Semiautomated Method for Serum Pepsinogen Determination

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An automated fluorometric determination of tyrosine has been adapted to the measurement of pepsinogen in body fluids. Preparation of the hemoglobin substrate and methods of incubation are similar to those previously described. The major technical improvement is the elimination of precipitation and filtration; instead, dialysis has been incorporated into the automated procedure for determination of tyrosine and tyrosine-containing peptides. This method provides a reliable, simple technique for use with large numbers of serum specimens.

Our method eliminates the need for precipitation and filtration; dialysis is incorporated into an automated procedure for the determination of the amino acids released from the substrate. The choice of a substrate depends in part on the peptides that pass through the dialysis membranes.

Method

Serum is incubated with acidified hemoglobin substrate (11–14), and the mixture is then dialyzed in the AutoAnalyzer. Since human serum contains several interfering substances, an unincubated control determination is required for each serum specimen. Each specimen is mixed with a neutral hemoglobin mixture (pH 7.0) and remains at 4°C until assay. Blanks containing distilled water are also incubated with the acid hemoglobin substrate.

The automated procedure is a modification of a manual fluorometric method (15). Tyrosine and tyrosyl peptides are coupled to 1-nitroso-2-naphthol to form a fluorescent compound. We remove excess nitroso-naphthol by extraction with dimethylsulfoxide, rather than with methylene dichloride as in the manual method (16).

Preparation of Substrate

A solution containing 2.5 g of bovine hemoglobin substrate powder (Pentex Inc., Kankakee, Ill.) per 100 ml is prepared in distilled water containing 0.5 g neomycin sulfate per liter (to inhibit bacterial growth). After the solution is stirred for 3 to 5 h with a magnetic stirrer it is kept frozen at −20°C in plastic bottles. Five volumes of this hemoglobin...
solution is mixed with four volumes of acidified water to give a substrate with a pH of 1.5 (13).

Incubation of Samples

Serum specimens are stored at −20°C until studied; gastric juice requires additional preservatives (17). To 0.5 ml of serum is added 4.5 ml of acidified substrate mixture, measured with an Autodilutor. Disposable glass test tubes are used to avoid inadvertent contamination by detergents. Each test tube is stoppered and incubated for 24 h at 37°C. Blanks of distilled water, serum specimens of known strength, and two commercial reference serum specimens are processed similarly. After incubation, all tubes are placed in ice water until analysis. The neutral hemoglobin specimens are not incubated, but are stored at 4°C until the time of assay.

Reagents

All reagents are reagent grade except as noted.

Diluent. To 250 ml of dimethylsulfoxide, add 750 ml of distilled water and 2.0 ml Triton X-405 (Technicon Corp., Tarrytown, N. Y.), mix, and filter. Stable for two weeks at room temperature.

Recipient solution. To 250 ml of dimethylsulfoxide, add 0.25 g of 1-nitroso-2-naphthol (P grade, Eastman Organic Chemicals, Rochester, N. Y.), mix, and add 750 ml distilled water and 2.0 ml of Triton X-405. Mix and filter. Stable for two weeks at room temperature.

Nitrous acid-sodium nitrite mixture. Dilute nitric acid is made by adding 1 volume of nitric acid to 5 volumes of distilled water. To 98 ml of this, add 2.0 ml of sodium nitrite, 2.5 g/100 ml, which must be freshly made each day.

Sodium metabisulfite. A solution of 15 g/100 ml is made in distilled water, filtered, and stored for up to one week at 4°C. More rapid and efficient filtration is possible by use of a Millipore filter and a vacuum pump. This solution is stable only for one day.

Wash solution. Add 2.0 ml of Triton X-405 to each liter of distilled water.

Standards. A stock standard is prepared by dissolving 0.05 g L-tyrosine (Merck) in 500 ml 0.1N HCl. This solution is stable indefinitely when stored at 4°C. Working standards may be diluted and prepared as needed and discarded at the end of the day or may be frozen in stoppered AutoAnalyzer cups and thawed as needed. The following standards are prepared by dilution of the stock

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**Fig. 1.** Flow diagram for automated tyrosine determination applied to the AutoAnalyzer

The tubing specified permits aspiration of a 1.6 ml of wash solution.
standard with distilled water: 100, 200, 300, 400, 500, 1000, 1500 μg/100 ml.

Normal clinical chemistry reference serum, un-assayed, (Hyland Div., Travenol Laboratories, Los Angeles, Calif.).


**Equipment**

*AutoAnalyzer Sampler II*. Equip with a cam so that it aspirates 30 specimens per h and follows the specimen with an equal volume of wash solution.

*AutoAnalyzer fluorometer*. Primary filters are 47 B and 2 A (460 nm), secondary filter No. 16 (570 nm), aperture setting 30X. Light source No. 110-853 lamp with No. 110-856 lamp adaptor (G. K. Turner Associates, Palo Alto, Calif.). Slits and (or) neutral density filters may vary, but a No. 3 slit with a 90% neutral density filter seemed optimal.

