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A SIMPLE AUTOMATED METHOD FOR THE ASSAY OF PANCREATIC LIPASE

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Summary

A simple potentiometric method is described for the automated assay of lipase in duodenal juice. The procedure utilizes a pH meter connected to a classical blood pH assembly made of a reference and a capillary electrode. A potentiometric recorder connected to the pH meter is adjusted for a full scale deflection corresponding to 0.5 pH unit.

Samples containing 0.4 to 2 enzyme units in 2 ml are analyzed at a rate of 30 assays per hour. Each sample is incubated for 4 min with a tributyrin emulsion prepared in a mixed buffer at pH 8. The buffer composition ensures a linear response of pH versus H^+ liberated by lipolysis.

Introduction

Recently in comparative assays of various pancreatic hydrolases in human duodenal juices, there has been some preference for the lipase assay in clinical tests of pancreatic insufficiency [1]. A new interest for a simple and rapid automated method for lipase determination arose from these comparative studies.

Our goal was to estimate the lipolytic activities, in physiological samples or in chromatographic eluates, by a method as efficient as that recently described for two other pancreatic hydrolases i.e. trypsin and chymotrypsin [2]. The usual method for the assay of lipase is by constant pH titration. This method has already been automated [3]. Its disadvantage is the relative slowness which is characteristic of all kinetic assays automated by an interrupted-flow method. It was, therefore, felt to be of interest to perfect an automated method based on continuous single-time-point assays of lipase. This new method would replace the constant pH titration whenever a large number of samples must be submitted to routine analysis.

Materials and Methods

1. Reagents

Buffer. During preliminary testing, the mixed buffer used for enzymatic incubations was composed of 6 mM acetic acid, 4.5 mM maleic acid, 5 mM *N*-morpholinopropanesulfonic acid (MOPS), 5 mM *N*-Tris-(hydroxymethyl)-methyl-3-aminopropanesulfonic acid (TAPS), 5 mM glycine and 50 mM NaCl. The pH of this solution was adjusted to the desired value between pH 6 and 8.5 by use of 1 M NaOH. The components of this buffer were purchased from Serva (Heidelberg, Germany).

The buffer which was found useful for routine analysis in the optimal pH range (pH 7.5 to 8) consisted only of TAPS and MOPS, in equimolecular proportions (adjusted to pH 8.0), and NaCl. After dilution the final concentrations of the acids were 5 mM and NaCl was 50 mM.

Substrate. Tributyrin (from Fluka, Buchs, Switzerland) was distilled under a vacuum and stored in a cold room. A 5% tributyrin emulsion was prepared daily in the buffer which was enriched with sodium taurodeoxycholate (from Sigma, St. Louis, U.S.A.) in a 1 mM final concentration. This medium was emulsified for 5 min at 10000 rev./min using a Ultra-Turrax type TP 18 homogenizer from Janke and Kunkel KG (Staufen, Germany). The resulting emulsion was continuously mixed with a magnetic stirrer during the duration of the assays.

2. Equipment

1. A sampler for 400 samples (Plate SC 15 with TD 15 T 3 timer from Gilson Medical Electronics, Villiers-le-Bel, France) was kept at 4° in a miniature cold room (Gilson Model RCA) in order to prevent enzyme denaturation by proteolytic enzymes.

2. Fluid flow was maintained by a proportioning pump (Model I from Technicon Auto Analyzer).

3. The instruments for pH measurements were purchased from Metrohm (Herisau, Switzerland). A capillary glass electrode (EA 138) and a reference electrode (EA 402) were inserted in a rubber stopcock which closed a 25-ml beaker. A teflon tube, also inserted in the stopcock, was connected to the pump which continuously aspirated the liquid through the capillary electrode. The two electrodes were coupled with a pH meter (E 300) and were connected to a potentiometric recorder (PM 8100 from Philips, Eindhoven, The Netherlands).

3. Procedure

The basic principle of the method proposed in view of automation consists of measuring, by means of a simple pH reading, the H^+ liberated after a fixed-time esterolysis.

Fig. 1 represents the flow diagram for the automated lipase assay. The rate of analysis was set at 30 samples per hour using equal periods of 1 min each for the sampling and washing operations.

The enzymatic reactions were developed during 4 min in a teflon mixing coil maintained at 30°. The reactions were stopped by mixing the incubates

Fig. 1. Flow diagram for automated lipase assay. The glass and reference electrodes and the aspirating tube are inserted in a rubber stopcock and extended into a 25-ml hermetically closed beaker.

with 2% Triton X-100—1 M NaCl. This concentration of Triton completely inhibited lipase activity.

After debubbling, the stream was pumped through the flow electrode. The sensitive scale of the pH meter was used, and the potentiometric recorder was adjusted so that 10 mV full scale was equivalent to 0.5 pH unit. Distilled water was used as the washing solution.

Results and Discussion

1. Choice of the substrate

Tributylin has been reported to be a specific substrate for lipase when used with the pancreatic juice of rat and man, or the duodenal content of man [4]. We have personally verified that tributyrin was as specific as the more widely used triolein. The ratio of the rate of tributyrin hydrolysis versus triolein hydrolysis was 12 whether we used duodenal juice or pure human lipase isolated from pancreatic juice. The tributyrin emulsion was stable enough to allow a large number of assays since the interphase area was never critical. The Michaelis constant measured for the lipase—colipase system present in a crude rat pancreatic extract was 0.25 g/l at 30° with freshly emulsified tributyrin. This value was unchanged 6 h later. It increased to 0.37 g/l after 24 h of magnetic stirring at 25°. At that time, the substrate concentration (50 g/l) was still 135-fold higher than the K_m .

