Three Methods Compared for Determination of Pancreatic and Salivary Amylase Activity in Serum of Cystic Fibrosis Patients

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We evaluated three methods for serum amylase (EC 3.2.1.1) isoenzymes to determine whether they are interchangeable and to test their ability to discriminate between cystic fibrosis patients with and without pancreatic insufficiency. One method involved salivary amylase inhibitor (O), and two were polyacrylamide gel electrophoresis separations differing in method of detection—either direct zymogram (G) or gel slicing followed by activity estimates per slice (W). Results for percentage pancreatic amylase differed significantly. Reproducibility for percentage pancreatic amylase was high, moderate, and low (r = 0.95, 0.53, and 0.02) for methods G, O, and W, respectively; moderate (r = 0.60) among the three methods; and moderate between pairs. Therefore, this result for a subject must be considered relative to the method used in its determination. The clinical diagnosis of pancreatic insufficiency was verified by 77.8%, 83.3%, and 94.4% correct classification rates for methods O, W, and G, respectively. Evidently, method G is the most efficient and may be the method of choice for measuring serum amylase isoenzymes in cystic fibrosis.

Additional Keyphrases: isoenzymes • enzyme inhibition assay • electrophoresis, polyacrylamide gel

Values for ß-amylase (EC 3.2.1.1) in serum have a long history of usefulness as a diagnostic adjunct in assessing exocrine gland function. More recently, the diagnostic specificity of serum amylase activity was improved by the fractionation of total serum amylase into isoenzymes of pancreatic (P) and salivary (S) origin based on chromatographic, electrophoretic, or inhibition methods (1–3). A The P isoenzymes are a post-translational product of the amy3 gene and are specific to the pancreas (4, 5). The S isoenzymes, post-translational products of the amy1 gene, are primarily a product of the salivary glands, but other organs also are known to produce them (6). Abnormal serum P activity (high or low) provides strong evidence for pancreatic exocrine dysfunction. Abnormal S activity usually indicates salivary gland involvement but also reportedly is increased in cases of severe lung disease (6) and ovarian cancer (7).

Methods for the quantification of serum P and S isomylase activity have been too time-consuming and exacting for routine clinical application (8, 9). A method developed by O'Donnell et al. (1) is now commercially available and gives promise of being more suitable for clinical application (10). Our aim in this study was to compare this method with the methods of Wolf et al. (11, 12) and of Gillard (2) for isomylase assay, for the purpose of rating their diagnostic value in cystic fibrosis (CF). The method of Wolf et al. (W), the first to be applied for evaluation of pancreatic exocrine function, separates isomylases by polyacrylamide gel electrophoresis (PAGE) and measures enzyme activity in gel slices (13). In the Gillard (G) method, amylase isoenzymes also are separated by PAGE, but enzyme activity is evaluated by direct staining and densitometry of the gel (2). The higher sensitivity of this method allows determination of normal reference intervals for isomylases in serum of infants and children (14, 15). The O'Donnell (O) method resolves pancreatic from salivary amylase isoenzyme activity by exploiting an inhibitor in wheat that is specific for the salivary isomylase.

In the present study three different laboratories assessed aliquots of the same serum specimens by the method routinely used in that laboratory, thereby more closely approximating normal clinical practice. Comparability of laboratories was assessed by having each laboratory report a value for total amylase for each sample, arrived at by each by the same method: if such values are closely similar, the likelihood is increased that any differences between methods are really method-related differences rather than laboratory-related differences.

We selected sera from control subjects and patients with CF, with and without clinical pancreatic insufficiency, to provide a range of pancreatic activity from low to above-normal, and to test the ability of each method to discriminate between samples from patients with and without pancreatic insufficiency.

Materials and Methods

Samples. A 20-mL specimen of blood was obtained by venipuncture from each of 35 subjects (ages six to 37 years) after obtaining informed consent. These were grouped by clinical criteria into three groups (16). Twelve of the subjects had no known abnormalities, 12 and 11 were patients diagnosed as having CF with and without pancreatic insufficiency, respectively. The serum from each sample was promptly divided into 1-mL aliquots, frozen, and stored at −40 to −70°C. Each aliquot was coded so as not to be identifiable by the assayers. Three sets, each with 70 coded-and-frozen 1-mL aliquots (i.e., each serum in coded duplicate), were collected. One set was sent to each laboratory for determination of total amylase activity and of the percentage of total amylase that was pancreatic enzyme (%P) by their method of expertise. The results were collected, decoded, and analyzed.

