We studied a new single-step direct chromolytic method (Behring D.A.T.) for measuring urinary amylase (1,4-\alpha-D-glucan glucoamylase, EC 3.2.1.1) activity, comparing results with those by a similar, but two-step, procedure that requires an auxiliary (coupling) enzyme. The two methods gave virtually identical relative responses to purified human pancreatic and salivary amylases. Assay of four quality-control materials to evaluate the total (day-to-day) precision of the new method yielded CVs of 4 to 7%, similar to those of the comparison method for each of the four quality-control samples. Amylase activity was measured by both methods in 110 random (i.e., untimed) urine specimens. Linear regression analysis provided a slope and y-intercept of 0.947 and 4 U/L (x = comparison method, y = direct method), respectively, and a standard error of the estimate of 25 U/L for specimens in which the amylase activities ranged from 11 to 1465 U/L (mean = 358 U/L) by the comparison method. The mean rate of amylase excretion in 2-h timed urine specimens from 95 healthy volunteers, as measured by the new method, was 7.18 (SD = 3.18) U/h, and the nonparametric (95% confidence interval) reference interval was 1.6 to 15.2 U/h. We consider the new method a promising alternative to other kinetic assays that require the use of auxiliary enzymes.

Additional Keyphrases: reference interval • two-step procedure compared

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Received November 16, 1987; accepted January 4, 1988.

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We compared the performance of the D.A.T. method with that of the Pantrak method by using both to measure amylase activity in 110 urine specimens from hospitalized patients. We also generated a reference interval for urine amylase from the results for timed urine specimens from 95 healthy volunteers.

Materials and Methods

Reagents. Pantrak and D.A.T. reagents (supplied by Behring Diagnostics, San Diego, CA 92112) were reconstituted with de-ionized water according to specifications in the manufacturer’s inserts. Reagent vials were gently agitated for 10 min after reconstitution, then stored at 5 °C until use. One drop of “Wetting Agent W” (Technicon, Tarrytown, NY 10591) was added per 7.5 mL of reagent, as specified by the manufacturer. Reagents were freshly prepared daily.

Quality control. Dade’s “Monitol ES” (American Dade, Miami, FL 33152) Levels I and II, Beckman’s “Triad” (Beckman Instruments, Brea, CA 92621) Levels 1 and 3, and Fisher’s “UriChem” (Fisher Scientific, Orangeburg, NY 10621) Level I were used as quality-control materials. Monitol was reconstituted with diluent according to the manufacturer’s specifications; UriChem was reconstituted with 25 mL of de-ionized water. Triad requires no reconstitution. All five materials were assayed in duplicate at the beginning and end of each set of patients’ urine samples.

Apparatus and analytical methods. All assays were performed at 37 °C in an RA-1000 analyzer (Technicon Instruments Corp., Tarrytown, NY 10591). Instrument settings for Pantrak and D.A.T. assays differed only with respect to sample volume (8 and 6 μL, respectively), reagent volume (335 and 355 μL, respectively), and read-delay time (5 and 1 min, respectively). All specimens were sampled and assayed in duplicate.

Specimens. For comparison of the two methods, we used untimed urine specimens from urines submitted to the University of Virginia Hospital Clinical Laboratories for routine or urgent (“stat”) urinalysis. Approximately half of the 110 specimens were selected without conscious bias; the rest were screened for above-normal amylase activity in order to expand the distribution of data for comparison. Screened specimens were selected on the basis of either above-normal relative density (specific gravity) or a clinical history of pancreatitis. Specimens were stored at 5 °C until analysis (no longer than two days). Before assay, we centrifuged the specimens at 1000 × g. Amylase activity was measured concurrently by the direct and comparison methods in a single RA-1000 instrument.

Subjects for the reference-interval study were selected from hospital employees who were without acute or chronic illness and were in apparent good health. The reference population consisted of 50 men and 45 women, with a mean age of 32 (SD 8) years (range: 21–59). Subjects were instructed to void and then collect the entire urine specimen approximately 2 h later, recording the exact time interval.

Table 1. Estimates of Total Imprecision in Our Day-to-Day Precision Study

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pantrak (n = 24)</th>
<th>D.A.T. (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monitol I</td>
<td>46 (7.3)</td>
<td>42 (5.0)</td>
</tr>
<tr>
<td>Monitol II</td>
<td>351 (3.5)</td>
<td>334 (3.7)</td>
</tr>
<tr>
<td>Triad 1</td>
<td>71 (7.0)</td>
<td>50 (6.0)</td>
</tr>
<tr>
<td>Triad 3</td>
<td>406 (3.8)</td>
<td>280 (4.2)</td>
</tr>
</tbody>
</table>

All specimen collections were begun and completed between 0600 and 1800. After the volume was noted, the amylase activity was measured by the D.A.T. method and amylase excretion was expressed as U/h in order to compensate for variable degrees of hydration.

Human pancreatic and salivary amylases were purified as described earlier (11).

