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Immunoluminometric assay for human pancreatic amylase in feces as a new noninvasive test of pancreatic function

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The aim of the present study was to evaluate: 1) the sensitivity and specificity of a new immunoluminometric test (ILMA) to quantify fecal pancreatic amylase as a new noninvasive test of pancreatic function, 2) to compare these results with fecal fat excretion and the determination of pancreatic enzyme secretions such as fecal chymotrypsin, fecal elastase, and 3) to determine the practicability and clinical handling of the fecal pancreatic amylase test.
Materials and Methods
Light emission was measured with a Berthold LB 954 automatic luminescence analyzer and LBIS software using 50-tube racks. The same racks fit the automatic LB 9200 washer. Stool sample dilution buffer (pH 7.4) consisted of 800 ml of 67 mmol/l Na_2HPO_4 solution, 200 ml of 67 mmol/l KH_2PO_4 solution, 1 g sodium azide, 400 mg EDTA and 2 g gelatin. Assay buffer (pH 7.4) consisted of 800 ml of 67 mmol/l Na_2HPO_4 solution, 200 ml of 67 mmol/l KH_2PO_4 solution, 1 g sodium azide, 400 mg EDTA, 1 g human serum albumin and 50 g bovine serum albumin. Coating buffer (pH 9.5) was prepared by combining 250 ml of 0.1 mol/l Na_2CO_3 and 750 ml of 0.1 mol/l $NaHCO_3$. Saturation buffer was prepared with 1.84 g KH_2PO_4, 9.5 g of Na_2HPO_4 1 g of NaN_3, 0.4 g EDTA, 20 g BSA and 1 l aqua dest. The labeling buffer is 0.1 mol/l sodium

dihydrogen phosphate/disodium hydrogen phosphate buffer, pH 8.0, the quenching buffer is labeling buffer containing 5g/l lysine monohydrochloride.

Patients, sample collection and storage

A total of 231 subjects were included in this study: 184 were healthy subjects without any clinical signs of gastrointestinal disease, 78 females, 106 males, aged 3-77 years (mean 41 years). Ten patients were affected by cystic fibrosis with different grades of pancreatic insufficiency. Ten had severe chronic pancreatitis, five presented with celiac sprue, two of them with treatment, nine patients had steatorrhea of different causes and thirteen had increased fecal fat excretion without any other abnormal parameter.

Coating and labeling procedures

The new immunoluminometric assay (ILMA) employed monoclonal antibodies anti-hPancreatic Amylase from Oy Medix Biochemica. The monoclonal anti-hPancreatic Amylase antibody (6104) was used as assay tracer. The concentration was 100 µg acridinium ester labeled anti-Pancreatic Amylase/10 ml assay buffer, it was stored in aliquots at -20°C until measurement.

Assay procedure

The solid phase non-competitive luminescence immunoassay is performed in two incubation steps. Briefly, 50 µl standards, controls or patient samples and 200 µl assay buffer were pipetted into polystyrene tubes fitting the Berthold racks These racks fit the LB 9200 washer and the automatic luminescence analyzer LB 954. Coated beads were then added to each tube. The tubes were then incubated for 2 h at room temperature. Following incubation the tubes were washed, and 200 µl of acridinium ester-labelled human pancreatic amylase were added. The tubes were then incubated again, but this time overnight at 4°C, washed and counted for 2 s in the Berthold LB 954 analyzer.

Others methods used

Fecal pancreatic elastase 1 was determined immunologically with a commercial test kit (ScheBo Tech). Fecal chymotrypsin activity was determined according to Kaspar et al with a commercial kit manufactured by Boehringer Mannheim, Germany. Fecal fat excretion was analyzed according to the method of van de Kamer et al or by near-infrared analysis according to Stein et al.

Preparation of standards and controls

A urine sample with 32000 mU/L pancreatic amylase was diluted 15-fold in assay buffer to obtain the concentrations of 2632, 658, 164, 41, 10 mU/L.

Statistical analysis

The statistical differences were calculated with the Mann-Whitney *U* test, and regression analysis was performed with the Spearman rank test. Correlations were calculated with the method of least squares. Limits of significance were $P<0.05$ in all tests. The calculation of the area under the ROC curve was done by a computer program whereas the statistical comparison between the different areas was done on the basis of the mathematical expression defined by Hanley and McNeil.

Results

1. The normal range for pancreatic amylase in feces was studied using specimens from a healthy population of 184 volunteers: 78 women and 106 men, age 3 to 77 years, average 41 years. Fecal pancreatic amylase concentrations ranged from 11.8 to 103710 mU/g. The normal range was determined as 800 to 50000 mU/g, with 95% of all values within the 2.5^{th} to 97.5^{th} percentiles.