Total amylase determination. The total amylase activity of each serum aliquot determined on the "Phadebas® Amylase Test" method (Pharmacia Diagnostics, Pisc
cataway, NJ; batch no. FQ71962) scaled to one-fourth of prescribed volume. The same lots of Phadebas tablets and of amylase control serum (SeraChem, lot no. 306-010; Fisher Scientific Co., Orangeburg, NY) were used by all laboratories.

%P determinations. A. Method O (inhibitor method). A commercial kit (Phadebas Isoamylase Kit, gift of Pharmacia Diagnostics, Piscataway, NJ) was used as directed by the manufacturer. This method is based on the work of O’Donnell et al. (1) and involves an inhibitor protein in wheat extract with a 100-fold greater inhibitory effect on S isoamylase than on P isoamylase.

B. Method W. Serum amylase isoenzymes in aliquots containing 8 mU of total amylase activity were resolved by tube PAGE as previously described (13). The gels were then sectioned and the amylase activity in each slice was determined by using the Phadebas assay for total amylase.

C. Method G. Amylase isoenzymes in 10 µL of serum were resolved by PAGE and quantified by scanning the gels with a densitometer after specific staining for amylase activity as previously described (2).

Statistical analysis. All aliquots were analyzed in duplicate for both total amylase and %P. In addition to %P, the ratio P/S (where S = 100 - %P) was used in the analysis. For a few subjects, values were missing for either total amylase or %P: two normal persons, three CF patients with pancreatic insufficiency, and one without. This necessitated randomly excluding two additional subjects (one normal person and one CF patient without pancreatic insufficiency) to obtain equal numbers of subjects in the three groups, thus making the analysis much easier to perform and interpret. Consequently, we had complete data for nine subjects per group, on which all statistical analyses are based.

We used analysis of variance methods to compare the precision and degree of reproducibility of each laboratory and each method and to evaluate the interchangeability of methods. A complete analysis of sources of variation for each method and among the three methods will be presented elsewhere (Kingman et al., in preparation). We evaluated the comparability of participating laboratories by using their values for total amylase determination. The precision of a determination made within a laboratory is defined as the "repeatability standard deviation" of replicate determinations made in that laboratory (17). The reproducibility of a measurement was evaluated by using intraclass correlation coefficients (18). These correlation coefficients assess the magnitude of the variation between subjects as a percentage of the total variation in the data and are analogous to the "reproducibility standard deviation" given by Steiner (17).

The 18 CF patients were classified by their %P values for each method as compared with the normal (n = 9) %P mean and standard deviation (Table 4 below). Those CF patients having %P values that were significantly lower (>2 SD) than normal were classified as having pancreatic insufficiency and those in the normal range or above as not having pancreatic insufficiency. This classification based on %P values was then compared with the clinical assessment of pancreatic function.

Results

Total Amylase

Table 1 lists the means and standard deviations for total serum amylase obtained by each laboratory. The mean values for the normal individuals are within the expected range published by the test kit manufacturer (70–300 U/L). Although differences among mean values for total amylase for these laboratories were statistically significant (p = 0.001), the smallest mean value for total amylase was only 10% less than the largest mean value. The laboratories obtained consistent ordering in total amylase values for the three groups and also over the entire spectrum of total amylase values represented by these subjects (Figure 1). Figure 2 depicts the results for pairs of laboratories and subject type.

The within-laboratory precision levels for a single total amylase determination were 38.5, 34.0, and 11.0 U/L, respectively, for laboratories O, W, and G. The reproducibility values (intraclass r) for a laboratory making a single total amylase determination were 0.86, 0.92, and 0.99 for the laboratories using methods O, W, and G, respectively.

%P Amylase

The mean values for %P, and their standard deviations, are presented in Table 2 for all 27 subjects by method and subject type. Analysis of variance indicates the values differed significantly for the three methods (p = 0.021), but the differences were not of a consistent magnitude over individual subjects (p <0.001) or for a group (p <0.001). For subjects 1, 15, and 28, abnormally large differences were observed. Figure 3 illustrates %P values for individual subjects within each patient type.

