the SMAC, they are applicable to any LD assay in which a detergent is used. (Technicon recommends the use of serum rather than plasma in this assay.) We show that these results have clinical significance by comparing data obtained on plasma samples from a normal person and a patient with thrombocytosis (Figure 2). After differential centrifugation, LD was assayed with both the SMAC and System TR, and although for each patient these intersect at the same activity point, the basal activity is higher for the patient with thrombocytosis. Because of this higher basal activity, coupled with the difficulty in removing all the platelets from the thrombocytotic specimen, the possibility of obtaining an incorrect LD value is enhanced. The data from the SMAC also demonstrate that the rates of decrease in LD activity for the two samples are parallel, thus indicating that the LD activity is directly proportional to the number of platelets present and not a result of the thrombocytotic patient having a greater LD activity per platelet. In addition, the results on the System TR also confirm those of Rothwell et al. (8), who suggested that under conditions in which platelet number is high LD activity is inhibited.

References

Determination of Plasma Glucose With Use of a Stirrer Containing Immobilized Glucose Dehydrogenase

Jui-chang W. Kuan, Shia S. Kuan, and George G. Guilbault

A stirrer containing immobilized glucose dehydrogenase has been successfully used for determining glucose in plasma. The device is usable for at least two months and for about 500 assays. The reaction was measured kinetically, and linearity was observed to 4 g of glucose per liter. Tested with aqueous glucose and deproteinized plasma, within-day and day-to-day precision were good. Interference and method-comparison (hexokinase method) were examined. The performance of this system makes the technique useful and attractive for routine use in small-volume clinical laboratories.

Many enzymatic methods have been proposed for the selective analysis of glucose in body fluids (1). A specific onestep method in which glucose dehydrogenase (EC 1.1.1.47) is used has recently been proposed (2, 3). The new technique, which has good accuracy, specificity, and operational simplicity, appeared to be suitable for use in routine glucose assay. We found that glucose dehydrogenase in buffer solution is stable for only about two weeks under refrigeration. For better stability and reusability, we have successfully immobilized this enzyme and have adapted this system to an enzymeon-stirrer technique recently developed in our laboratory. We evaluated this technique and compared our results with those obtained by the conventional method in which the enzyme is used in solution.

Materials and Methods
Reagents
Glucose dehydrogenase/mutarotase enzyme mixture (stock No. 14053, a gift of E.M. Laboratories, Elmsford, N. Y. 10523, an associate of E. Merck, Darmstadt, Germany). The glucose dehydrogenase (GDH), from Bacillus cereus M 1020, had an activity, as measured by the method of Banauch et al. (2) of 5.1 U/mg of solid. The mutarotase (EC 5.1.3.3),

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from bovine kidney, had an activity of about 0.1 U/mg of solid. The enzyme mixture was immobilized on cyanogen bromide-activated cellulose as described below.

Phosphate buffer, 0.1 mol/liter, pH 6.8.
Perchloric acid, 0.32 mol/liter.
Zinc sulfate/barium hydroxide solutions (ZnSO₄/Ba(OH)₂) for deproteinization are prepared as described in the Somogyi–Nelson method for glucose (4).

Standard glucose stock solution, 10 g/liter, β-nicotinamide adenine dinucleotide (NAD⁺, grade III, from yeast), and glutaraldehyde were obtained from Sigma Chemical Co., St. Louis, Mo. 63178. Ethylenediamine was purchased from Matheson, Coleman and Bell, Norwood, Ohio 45212. All other materials for the enzyme stirrer were obtained from the same sources as in the previous report (5).

Immobilization of Glucose Dehydrogenase

An attempt at direct coupling of glucose dehydrogenase to the imido groups on cyanogen bromide (CNBr)-activated cellulose as described in a previous paper (5) was unsuccessful. The low retention of enzyme activity was mainly due to conformational change of the enzyme molecules after immobilization, since most of the enzyme protein was bound onto the cellulose surface. We therefore modified the procedure by adding ethylenediamine and glutaraldehyde to provide spacing groups. The activation of cellulose was essentially the same as described previously. After washing with ethanol, 0.1 g of the drained CNBr-activated cellulose was transferred into a beaker containing 30 ml of a solution of equal volumes of ethylenediamine and water and the mixture was stirred for 30 min at room temperature. The slurry was filtered and washed four times with 50-ml portions of distilled water to remove unreacted diamine.

