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Time-Resolved Immunofluorometric Assay of Human Pancreatic Isoamylase in Serum, with Use of Two Monoclonal Antibodies

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This new method for determining pancreatic isoamylase (EC 3.2.1.1) in serum involves two monoclonal antibodies: one immobilized in a microtitration well (the capture antibody), the other biotinylated. After the sample is incubated with the two antibodies, the captured immunocomplex is quantified by adding streptavidin labeled with a europium chelator and measuring the specific Eu^{3+} fluorescence in a time-resolved mode. Three assay protocols are proposed, involving incubation times of 90, 45, or 25 min. The assay has low (0.005%) cross-reactivity with the salivary isoenzyme. Analytical performance was satisfactory. Results correlate well with results obtained by measuring total amylase activity or by measuring pancreatic isoamylase activity after immunoinhibition. Unlike numerous current amylase assays, this method measures enzyme mass rather than enzyme activity. Potentially, this is a highly specific assay.

Additional Keyphrases: *pancreatitis · europium chelates · biotin–streptavidin system · distinguishing pancreatic and nonpancreatic increases in serum amylase · amylase isoenzymes · noncompetitive immunoassay · reference interval · pancreatic disease*

Total amylase (EC 3.2.1.1) activity in serum has been assayed for many decades as a confirmatory test for acute pancreatitis, but it has low clinical specificity in the diagnosis of pancreatic disorders (*1–3*). By contrast, determinations of pancreatic isoamylase have proved considerably more specific than total amylase determinations (*4, 5*), and they are becoming more popular. Amylase isoenzymes can be determined by using a separation technique to distinguish the pancreatic from the nonpancreatic amylase—i.e., column chromatography (*6, 7*), electrophoresis (*8, 9*), or isoelectric focusing (*3, 10, 11*). These methods are reliable but time-consuming and technically demanding. A method for pancreatic isoamylase determination based on selective inhibition of mainly salivary isoamylase by an inhibitor isolated from wheat germ (*12, 13*) does not give reliable results if the pancreatic amylase fraction is <10% or >90% of the total amylase (*14*).

Recently, increasing interest in applying antibodies in amylase isoenzyme determination has led to development of monoclonal and polyclonal antibodies raised against pancreatic and salivary amylases, both of which are now commercially available in pure form. Among the immunological approaches reported for determining isoamylases are radioimmunoassay (*15, 16*), immunoinhibition (*17–20*), and immunoextraction (*21, 22*).

Here we report our evaluation of the use of three commercially available monoclonal antibodies to develop a "two-site, sandwich-type" noncompetitive immunoassay of pancreatic isoamylase in serum. We selected a suitable antibody pair, then immobilized one of the pair onto white microtitration wells to be used as "capture" antibody; the other antibody was biotinylated and used for detection. We used labeled streptavidin to quantify the final immunocomplex, as detailed elsewhere (*23–25*). To our knowledge, this is the first report of a specific immunological assay of pancreatic amylase based on using two monoclonal antibodies in a noncompetitive immunoassay design.

Materials and Methods

Instrumentation

For solid-phase time-resolved fluorometric measurements we used the Model 615 Immunoanalyzer (CyberFluor Inc., Toronto, Ontario), which performs data reduction. We used a single-beam Model 240 spectrophotometer (Gilford Instruments, Oberlin, OH) to perform manual assays for total and pancreatic amylase and lipase, as recommended by the kit manufacturer (see below).

Materials

The europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) was synthesized as described elsewhere (*26*). Streptavidin and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO 63178. $EuCl_3$ hexahydrate was from Aldrich Chemical Co., Milwaukee, WI 53201. Sulfosuccinimidyl-6-(biotinamido)-hexanoate was from Pierce Chemical Co., Rockford, IL 61105. All other chemicals were from Sigma. White, opaque, 12-well microtiter strips (Immulon II) were products of Dynatech Labs, Alexandria, VA 22314.

The coating buffer was 50 mmol/L Tris solution, pH 7.80. The blocking buffer was 50 mmol/L sodium phosphate solution, pH 7.4, containing 9 g of NaCl and 10 g of albumin per liter. The assay buffer was 50 mmol/L Tris hydrochloride solution, pH 7.8, containing 10 g of albumin, 1 g of bovine globulins, 9 g of NaCl, and 0.5 g of sodium azide per liter. The streptavidin–europium dilution buffer consisted of 50 mmol/L Tris hydrochloride solution, pH 7.8, containing 9 g of NaCl, 40 g of albumin, 0.5 g of sodium azide, and 40 μmol of Eu^{3+} per liter. The wash solution was 9 g/L NaCl solution containing 0.5 mL of Tween 20 surfactant per liter.

