Automated Determination of Serum Ceruloplasmin Activity with o-Dianisidine Dihydrochloride as Substrate

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An automated method for the enzymatic determination of ceruloplasmin with o-dianisidine dihydrochloride as substrate is described. The method enables the measurement of 30 samples per hour with a coefficient of variation (day-to-day) of 2.8%. Results correlate well (r = 0.99) with those obtained by the corresponding manual method.

Additional Keyphrases: continuous-flow analysis • assessment of fetal status • enzyme activity

We have recently developed and described an enzymatic method for measuring serum ceruloplasmin (ferroxidase, EC 1.16.3.1) activity, in which o-dianisidine dihydrochloride was the substrate (1). This compound offers several advantages over the widely used substrate for ceruloplasmin assay, p-phenylenediamine (2), in that it is not sensitive to nonenzymatic catalytic oxidation, it is stable over long time periods, and the final colored product for the reaction is stable, thereby greatly facilitating accurate spectrophotometric measurement.

Sharp changes in sequential serum ceruloplasmin concentrations in “high-risk pregnancies” indicate changes in fetal status (3). In a preliminary clinical study to evaluate the feasibility of serum ceruloplasmin assays as a means of monitoring fetal status, we made such measurements on 67 women whose pregnancies ultimately resulted in premature births, stillbirths, fetal distress, or pre-eclampsia (4). Abnormally high serum ceruloplasmin activities (as compared to values at equivalent stages of gestation in normal pregnancies) were found to occur early in a significant number of these “problem” pregnancies, in some cases as early as the third month of pregnancy. The present method of choice used by obstetricians to monitor “high-risk pregnancies” is determination of urinary estriol (5). The amount of estriol in urine increases during pregnancy but only by the third trimester reaches values from which clinically significant conclusions may be drawn (6).

Our preliminary data indicate that measurement of ceruloplasmin activity in serum during the first trimester of pregnancy may help detect problems very early in gestation, and has the added advantage of being technically easier to perform than urinary estriol. We were therefore interested in developing a method that would be suitable as a screening method. Here, we show how the manual serum ceruloplasmin assay (1) can be adapted to continuous-flow automation on a single-channel AutoAnalyzer.

Materials and Methods

Reagents

Acetate buffer solution was prepared as previously described (1), except that 0.2 ml of isooctyl phenoxypolyethoxethanol (Triton X-100) was added to each liter of buffer.

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Sulfuric acid (9 mol/liter) and o-dianisidine dihydrochloride reagent (7.88 mmol/liter) were prepared as described previously (1).

Calibration sera. High- and low-activity serum pools can be prepared from sera collected from pregnant women and from normal subjects, respectively. A pool of intermediate activity should then be prepared by mixing different volumes of these pools to obtain the desired activity.

Method

The manifold and schematic flow diagram for the AutoAnalyzer I (Technicon Instruments Corp., Tarrytown, N. Y. 10591) are shown in Figure 1.

A Technicon Sampler II is used, sampling at a rate of 60 specimens per hour, alternating samples with water, and with a sample-to-water ratio of 2:1. The recorder baseline is adjusted to 99% transmittance with the reagents pumping through the system. Specimens are mixed with buffer in a 14-turn standard mixing coil before they are combined with the substrate. The buffer, sample, and substrate are then incubated at 45 °C in a previously siliconized 14-turn standard mixing coil. The enzymatic reaction is stopped by adding sulfuric acid, 9 mol/liter, which also increases the sensitivity, dissolves the yellow-brown colored product (7), and decreases turbidity. The purple-red product (7) formed after acidification is measured at 550 nm in a 15-mm flow cell. After each run the system is cleaned by pumping sulfuric acid (9 mol/liter) through all the lines and coils, followed by de-ionized water.

An analytical run consists of three calibrating sera (in duplicate) followed by a control serum and the samples. We recommend that the control serum be run after every five samples to monitor baseline drift. We found it necessary to insert a water cup between the serum samples to prevent carryover and to help minimize baseline shift (see Discussion).

Results

Figure 2 shows a typical calibration curve with nondiluted sera of different activities. No significant variation between peaks of replicate samples was noticed.