Figure 4 shows correlations of %P values for pairs of methods. Values obtained by the O method were consistently larger than by the W method for normal individuals and CF patients without pancreatic insufficiency, but were similar to values for CF patients with pancreatic insufficiency (Figure 4a). The O method generally produced larger values for %P than did the G method (Figure 4b). The W method produced larger values for %P than did the G method for CF patients with pancreatic insufficiency, but similar values for

Table 1. Total Amylase (U/L) by Laboratory and Subject Type

<table>
<thead>
<tr>
<th>Method</th>
<th>Normal</th>
<th>CF with PI</th>
<th>CF without PI</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>257 (51)*</td>
<td>299 (72)</td>
<td>329 (131)</td>
<td>265 (101)</td>
</tr>
<tr>
<td>W</td>
<td>245 (60)</td>
<td>201 (81)</td>
<td>327 (152)</td>
<td>257 (114)</td>
</tr>
<tr>
<td>G</td>
<td>274 (67)</td>
<td>219 (81)</td>
<td>378 (175)</td>
<td>290 (132)</td>
</tr>
</tbody>
</table>

*Mean (and SD) of the average total amylase values per subject. PI, pancreatic insufficiency.
normal persons and CF patients without pancreatic insufficiency (Figure 4c). Statistical analysis indicates no pair of methods is interchangeable: the correlation coefficients (r) for pairs of methods were 0.44, 0.76, and 0.59, respectively, for O and W, O and G, and W and G.

Table 3 shows the within-method precision for making a %P isoenzyme determination, by type of subject and overall for each method. Within-method precision was best for method G, which also produced the most consistent precision over the three types of subjects.

The reproducibility levels within a specific method for a %P determination were 0.95, 0.53, and 0.02 for the G, O, and W methods, respectively.

%P as an indicator of pancreatic insufficiency. The correct classification percentages for the specific methods are presented in Table 4. The highest correct classification rate, 94.4%, was with the G method.

### Table 2. %P Amylase by Method and Patient Type

<table>
<thead>
<tr>
<th>Method</th>
<th>Normal</th>
<th>CF with Pi</th>
<th>CF without Pi</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>54.8 (13.6) *</td>
<td>25.8 (17.8)</td>
<td>53.4 (20.5)</td>
<td>44.7 (21.7)</td>
</tr>
<tr>
<td>W</td>
<td>47.1 (10.8)</td>
<td>24.9 (13.7)</td>
<td>47.8 (7.8)</td>
<td>39.9 (15.1)</td>
</tr>
<tr>
<td>G</td>
<td>45.8 (9.8)</td>
<td>15.0 (14.0)</td>
<td>48.5 (6.4)</td>
<td>36.8 (18.7)</td>
</tr>
</tbody>
</table>

*Mean (and SD) of the %P values per subject.

### Discussion

Values obtained for amylase isoenzyme activity in serum are affected both by factors influencing the balance between P and S amylase in serum and by factors related to the method of assessment. The former include the site and rate of production or endosecretion, renal excretion (known to be different for P and S isoamylases), and catabolic rate, primarily in the liver (19). Variables associated with the assessment of amylase activity include choice of substrate, detection method (amylolytic, saccharogenic, etc.), presence of interfering enzymes (20), and the presence of inhibitors or enhancers (21). The profusion of assay methods in use [since 1831, more than 200 methods have been reported for measuring amylase activity (22)], coupled with the variety of units for reporting amylase activity, makes interlaboratory comparison of amylase values difficult. Comparisons of P and S ratios determined by different methods may be more difficult because of the variety of methods used to resolve P and S isoamylases.

The statistical comparisons of the three methods indicate that the %P values obtained differ both in precision and in absolute values. Although the two electrophoretic methods (G and W) gave the most similar average values, method G was significantly more precise and reproducible. Method O
produced %P values significantly higher (18%) than those for G. Thus, no pair of methods produced interchangeable results.