Pancreatic amylase standards. Highly purified pancreatic human amylase was purchased from either Calbiochem–Behring Diagnostics, La Jolla, CA 92037, or from Aalto Scientific, Escondido, CA 92025 ("Immunopure" grade). These two preparations performed identically. We routinely used the latter, which was less expensive. The highly purified salivary amylase used for cross-reactivity studies was also obtained from Aalto Scientific. We pre-

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pared pancreatic amylase standards by dissolving the pure substance in 50 mmol/L Tris buffer, pH 7.80, containing 60 g of albumin per liter. Salivary amylase was dissolved in the same matrix. The concentrations of pancreatic amylase standards routinely used were 0, 20, 100, 200, 500, and 1000 μg/L.

Monoclonal antibodies. We purchased—from Medix Biochemica AB, Kauniainen, Finland—three newly developed monoclonal antibodies for pancreatic amylase purified by ion-exchange chromatography (Code 6103) or Protein A affinity chromatography (Codes 6104 and 6105). The affinity constants (L/mol) were: 4×10^9 (for 6103), 1×10^9 (for 6104), and 1×10^{10} (for 6105). The stated (by the supplier) cross-reactivities with salivary amylase in an RIA assay were <0.1% (for 6103 and 6104) and ~5% (for 6105).

Specimens. We obtained human sera from individuals being investigated for gastrointestinal abnormalities, including acute pancreatitis, and we stored these sera at 4 °C for no longer than four weeks until assay. Sixty-two samples were obtained from a mixed population of apparently healthy subjects, ages 20–48 years, for estimation of the normal reference interval.

Procedures

Biotinylation of pancreatic amylase antibodies. The antibody solution (1 mL, containing 1 mg of antibody) was dialyzed twice in 5 L of isotonic (9 g/L) saline at 4 °C. We then diluted the dialysand with an equal volume of 0.5 mol/L carbonate buffer, pH 9.1. To this solution we added 2 mg of sulfosuccinimidyl-6-(biotinamido)-hexanoate dissolved in 50 μL of dimethyl sulfoxide and incubated the mixture for 2 h at room temperature. The solution was then dialyzed twice in 5 L of isotonic NaCl solution at 4 °C. For the assay, we diluted the stock antibody solution 1000-fold in the assay buffer (working antibody solution). The stock antibody solution is stable for at least six months, and the working antibody solution is stable for at least two weeks at 4 °C.

Preparation of the streptavidin–thyroglobulin–BCPDA–Eu^{3+} tracer. The preparation of BCPDA-labeled streptavidin–protein conjugates is detailed elsewhere (*25*). The concentration of streptavidin in the working solution of BCPDA-labeled streptavidin–thyroglobulin conjugate was 0.30 mg/L; that of $EuCl_3$ was 40 μmol/L.

Coating microtiter wells. We prepared the coating antibody solution by diluting the monoclonal pancreatic amylase antibody in the coating buffer to give a concentration of 5 mg/L. We then added 100 μL of the antibody solution per well into wells of microtiter strips and allowed the antibody to adsorb at 4 °C. After they had incubated overnight, we washed the wells twice with the wash solution, added 200 μL of the blocking buffer per well, and incubated for 1 h at room temperature. Just before use, the wells were washed twice with the wash solution.

Comparison Methods

We used two methods for total amylase determination in serum: (*a*) the Phadebas total amylase kit (Pharmacia, Piscataway, NJ) and (*b*) the total a-Amylase EPS kit (Boehringer Mannheim Canada, Montreal, Quebec). We have also used the Pancreatic a-Amylase EPS kit (*20*) and the Lipase kit from Boehringer Mannheim. All Boehringer Mannheim kits were used at 25 °C. All kits were used as recommended by the manufacturers.

Assay Procedure

Before the assay, the strips are washed twice with the wash solution. Ten microliters of standard or serum samples (in duplicates) is pipetted into each well, and 200 μL of the working biotinylated antibody solution is added. The strips are then shaken in an automatic shaking device for 2 min, then incubated at 37 °C for 1 h (protocol A), 30 min (protocol B), or 15 min (protocol C). The strips are then washed three times with the wash solution and 100 μL of the working tracer reagent (streptavidin–BCPDA–Eu^{3+}) is added. After incubation at 37 °C for 30 min (A), 15 min (B), or 10 min (C), the strips are washed three times with the wash solution and dried with a stream of air. Surface fluorescence is measured in a CyberFluor 615 Immunoanalyzer. The instrument can automatically reduce the data, and results along with the calibration curve are printed automatically as soon as the readings are complete (about 5 min for a 96-well plate).