Results

Table 1 gives day-to-day precision data for the direct (D.A.T.) method and for the comparison method. Precision was calculated by using the first of duplicate determinations for each of the four quality-control materials. Within-run CVs, based on the duplicates, were 3.5% or less in all cases (not shown). Fisher’s UriChem was deemed unsuitable as a quality-control material for urine amylase because of its instability over one or two days. The manufacturer provides no assay data for amylase activity in this product, although our results suggested that amylase activity in the freshly-prepared material was 100 to 130 U/L (by D.A.T.). Within-run precision for the urine control was acceptable by each method (for Pantrak: CV = 3%, n = 12; for D.A.T.: CV = 6%, n = 14).

Regression analysis of results from determination of amylase in untimed urine specimens by the direct (y) and comparison (x) methods yielded a slope of the regression line of 0.947 and a y-intercept of 4 U/L. The standard error of the estimate was 25 U/L. Amylase activities ranged from 11 to 1465 U/L, and the mean activity was 358 U/L (SD = 275 U/L) as measured by the comparison method. The mean and SD of the y-assay (direct method) were 343 and 267 U/L, respectively. Twenty-five of the urine specimens had amylase activities exceeding 500 U/L, for 15 it was >700 U/L, and for three it was >1000 U/L.

To assess the relative activities of the D.A.T. and Pantrak reagents toward the salivary and pancreatic amylase isoenzymes, we measured the activities of purified human isoenzymes by the direct and comparison methods. The activities of the salivary isoenzyme preparation were 346 and 322 U/L as measured by the direct and comparison methods, respectively. Corresponding values for the pancreatic isoenzyme preparation were 276 and 274 U/L.

Results of the reference-interval study are summarized in Figure 1. The mean units of amylase activity excreted per hour for the reference population was 7.2, with a standard
deviation of 3.2. The central 95% tendency of the range, assuming a gaussian distribution, was 0.9 to 13.5 U/h. The central 95% of the reference data, estimated nonparametrically, was 1.6–15.2 U/h. One value (35.2 U/h) was removed before data analysis; no pathological or pharmacological cause for the high urine amylase activity in this individual was apparent. The mean (and SD) urinary amylase activity (U/L) in the 95 specimens was significantly correlated with the calculated excretion rate (U/h): $r = 0.58$, $P < 10^{-9}$.

The stability of urine amylase was evaluated in urine specimens from three healthy individuals and from two individuals in whom above-normal amylase activity in serum and urine resulted from acute pancreatitis and a pancreatic abscess, respectively. Urine from healthy individuals retained at least 80% (mean = 89%) of its original amylase activity during five days of storage at 5 °C. Urine from the two patients with pancreatic disorders retained at least 84% (mean = 87%) of original amylase activity after five days at 5 °C.

Discussion

The present study indicates that the direct amylase test provides results that compare well with those obtained by use of the more familiar approach that requires a coupling (or auxiliary) enzyme. The elimination of auxiliary enzyme(s) in amylase assays and the use of a defined substrate have been recognized as desirable features for an amylase reference method (12). The direct and comparison methods were similarly precise (Table 1) and gave similar values for urinary enzyme activities. Moreover, their relative responses to purified human pancreatic and salivary amylases were essentially identical. It is not known, however, from these studies whether the new substrate in a different buffer would produce this same pattern of response to the two human isoenzymes. It is clear from the data in Table 1 that the two methods may give very different results with some control materials. Such differences in amylase activity measured in control materials by different methods have been noted before (13–15), and probably result from the biological sources of the amylase enzyme in the quality-control materials.

The stability of amylase activity in various samples is often important. An incidental finding of this study was that the amylase activity in the quality-control materials (with the exception of UriChem) was stable at 5 °C for at least 48 h, and amylase activity in several selected spot urine specimens from both healthy individuals and patients with pancreatic disorders appeared to be stable at 5 °C for at least five days after collection. Reportedly (16), amylase activity in serum from pancreaticitic patients is unstable, whereas activity in serum from healthy individuals is stable for 22 h at various storage temperatures. The data from our small sample did not indicate any difference in the stabilities of amylase activity in urine specimens from healthy subjects or individuals with pancreatic disease.

With few exceptions, amylase activities measured in fresh urine specimens by the direct and comparison methods agreed within a few percent, and linear regression analysis produced equivalent correlation data whether individual measurements or the means of duplicates were used in the analysis. In this study, regression analysis was based on the first of duplicate determinations. We conclude that there is no need, in routine use, to measure urinary amylase in duplicate.

Few recent reports include reference intervals for urinary amylase excretion rate, but our results compare closely with results previously obtained in our laboratory and with a reference range reported by Tietz (17) for a method that measures production of NADH in a multi-step reaction scheme. The non-gaussian distribution of reference values for urinary amylase excretion is in contrast to a previous study (18), which reported a normal (i.e., gaussian) distribution for 2-h urinary amylase activity in normal individuals.