2. Wash characteristics and rate of analysis

Care must be taken in the pumping of the glyceride emulsion so as to avoid poor wash characteristics which might impair the efficiency of the analysis. The use of tributyrin instead of triolein was decisive from this point of view. Indeed, the choice of triolein as the substrate during preliminary assays gave rise to real problems for automation. With triolein, Ca^{2+} , which was necessary for a quantitative deprotonation of liberated oleic acid (even at pH 9), precipitated as calcium oleate in the incubation vessel. Neither the use of high

Fig. 2. Wash characteristics of the flow system. Successive samples of the same lipase solution were pumped for varying times. S: steady state attained with a sample probing of 3.5-min duration; 1 and 2 peaks obtained with only 45-s sampling and 45-s rinsing time; 4 to 7: peaks obtained with 1-min sampling and 1-min rinsing time.

flow rates, nor that of teflon coils and tubings, was adequate to avoid a progressive accumulation of these salts on the walls of the manifold. This complication was avoided by use of tributyrin. A further advantage was that, in comparison to triolein, tributyrin was more rapidly hydrolyzed by pancreatic lipases. The rate of analysis of 30 assays per hour became possible by using a capillary pH electrode with minimal dead space, allowing continuous pH readings without turbulence after debubbling (Fig. 2).

3. Calibration of the method

The simple pumping of HCl samples of known concentrations, instead of enzymatic solutions, allowed the calibration of this system in equivalents H^+ liberated by lipolysis. The complete buffer mixture used in this study has a constant buffer capacity from pH 6 to pH 9. As a result of this property a linear relationship was obtained between pH 6 and pH 9 in plotting pH against H^+ concentration (Fig. 3). The simpler buffer described under Materials and

Fig. 3. Calibration curve of the assay. 23 tubes of increasing HCl concentration (from 0 to 22 μ equiv/ml) were placed on the sampler instead of enzyme samples. The emulsion of tributyrin ("substrate" in Fig. 1) in the mixed buffer described under Materials and Methods (6 mM acetic acid; 4.5 mM maleic acid; 5 mM MOPS; 5 mM TAPS; 5 mM glycine and 50 mM NaCl) was initially adjusted to pH 9.5. The experimental pH curve obtained after increasing H^+ additions was linear from pH 9.0 to pH 6.0.

Methods, and used for routine analysis, had a similar buffer capacity from pH 7.5 to 8. Thus, it was sufficient to test daily only one H^+ concentration (1 or 2 $\mu\text{equiv } H^+/\text{ml}$) before each series of assays.

4. Sensitivity

Using a 10-inch potentiometric recorder adjusted to 0.5 pH unit at full scale, the observed deviation for a given measurement was less than 0.005 pH unit (i.e. less than a 1/10 inch paper graduation). It may thus safely be stated that the method allows the measurement of a fall of 0.1 pH unit with excellent accuracy. Such a value was obtained when approximately 0.4 $\mu\text{equiv } H^+$ were liberated per ml of buffer reaction mixture. As a consequence, the enzyme concentrations which allowed accurate determinations ranged from 0.2 to 1 unit per ml of sample. This range may, of course, be shifted to lower or higher enzyme concentrations simply by modifying the incubation time or the buffer concentration.

5. Effects of pH, bile salts and colipase on the conditions of the assay

Originally the buffer composition was chosen to allow lipase assays at any pH between 6 and 9. This choice was made because the pH optimum for rat lipase activity had been reported to be shifted from 9 to 6 when bile salts were added to the incubating media in the presence of colipase [5]. However, the pH optimum for porcine lipase activity has recently been demonstrated to remain around pH 8 in the presence of colipase whether bile salts were added or not [6]. Similarly, the pH-activity curve obtained in our laboratory with pure human lipase showed an almost constant rate of lipolysis between pH 7 and 8 for sodium taurodeoxycholate concentrations varying between 0 and 5 mM. In conclusion, using samples of human enzyme, a strict proportionality existed between lipase concentrations and pH decays between 8.0 and 7.0.

6. Application to lipase assays in human duodenal juice

With our system, based on tributyrin and taurodeoxycholate, lipase assays in chromatographic eluates or in pancreas homogenates in a known buffer of fixed pH did not present any difficulty provided that colipase was present in saturating amounts. However, a problem can arise when working with specimens of duodenal juice which can vary in acidity and ionic strength. We wanted to investigate if the base line and the calibration of the system would vary from one sample to another. In this regard, it was first verified that the endogenous buffer capacity of samples of human duodenal juice diluted 100-fold in water was always negligible when compared to that of the MOPS-TAPS buffer used in the automated method. Since the mean lipase concentration in duodenal juice from normal subjects ranged from 400 units/ml under conditions of secretin stimulation to 1200 units/ml after pancreozymin injection [7], the duodenal samples must be diluted 1000-fold in order to obtain a suitable lipase concentration for the automated method. As a result of this dilution factor, it is obvious that normally no special problem will be encountered when lipase is assayed in human duodenal juice.

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