in plasma (11), laboratorians frequently calculate "adjusted" calcium concentrations (12), based on this pair of results.

The conditions selected here could be readily adaptable to instrumentation other than Hitachi 704, provided the instrumentation has the software capability for dual testing.

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We investigated the ability of current immunometric methods for thyrotropin (TSH; thyroid-stimulating hormone) to distinguish between low-normal and subnormal hormone concentrations by using the data from an external quality assessment (EQA) survey in 1990. We computed the interassay (between-run) precision profiles from results from 101 laboratories, which used the five most popular kits in the survey; during the control period (one year) each laboratory assayed 4 EQA pools distributed (as hidden replicates) in five occasions. The interassay CV was relatively low (9–13%) for three pools in the normal TSH range (>0.8 milli-int. unit/L) but markedly higher (30–40%, except for one more precise kit) in the subnormal range (0.2 milli-int. unit/L). We calculated the effect of the between-run variability on the diagnostic accuracy (discrimination between normal and subnormal values) for three representative TSH concentrations: 0.2, 0.4, and 0.5 milli-int. unit/L (0.3 milli-int. unit/L was considered the lower normal limit). The three concentrations were reasonably discriminated (P<5%), and only one kit showed a between-run CV <18% at 0.2 milli-int. unit/L. For the other four less-precise kits, only the higher TSH value (0.5 milli-int. unit/L) could be classified with an acceptable diagnostic reliability. With the most precise kit, one can distinguish two TSH concentrations in the 0.3–0.5 milli-int. unit/L range that differ by at least 30%; with the other kits, differences greater than 50–60% are needed for reliable discrimination. Thus many laboratories fail to achieve the functional sensitivity of a second-generation assay, even if they use immunometric methods. TSH assays with a better interassay precision in the low concentration range are needed.

Additional Keyphrases: distinguishing low-normal from below-normal thyrotropin concentrations · thyroid status

During the past five years, the traditional competitive RIA methods for thyrotropin (thyroid-stimulating hormone, TSH) determination have been increasingly replaced by the so-called ultrasensitive or supersensitive immunometric assays (IMAs) based on noncompetitive “two-site” technology. Almost all of these methods use monoclonal antibodies to extract TSH from the serum samples (solid-phase “capture” antibody) and to quantify the bound analyte (“tracer” antibody). The label for the tracer antibody may be either 125I or a nonradioactive molecule, e.g., enzymes, fluorescent dyes, and chemiluminescent compounds. Commercially available kits based on IMA technology are generally presented as being ~10-fold more sensitive than the traditional competitive RIAs (detection limits of ≤0.1 milli-int. unit/L versus 0.6–1.0 milli-int. unit/L, calculated either from within-run replicate

References