Total urinary amylase (U/L) activity was a reasonable predictor of amylase excretion rate (standard error of the estimate = 2.6 U/h) in a healthy reference population; however, a greater error in such an estimation can be expected for a patient population, whose hydration status is much more variable.

The direct method for amylase appears to be a simple and precise alternative to other methods that require the use of auxiliary (or coupling) enzyme(s). The present studies of this method demonstrate that results for urine are comparable with those of the comparison method and provide a reference interval for the excretion rate for amylase in urine. These findings suggest that the new method will prove to be a useful aid in the differential diagnosis of hyperamylasemia and in the laboratory diagnosis of acute pancreatitis.

Regents and financial support for a part of this evaluation were provided by Behring Diagnostics, Division of American Hoechst Corporation.

References

Factors Influencing Fluorescence Spectra of Free Porphyrins

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We recorded fluorescence excitation and emission spectra of uro- and coproporphyrin under different experimental conditions, to see how these conditions influence quantifications based on measurement of fluorescence intensity. We found that, for bands \( \alpha \) and \( \beta \) of the emission spectra and the main peak of the excitation spectra, fluorescence depends on pH and is minimal near pH 5 and near pH 7–7.5 for copro- and uroporphyrin, respectively. For band \( \gamma \) of the emission spectra there was a constant decrease of fluorescence with increasing alkalinity of the solution. The intensity of porphyrin fluorescence also depends on ionic strength, reaching sharp maxima at 0.1 mol/L (for uroporphyrin) and 1 mol/L (for coproporphyrin). The organic mixture ethyl acetate:acetic acid (4:1 by vol), commonly used to extract porphyrins from biological samples, markedly diminishes the fluorescence of both porphyrins as compared with the same concentration of each porphyrin in aqueous acidic solvent. Furthermore, when we measured different ratios of uro:copro mixture at three distinct pHs and buffers, we found that at pH 10.5 (in carbonate buffer) the measured units of fluorescence depend only on total porphyrin concentration and not on the composition of the mixture.

Additional Keyphrases: diagnosis of porphyrias · determination of porphyrins in biological samples

The porphyrias are a group of diseases that have in common fundamental abnormalities in the heme biosynthetic pathway (1–5). Diagnosis demands a combination of characteristic history and physical signs plus excess porphyrin excretion or accumulation in the body, so measurement of these tetrapyrroles acquires clinical significance (6, 7). There are several methods for separating, identifying, and quantifying porphyrins, metalloporphyrins, and their derivatives, extensively reviewed by Falk (8) and Smith (9). The presence of a conjugated double-bond system in the tetrapyrrole nucleus is the structural feature responsible for the strong and characteristic absorption bands in the visible and near-ultraviolet. This useful property is widely applied in clinical laboratories to quantify porphyrins, its major limitation being the relatively high concentration necessary for accurate spectrophotometric measurement.

Another characteristic and most important property of free porphyrins and their esters is the intense red fluorescence emitted on radiation with light of wavelength near 400 nm, which allows their detection in concentrations of about 10⁻³ mol/L. Because of their sensitivity and specificity, fluorometric methods are now often used to quantify tetrapyrroles. In our laboratory this is the method of choice for samples both from patients and from any other related investigation. Therefore, we undertook a study using different experimental conditions to determine the effect of pH, ionic strength, and solvent composition on measurement of porphyrin fluorescence.

Materials and Methods

Chemicals

Coproporphyrin III methyl ester was from Porphyrin Products, Logan, UT. Uroporphyrin III was prepared from turaco (Turacus sp.) feathers (10), a gift from C. Rimington. Free porphyrins were obtained from their respective methyl esters by acid hydrolysis (HCl 6.85 mol/L, 24 h, in darkness and at room temperature). Stock solutions were prepared in HCl at concentrations of 0.1 mol/L (for coproporphyrin) or 0.5 mol/L (for uroporphyrin), measured spectrophotometrically. Both stocks were then diluted as indicated.

All other chemicals used were reagent grade.

Solutions

Buffers. To study the effect of pH, we used buffers covering the pH range from 1 to 11 (Table 1). The pH was carefully adjusted at room temperature (25 °C), to within ±0.02 unit.

Ionic strength. Because KCl dissociates completely in solution, we used it to adjust solution ionic strength. Taking into account the presence of HCl and knowing the concentration of the ionic species, the ionic strength (\( \mu \)) of the solution was calculated by using the equation \( \mu = \frac{1}{2} \sum i^2 \zeta^2 \), where \( C \) is the concentration and \( \zeta \) the charge.

Apparatus

We used a Model RF-510 spectrofluorophotometer (Shimadzu Co., Kyoto, Japan) with an optional Shimadzu R928 photomultiplier (wavelength measuring range, 220 to 900 nm) and with an Omnigraphic 2000 recorder (Houston Instruments, Austin, TX). For spectrophotometric measurements we used a DB Model 35 (Beckman Instruments Inc.,