2. The specificity of the assay was studied by measuring standards and internal control samples with added human saliva amylase, porcine pancreatic amylase, and a selective inhibitor. A standard curve was prepared by mixing standards and controls with porcine amylase in the presence and absence of the amylase inhibitor and comparing the obtained curves with the reference curve given for pure pancreatic amylase. The inhibitor concentration used was 50 μg/L. A good parallelism was found, thus suggesting the absence of interfering reactions.

3. Sensitivity was defined as the concentration corresponding to a light emission of the mean counts plus 3 SD of 12 determinations of a zero standard containing pure assay buffer. It was found to be 0.76 mU/g.

4. The intra-assay variation of pancreatic amylase was measured by assaying 4 different concentrations of controls twelve times within one assay; the results showed a coefficient of variation of 5.64 % -6.72 % (mean 6.18 %).

5. The inter-assay coefficient of variation of pancreatic amylase, calculated from twelve consecutive assays of 4 different controls on three different days, ranged from 6.21% to 9.85% (mean 7.35 %).

6. The analytical recoveries were evaluated by mixing equal volumes of one patient sample with a known concentration of pancreatic amylase to 5 assay standards, and these mixtures were measured in one assay. The calculated recoveries (measured values/theoretical values) ranged from 88 to 106% (mean 95%).

7. The stability of the standards and stool samples was tested under different storage conditions (4°C, 20°C and 37°C). The standards and the stool samples were stable for at least 3 days at all the temperatures tested. At room temperature, pancreatic amylase in stool samples showed no decay and no difference when stored in darkness or in daylight.

8. Linearity: no deviation of the measured values from the expected values was observed using three high samples with concentrations up to 80000 mU/g, serially diluted from 1:2 to 1:20 and assayed in duplicate. Repeating the experiment the linearity did not change even after 8 days since the preparation of reagents which had been stored at –4°C.

9. The compound-precision-profile coefficients of variations for the assay standards were less than 10% at all concentrations.

10. No high-dose-„hook"-effect could be recognized up to a concentration of 32 000 mU/g.

Clinical evaluation

Fecal pancreatic amylase concentrations in patients with severe exocrine pancreatic insufficiency were considerably lower (28 mU/g-6936 mU/g, mean 1676 mU/g) compared with healthy controls (mean 22144 mU/g) or patients with non-pancreatic gastrointestinal diseases (mean 17844 mU/g). In contrast, the elastase concentrations were less reduced in patients with severe exocrine pancreatic insufficiency (1 µg/g-405 µg/g, mean 141 µg/g) according to the cut-off of 200 µg/g recommended by the manufacturer (ScheBo Tech) and compared with healthy controls (mean 342 µg/g) or patients with other gastrointestinal diseases (mean 300 µg/g). Fecal fat excretion in the group of patients with severe exocrine pancreatic insufficiency was elevated (6.3 g/day-60 g/day, mean 18.5 g/day) compared with the group of patients with sprue (mean 8 g/day). However, patients with severe pancreatic insufficiency had not significantly lower fecal chymotrypsin activities 0 U/g-13 U/g, mean 6.4 U/g, with a cut off value of 3 U/g, Furthermore in patients with cystic fibrosis, the pancreatic amylase concentrations were also lower (mean 9429 mU/g) than in the controls.

Linear regression analysis showed a significant inverse correlation between pancreatic amylase concentrations and fecal fat in patients with severe exocrine pancreatic insufficiency, and a significant correlation with pancreatic elastase in patients with cystic fibrosis.

The receiver-operating characteristic (ROC) curve for pancreatic amylase based on calculations of sensitivity and specificity at several cut-off values achieved a good diagnostic accuracy (true positive rate divided by the true negative rate) at a cut-off value of 800 mU/g, with an area under the ROC curve of 0.8744 (specificity 91 %, sensitivity 70 %) compared with the discrimination achieved with pancreatic elastase at a cut-off value of 200 µg/g, with an area under the ROC curve of 0.7173 (specificity 70%, sensitivity 65 %).

Conclusion

The immunoluminometric assay for fecal pancreatic amylase is a simple, non-invasive exocrine pancreatic function test, which may be established in several laboratories. The test is highly specific for human fecal pancreatic amylase because it utilizes monoclonal antibodies; the test can be used with pancreatic enzyme therapy. The observed sensitivity was good in comparison to the elastase, fecal fat and fecal chymotrypsin tests. The immunoluminometric test showed a good reproducibility, both in intra-assay variation and inter-assay coefficient of variation. Good analytical recovery was also observed. The good stability of the standards and the stool samples of the test for at least 3 days allows the mailing of the test stool samples from out-patients to diagnostic centers.