Results

Assay Optimization

We used the three available antibodies either as "capture" (immobilized on microtiter wells) or "detection" (biotinylated) antibodies. We determined early in these studies that none of the pairs 6103–6103, 6104–6104, or 6105–6105 produced any dose–response curves, indicating that the pancreatic amylase molecule does not possess more than one copy of the antigenic determinant recognized by these antibodies. We also verified, by using either 6103 as coating and 6104 as detection, or vice versa, that these two antibodies bind to the same antigenic determinant or to antigenic determinants positioned in such a way that the binding of the one antibody prevents binding of the other. A 6103–6104 pair could not produce a dose–response curve. The antibody combinations that produced dose–response curves were (coating antibody first) 6103–6105, 6104–6105, 6105–6103, and 6104–6105. From all these systems, we selected the combination 6104 (coating)–6105 (detection), because it gave the steepest dose–response curve.

We have studied the time course of both the first and second incubation steps. Using a constant 30-min incubation of the second step, we constructed calibration curves by varying the antibody binding step from 10 to 120 min. Figure 1*a* shows that antibody binding increases continuously, even after 120 min of incubation at 37 °C. We also used a constant 60-min antibody incubation step and varied the streptavidin incubation step from 5 to 45 min (Figure 1*b*). Streptavidin binding reached a plateau at 30 min of incubation only at amylase concentrations <500 μg/L.

On the basis of these observations, we devised three different assay protocols: A, 60 min–30 min; *B*, 30 min–15 min; and C, 15 min–10 min. The times refer to the first and second incubation steps, respectively. Because the above procedures do not operate under equilibrium conditions, pipetting steps for one plate should be completed within 5 min for protocols B and C and within 10 min for protocol A to avoid unacceptable errors (>10%) caused by between-well variations in incubation time.

Figure 2 depicts dose–response curves for the three assay protocols established (log-log plots). These curves are nearly linear up to 100 μg/L (C) or 500 μg/L (A, B); they deviate from linearity but they are still useful in the 500 to 1000 μg/L range. The detection limit of the three protocols

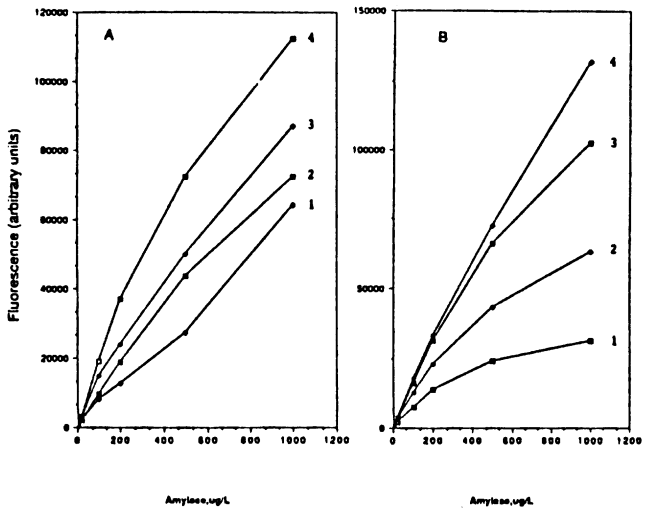

Fig. 1. Effect of incubation time of (*A*) the first incubation step (antibody binding) and (*B*) the second incubation step (streptavidin binding) on the fluorescence observed at various pancreatic amylase concentrations

A: (*1*) 10 min, (*2*) 30 min, (*3*) 60 min, and (*4*) 120 min, with the second incubation kept at 30 min. *B:* (*1*) 5 min, (*2*) 15 min, (*3*) 30 min, and (*4*) 45 min, with the first incubation kept at 60 min

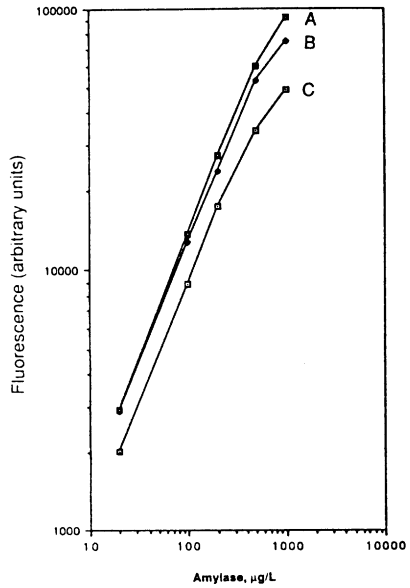

Fig. 2. Calibration curves (log-log plots) of the proposed assay with use of protocols A, B, and C

(defined as the concentration corresponding to the mean plus three standard deviations of the zero standard signal for 12 replicates) was found to be essentially the same, 1.1 µg/L. Protocols having a steeper calibration curve (e.g., A in comparison with B and C) do not have a better detection limit, because when the incubation times are increased the background signal also increases, along with the specific signal. The background signal was around 800, 400, and 190 arbitrary units for protocols A, B, and C, respectively.