The amine-substituted cellulose was then activated with 20 ml of glutaraldehyde/water (10/90 by vol) for 20 min, and washed thoroughly with distilled water. The drained activated carrier was immediately mixed with 20 mg of the glucose dehydrogenase/mutarotase enzyme mixture in 2 ml of potassium phosphate buffer (0.1 mol/liter, pH 7.0) with shaking at 4 °C for two to three days under a nitrogen atmosphere. The immobilized enzyme was washed with the same buffer several times before it was loaded onto the stirrer.

The immobilized enzymes were placed in contact with a stirring bar, which we modified from a previous version for easier mounting of immobilized enzyme and better performance (Figure 1). An AminoCo filter fluorometer was modified to accommodate the enzyme stirrer as previously reported (5).

Assay Procedure

Mix 0.2 ml of plasma sample with 0.8 ml of 0.32 mol/liter perchloric acid. Let the mixture stand for 5 min, then centrifuge for 5 min and separate the supernatant fluid. Place into a test tube 3 ml of phosphate buffer (0.1 mol/liter, pH 6.8), 0.3 ml of 5.0 g/liter NAD⁺, and either 10 µl of standard solution of glucose plus 40 µl of HClO₄ solution, or, for sample analysis, 50 µl of deproteinized plasma. Shake for 10 s with a mixer, then transfer this mixture into the cell that contains the enzyme–stirrer device. Stir the mixture at 1200 rpm and measure the change of fluorescence (ΔF/2 min) at 460 nm (λex = 370 nm) for 4 to 5 min. Obtain the glucose value for an unknown or plasma sample from a calibration curve of ΔF/2 min vs. standard glucose concentration. After each run, wash the stirrer twice on a vortex-type mixer for 30 s each with 3 to 4 ml of water and pat it dry with a Scott disposable wiper tissue.

If ZnSO₄/Ba(OH)₂ is used as deproteinizing reagent, add 2.5 ml of water and 1 ml of barium hydroxide to 0.5 ml of the standard solution of glucose or the plasma sample. Wait about 5 min, then add 1 ml of the zinc sulfate solution. Centrifuge for 5 min. Use 0.2 ml of the resulting protein-free supernate for glucose assay as outlined above.

Results and Discussion

We optimized each variable of the glucose assay, with use of 50 µl of a 1 g/liter glucose solution. The effects of NAD⁺ and pH of phosphate buffer on the overall reaction rate are shown in Figures 2 and 3. The optimum NAD⁺ concentration is 0.6 mmol/liter; with higher concentrations there is a slight inhibitory effect. Both tris(hydroxymethyl)aminomethane and phosphate buffer were investigated for the system; with the latter the reaction curve was linear over a broader range and the reagent background was lower. The optimum pH for glucose dehydrogenase in solution is 7.0, but this shifts to 8.8 with the immobilized enzyme. The optimum stirring speed was found to be about 1200 rpm; we used a stronger magnet in our new stirrer. The calibration curve is linear to 4.0 g of glucose per liter.

Long-Term Stability of the Enzyme–Stirrer

We observed that glucose dehydrogenase is stable for less than 4 h in water at room temperature, although the manu-
facturer claims it is stable for about two weeks in phosphate buffer (0.12 mol/liter, pH 7.8). The immobilized enzyme was much more stable. The first enzyme--stirrer device we made was used in most of our studies. It was used continuously at room temperature during weekdays and was stored refrigerated overnight and on weekends in a storage solution consisting of 3 ml of phosphate buffer (0.1 mol/liter, pH 6.8), 0.3 ml of 5.0 g/liter NAD+, and 20 μl of 4 g/liter glucose. Before storage, the stirrer was thoroughly cleaned with water or physiological saline solution. The activity of the enzyme--stirrer had declined to about 15% of its original value after 644 runs in 70 days. Much of this loss occurred during studies of interfering substances and in the analysis of non-deproteinized blood samples. Three more enzyme--stirrers were then made and used for serum sample analysis to investigate deproteinization reagents. The useful life of these stirrers varied, depending on the success in immobilizing the enzyme and the kind of deproteinization reagent used. From our experience, we estimate that under normal conditions and with proper care a stirrer should be useful for routine analysis for two months or about 500 runs.