The lowest within-method reproducibility value—0.02, obtained for method W—is explicable, in part, by two factors inherent to this method. First, the 1.6-mm gel sections often divided a single isoamylase band between two or more sections, there being no exact method of indexing the polyacrylamide gel column with respect to the invisible amylase bands. Secondly, the constant current used in the electrophoresis produced an internal V-shaped protein configuration (post-study observation on longitudinally sliced tube gels), with the apex at the longitudinal center pointed in the direction of migration. Even for a slice perfectly oriented with respect to the outer gel surface, the inner portion could contaminate the adjacent slice. Method G, which involves no gel slicing, avoided these complications; and because the stained starch is only on the outer portion of the gel, resolution is better.

The higher %P values obtained by method O vs G are probably related to the different properties being assayed. The %P in the O method is obtained by partial inhibition of the S amylase activity and estimates the pancreatic portion as the difference between total and S activity. The mechanism of amylase inhibition by the wheat albumin is not known but is thought to involve preferential binding to the S molecule at a site other than one of the catalytic sites (22-24). Rammeloo et al. (25) have reported that the changes in serum isoamylases after aging for three weeks at 4 to 8 °C were recognized as altered migration by electrophoresis (method G and W) but were not detected by the inhibition method (method O). It is then not surprising that a method based on inhibitor binding (probably to noncatalytic sites) vs one involving physical separation based on molecular charge, size, and shape of the P and S isoamylases might give different estimates of %P.

Because method O is a simple, easily executed method, additional studies comparing it with method G may be warranted, to correct or adjust the %P value one obtains for a subject by the O method as it becomes equivalent to the corresponding value that would have been obtained from the more time-consuming G method (Figure 4b). Rammeloo et al. (25) reported a 10% higher value for method O relative to agarose electrophoresis. Good correlations, but not necessarily higher values, were reported for the O method vs cellulose electrophoresis (8, 9), isoelectric focusing (5), and chromatography (10, 26).

In our hands method O agreed with the clinical diagnosis in 77.8% of the observations, whereas methods W and G demonstrated 83.3% and 94.4% agreement (Table 4). By comparing results by three methods for the same samples, we were able to show that explicable differences exist between two closely related methods (G and W) and that the higher values obtained by method O might, with additional data, be usable for predicting the G method values. The concept of fitting the method to the task, as suggested by the work of Tuzhilin et al. (20), may be a reasonable step in using determinations of serum amylase P and S isoenzymes for clinical evaluation of exocrine gland function.

This research was supported in part by the National Institutes of Health NIH AM 25107, Cystic Fibrosis Foundation G 721 B, the Ramer Cystic Fibrosis Research Fund, and the Gwynne Hazen Cherry Memorial Laboratory. We thank Lee Goodglick and Loretta Licini Duff for excellent technical assistance, Barbara DeGraff and Sita Persaud for their most able assistance in manuscript preparation, and Pharmacia Diagnostics, Piscataway, NJ, for the gift of Phadebas Isoamylase Kita.

References

Table 3. Within-Method Precision Levels for %P isoamylase by Patient Type

<table>
<thead>
<tr>
<th>Method</th>
<th>Normal</th>
<th>CF with PI</th>
<th>CF without PI</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>7.8</td>
<td>11.2</td>
<td>7.4</td>
<td>9.0</td>
</tr>
<tr>
<td>W</td>
<td>6.9</td>
<td>20.7</td>
<td>5.4</td>
<td>13.0</td>
</tr>
<tr>
<td>G</td>
<td>1.9</td>
<td>2.1</td>
<td>2.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Table 4. Classification of 18 CF Patients by Isoamylase Method Compared with the Clinical Diagnosis

<table>
<thead>
<tr>
<th>Method</th>
<th>CF with PI</th>
<th>CF without PI</th>
<th>Total</th>
<th>% correctly classified</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>8/9</td>
<td>8/9</td>
<td>14/18</td>
<td>77.8</td>
</tr>
<tr>
<td>W</td>
<td>8/9</td>
<td>8/9</td>
<td>15/18</td>
<td>83.3</td>
</tr>
<tr>
<td>G</td>
<td>8/9</td>
<td>8/9</td>
<td>17/18</td>
<td>94.4</td>
</tr>
</tbody>
</table>

Fig. 4. Mean %P amylase for pairs of methods

CLINICAL CHEMISTRY, Vol. 32, No. 2, 1986 299