Within-day precision of the automated method was assessed by using three sera having high, intermediate, and normal activities. Each run consisted of the three sera assayed in a different order, in duplicate. In this way we could determine the effect of different concentrations of ceruloplasmin on the peak heights of the samples that followed. The results (Table 1) illustrate that carryover is negligible. Baseline correction insignificantly improves the precision, so it is not necessary to perform this correction.

Day-to-day precision was assessed by running the same three samples on each of 12 consecutive days. The results (Table 1) show good precision over this time period.

The method was "standardized" on the AutoAnalyzer by assaying three pooled human sera (nonhemolized, nonicteric and nonlipemic) having normal, medium, and high ceruloplasmin concentrations, as measured (in triplicate) by the manual method (1). Figure 3 shows a typical calibration curve. The relationship was linear to at least 450 U/liter.

2 The mixing coil was cleaned by introducing a 0.5 mol/liter solution of NaOH into the coil. After rinsing thoroughly with water, a hot aqueous solution (10 ml/liter) of "Siliclad" (Clay-Adams, Parsippany, N. J. 07054) containing 3 drops of NH₄OH was aspirated into the coil for 5 s, followed by rinsing thoroughly with water and drying in an oven (100 °C for 10 min). The process should be repeated when needed (usually indicated by baseline instability).

Fig. 1. Flow diagram for continuous-flow enzymatic determination of serum ceruloplasmin

D1 and H2 are connectors as designated by Technicon Corp. Standard mixing coils used: 1, 28-turn; 2, 14-turn (siliconized); 3, 14-turn

![Diagram of flow diagram for continuous-flow enzymatic determination of serum ceruloplasmin](image)

Fig. 2. Recorder tracing obtained with the AutoAnalyzer for sera with different ceruloplasmin oxidase activities

![Recorder tracing obtained with the AutoAnalyzer for sera with different ceruloplasmin oxidase activities](image)

Fig. 3. Typical standard curve obtained for the automated method

![Typical standard curve obtained for the automated method](image)

Table 1. Precision of Ceruloplasmin Assay by the Automated Method

<table>
<thead>
<tr>
<th>Serum activity</th>
<th>Mean U/liter</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within-day</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>133</td>
<td>4.7</td>
<td>3.5</td>
</tr>
<tr>
<td>Intermediate</td>
<td>219</td>
<td>5.7</td>
<td>2.6</td>
</tr>
<tr>
<td>High</td>
<td>434</td>
<td>3.9</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Day-to-day</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>135</td>
<td>3.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Intermediate</td>
<td>219</td>
<td>4.1</td>
<td>1.8</td>
</tr>
<tr>
<td>High</td>
<td>351</td>
<td>4.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

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Fig. 4. Correlation between results by the manual (1) and automated methods for serum ceruloplasmin oxidase activity

Discussion

The advantages of o-dianisidine dihydrochloride over p-phenylenediamine as substrate for the enzymatic determination of ceruloplasmin have been described (1). Thus many of the precautions necessary to obtain clinically useful results from the automated p-phenylenediamine procedure (8) are not necessary in this case.

A major problem in using o-dianisidine dihydrochloride was deposition of the yellow-brown oxidation product in the incubation coil, resulting in baseline drift and significant carryover between samples. The use of a siliconized 14-turn mixing coil for the incubation, and placing cups containing de-ionized water between the samples corrected both of these deficiencies so that there was no significant deposition of the product on the coil walls and baseline drift and carryover were negligible. Washing the coil with sulfuric acid (9 mol/liter) after each run and changing the coil whenever an unstable baseline was observed ensured that results did not significantly differ from those obtained by the manual method.

The decreased sensitivity resulting from use of the short incubation coil was compensated for by increasing the incubation temperature to 45°C. At this temperature there is no heat inactivation of ceruloplasmin activity (4), and the rate of the enzymatic reaction was increased so that samples in the range 50–450 U/liter (90–40% transmittance) could be measured with good precision and without diluting high-value sera.

The close correlation (Figure 4) between results by the manual and the automated procedures for sera, including some that were slightly hemolysed and others that were slightly turbid, indicates that blanks are not necessary. The intercept at 5 U/liter on the abscissa in Figure 4 shows that the lag phase, which is significant in the manual procedure (1), is negligible in the automated procedure, probably because of the high temperature used here.

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References


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