Effect of Diverse Substances

We evaluated the specificity of the present method by adding potentially interfering substances to a 2 g/liter standard glucose solution. The following compounds in the concentrations (grams per liter) indicated did not show any interference: potassium oxalate (20), sodium fluoride (30), ascorbic acid (1.1), uric acid (1.0), creatinine (0.5), D(+)-xylose (1.1), fructose (6.0), D(+)-mannose (1.2), galactose (2.0), and bilirubin (0.12). Ethylenediaminetetraacetic acid in concentrations exceeding 5.0 g/liter depresses the enzyme reaction; we recommend that it not be used as anticoagulant in the blood sample. Hemoglobin in concentrations greater than 50 mg/liter significantly decreases the overall reaction rate, apparently because adsorption of hemoglobin onto the stirrer blocks the active site of the enzyme. (However, the activity of the enzyme stirrer recovers after overnight storage in the storage solution.) Hence, the blood sample must be deproteinized, as is also true of the enzyme used in solution. The activity of glucose dehydrogenase on 2-deoxy-D-glucose is about the same as on glucose. Other sugars that we tested do not react.

Reproducibility Studies

We assessed the reproducibility of the method by assay 10 times each day of a 4 g/liter standard solution of glucose for seven days and of a deproteinized plasma sample 10 times a day for three days. For the glucose standard solution, in terms of reaction rate (ΔA/min), the within-day standard deviation (SD) was 1.80 (equivalent to 0.205 g/liter), the coefficient of variation (CV) was 4.6%, mean 39.3; the day-to-day SD was 1.65 (equivalent to 0.188 g/liter), the CV 4.09%, and the mean 40.3. The within-day precision (CV) for plasma was 3.9%, SD of 39 μg/liter, and the mean 1.007 g/liter; the day-to-day SD was 17 mg/liter, the CV 1.7%, and the mean = 0.984 g/liter.

In general, a glucose standard solution and samples containing noninhibitory substances showed very good reproducibility. For example, two glucose calibration curves, made on two consecutive days under the same conditions, coincided. Excessive amounts of HClO₄ or ZnSO₄/Ba(OH)₂ reagents, used to deproteinize the plasma samples, inhibit the enzyme and cause a gradual decrease in reaction rate. However, the concentrations recommended for use above do not cause a significant effect and reproducibility is good.

Deproteinization Reagents and Comparison Studies

We assayed 18 plasma samples with which potassium oxalate was used as anticoagulant by the present method, without pretreatment. The correlation between results by this method and by the hexokinase method (6) was poor because of interference from protein. Deproteinization before the glucose determination is necessary. We surveyed four deproteinization reagents: trichloroacetic acid, ethanol, HClO₄, and ZnSO₄/Ba(OH)₂. The trichloroacetic acid-deproteinized filtrate was cloudy and recovery was poor. The ethanol-deproteinized supernate was transparent but very yellow, which causes a high fluorescent background and low glucose values as compared with other methods. Plasma samples deproteinized with HClO₄ or ZnSO₄/Ba(OH)₂ gave good results. The calculated linear regression and coefficient of correlation (r) between the present method (x) and the hexokinase method (y) are y = 0.980x + 0.068, r = 0.9978 for 18 HClO₄-deproteinized plasma; and y = 0.996x + 0.078, r = 0.9995 for 10 ZnSO₄/Ba(OH)₂ deproteinized plasma. All four deproteinization reagents cause some degree of inhibition of the enzyme activity. At the concentrations normally used for deproteinization, trichloroacetic acid shows the strongest inhibition, ZnSO₄/Ba(OH)₂ the weakest.