*AutoAnalyzer recorder*, *proportioning pump*, *dialyzer*.

**Procedure**

The flow diagram is presented in Fig. 1. A hood is necessary because of the hazard of fumes emitted by dimethylsulfoxide. The equipment is turned on and all lines are filled with water for 30 min. When temperature of the heating bath is 70°C, the reagent lines are placed in their proper containers and cold tap water is allowed to fill the jacketed mixing coil. When the baseline stabilizes, it is set at a transmission of approximately 5%. The peak line is set at approximately 85% transmission by continuous aspiration of the 1500 μg/100 ml standard, or by samples of this standard, separated by sampler cups of distilled water. Six or seven standards are then placed on the sampler plate followed by two cups of distilled water. A typical standardization record is illustrated in Fig. 2.

It is necessary to prime the machine with hemo-globin substrate mixture after the distilled water and to ignore the first few readings. No water should be added between specimens hereafter. All the neutral substrate specimens are placed on the
Table 1. Effect of Incubation Time on Serum Pepsinogen Determinations

<table>
<thead>
<tr>
<th></th>
<th>6 h</th>
<th>12 h</th>
<th>18 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum from a volunteer subject</td>
<td>147</td>
<td>324</td>
<td>413</td>
<td>560</td>
</tr>
<tr>
<td>Pooled serum 1 from hospital patients</td>
<td>412</td>
<td>585</td>
<td>760</td>
<td>1000</td>
</tr>
<tr>
<td>Pooled serum 2 from hospital patients</td>
<td>181</td>
<td>265</td>
<td>382</td>
<td>560</td>
</tr>
<tr>
<td>Normal commercial reference serum (Hyland)</td>
<td>118</td>
<td>206</td>
<td>353</td>
<td>441</td>
</tr>
</tbody>
</table>

* S, standard method; A, semiautomated method.

Sampler plate at once. Following this, 10 to 15 acid substrate specimens are placed on the sampler plate; the unused acid substrate mixture is returned to the ice bath (if the acid substrate mixture is kept cool, later readings for these specimens show no change in enzyme activity). Since peaks separate excellently, it is necessary to repeat only those samples preceded by very high readings. Samples with concentrations too high to read may be diluted with a mixture of 9 ml acid substrate and 1.0 ml of distilled water and serum. After all analyses are completed, water is pumped through the system until all lines are cleared of reagents. Figure 3 shows a typical record including controls and incubated specimens.

Calculations are simplified by expressing the tyrosine concentration in μg/100 ml. Since there is a 1 to 10 dilution of serum into the substrate mixture, the following equation is valid: \[ \text{[(μg acid sample - μg neutral sample) - (μg acid water blank - μg neutral water blank)]} \times 10 = \text{μg tyrosine liberated at pH 1.5 in 24 h at 37°C per ml serum}. \]

Results

Several techniques were used to compare the semiautomated method with the standard colorimetric method (13).

Various incubation times permitted such a comparison in four serum specimens of known enzyme concentration. The results (Table 1) are expressed in the standard manner for all methods that use a hemoglobin substrate: micrograms of tyrosine released per ml of serum, for a specified incubation period. These studies show a linear increase of tyrosine with increasing periods of incubation using both methods, after the initial 6-h incubation period. Other investigators report similar results (9, 14).

Results by this method were compared with the standard method by statistical analysis of duplicate determinations on specimens from 293 consecutive hospital patients (Fig. 4). The automated method appears to measure the same proteolytic enzyme in serum as the standard technique, but the mean results with the automated method are

![Fig. 4. Distribution of 293 consecutive serum pepsinogen determinations performed by standard colorimetric method](image)
approximately 100 µg lower than with the standard colorimetric methods (11–14).

Discussion

This automated method of tyrosine determination avoids the laborious step of protein precipitation and filtration to measure the concentration of amino acids released from the hemoglobin substrate. The substitution of dialysis for filtration provides simplicity, and the automated method supplies a written record of the result. As with other AutoAnalyzer techniques, the next result after a very high reading may be inaccurate. This can be remedied by reversing the order of specimens when duplicates are determined.

Results of these serum pepsinogen studies indicate that 6-h or 18-h incubation periods may be satisfactory for most clinical purposes. Our studies and those of others indicate that this time relationship is linear after the first 6 h. Recalculation of previously published results, using this information, might be useful. The mean value of the automated method is approximately 100 µg/ml lower than that of previously reported methods, but the standard deviations are comparable. In our studies, we assumed 240 to 700 µg pepsinogen per ml of serum to be the normal range, but studies of larger population groups are needed before we can confirm this.

The proteases in human serum have never been systematically studied. It is unlikely that all of the proteolytic activity measured by this method is produced by pepsinogen (8, 11, 12, 14). The origin of serum proteolytic enzymes from the gastric mucosa and their relationship to diseases of the stomach have been described by many authors. However, the simplicity of this method permits studies of serum proteolytic enzymes in large numbers of patients.

The assistance of Marian Sundy is gratefully acknowledged.

References