Our results indicate that this system performs satisfactorily in determining glucose. Besides, this technique has several advantages over use of the enzyme in solution:

- Fluorescent background interference originating from the presence of enzymatic protein in the reaction mixture is completely eliminated, thus increasing the sensitivity and lowering the detection limit.

- The stability of glucose dehydrogenase at room temperature is greatly improved after immobilization and in addition to the operational simplicity as well as the ease of handling and storage, the enzyme--stirrer device was stable for months and could be used for several hundred assays. All this makes the system an ideal technique for small-volume routine laboratory analysis.

- The rate of catalysis could be regulated by varying the rotating speed of the stirrer to obtain the best conditions for different measuring ranges when the kinetic initial rate is used (5).
However, the gradual loss of enzyme activity caused by the deproteinizing reagents somewhat shortens the useful life of the stirrer. Even so, our technique is much more economical than use of the enzyme in solution, in terms of cost per assay.

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References

Amylase Activity in Serum and Urine: Comparison of Results by the Amyloclastic, Dyed-Starch, and Nephelometric Techniques

Derek P. Lehane, Paul J. Wissert, Gifford Lum, and Arthur L. Levy

We assayed serum and urine specimens for amylase activity by the nephelometric (I), dyed-starch (Amylochroic®) (II), and amyloclastic (III) techniques. For serum, the correlation coefficients of the regression lines were: I vs. II, 0.978 (n = 106); I vs. III, 0.736 (n = 110); and II vs. III, 0.736 (n = 108). For urine, they were I vs. II, 0.938 (n = 49); and I vs. III, 0.752 (n = 48). Because calculation of the Kolmogorov–Smirnov statistic showed the distributions to be nongaussian, Spearman rank correlation coefficients were determined and showed that I and II correlated well but neither method correlated with III. The clinical data show that I and II gave above-normal activities in every case of pancreatitis, but III gave normal values in two of eight cases. In all cases, I and II were more sensitive, giving higher amylase activities (as compared with the upper limit of normal) than did III. The nephelometric procedure is most suitable for routine and emergency testing; the dyed-starch assay is equally sensitive and reliable, but less convenient. The amyloclastic procedure appears to be less reliable.

Additional Keyphrases: pancreatitis • intermethod comparison • normal values

Measurement of amylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.1) activity in serum remains the most important single clinical laboratory determination in acute pancreatitis (1). Because amylase assays are usually emergency determinations, there has been a continuing effort to develop a routine procedure that is simple, rapid, reproducible, and capable of differentiating normal and above-normal enzyme activity. Approaches to measurement of amylase activity include (2): amyloclastic (starch–iodide) methods, dyed-starch (“chromolytic”) methods, saccharogenic-reducing methods, coupled enzymatic assays, and light-scattering (nephelometric) procedures.

Amyloclastic procedures, such as that of Caraway (3), have been widely applied and have provided valuable clinical information. Such a procedure is quite suitable as an emergency method, because it is simple, rapidly performed, and only inexpensive colorimeters are required. On the other hand, substrates may vary from lot to lot, such methods are difficult to standardize, and their ability to discriminate between normal and increased activity is limited (2).

Dyed-starch procedures (4–6) differentiate clearly between normal and abnormal amylase activity (2), but have manipulative drawbacks as emergency procedures, because blanks, centrifugation, decanting, and spectrophotometric measurements are required. The time-consuming saccharogenic techniques require blanks for endogenous interfering substrates and can be unreliable due to small net changes in absorbance. Reagents for coupled enzymatic assays (7, 8) are expensive, and three or four additional enzymatic reactions, which are possibly subject to in vivo interferences, are involved.

Light-scattering techniques offer a multi-point approach, but until recently have been difficult to standardize, lack precision, and the substrates are not adequately stable (9). An instrument (10) designed for the nephelometric measurement

Clinical Chemistry Laboratory, Saint Vincent’s Hospital and Medical Center of New York, 153 West Eleventh St., New York, N. Y. 10011.

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