Other Analytical Variables

Precision. Within-run precision (Table 1) was evaluated by assaying human control sera (supplemented serum samples) at three concentrations, 24 times each. We also assessed day-to-day precision at the same analyte concentrations for a month (12 runs). The CVs were between 9% and 13%.

Dilution test. We evaluated the dilution linearity of the procedure by assaying samples serially diluted with the zero standard (Table 2). The expected values were derived from the initial concentrations of amylase in the undiluted samples. The results show reasonable agreement between expected and observed values in all cases.

Analytical recovery. Amylase-supplemented serum samples were prepared by adding known concentrations of exogenous pancreatic amylase to aliquots of pooled serum samples. Analytical recovery was assessed by analyzing the samples before and after the additions and subtracting the estimated endogenous pancreatic amylase concentrations. As shown in Table 3, recovery ranged from 78% to 97%, averaging 86%.

Specificity. The proposed procedure was tested for cross-reactivity by salivary amylase. At salivary amylase concentrations <25 000 µg/L, the equivalent pancreatic amylase response was <1 µg/L. Cross-reactivity, calculated at salivary amylase concentrations between 100 000 and 1 000 000 µg/L, was 0.005%.

High-dose "hook" effect. We constructed dose–response curves with all three procedures we developed, using doses of pancreatic amylase as high as 100 000 µg/L. We found that the fluorescence signal continues to increase with increasing dose up to about 10 000 µg/L, then reaches a plateau, which is maintained at doses between 10 000 and 100 000 µg/L. Thus, the assays are free from the high-dose hook effect, at least up to 100 000 µg of pancreatic amylase per liter.

Reference Interval

Using the proposed protocol A, we analyzed samples obtained from a mixed population of 62 apparently healthy individuals ranging in age from 20 to 48 years. Figure 3 shows the frequency distribution obtained. The minimum and maximum concentrations observed were 23 and 229 µg/L, respectively. The mean was 126 (SD 44) µg/L; the median, 124 µg/L. The range (i.e., the mean ± 2 SD) was 38–214 µg/L, which is proposed as the reference interval for this assay.

Table 1. Within-Run Precision of the Proposed Assay[a]

	Protocol A		Protocol B		Protocol C	
Control	Mean (and SD)	CV, %	Mean (and SD)	CV, %	Mean (and SD)	CV, %
1	198 (14)	6.9	198 (14)	7.3	185 (15)	8.0
2	315 (13)	4.0	349 (21)	6.0	301 (34)	11.4
3	395 (23)	5.9	453 (28)	6.1	383 (37)	9.6

[a] Concentrations in µg/L; n = 24 each.

Table 2. Dilution Linearity of Samples with High Amylase Concentrations

Samples	Undiluted	2×	4×	8×	16×	32×
Protocol A						
1 Expected	—	203	102	51	25	13
Observed	405	164	99	51	26	13
2 Exp	—	272	136	68	34	17
Obs	544	199	119	64	31	18
3 Exp	—	264	132	66	33	16
Obs	529	349	199	107	54	30
4 Exp	—	163	82	41	20	10
Obs	326	164	86	41	21	12
5 Exp	—	402	201	100	50	25
Obs	804	427	192	99	50	24
Protocol B						
1 Exp	—	210	105	52	26	13
Obs	419	176	95	51	23	13
2 Exp	—	276	138	69	34	17
Obs	552	210	131	66	28	19
3 Exp	—	370	185	92	46	23
Obs	740	413	196	102	48	29
4 Exp	—	184	92	46	23	12
Obs	367	153	82	39	20	11
5 Exp	—	430	215	108	54	27
Obs	861	422	204	100	46	27
Protocol C						
1 Exp	—	230	115	58	29	14
Obs	459	213	94	50	21	11
2 Exp	—	295	148	74	37	18
Obs	590	309	126	59	31	16
3 Exp	—	358	179	90	45	22
Obs	717	423	220	98	48	27
4 Exp	—	200	100	50	25	12
Obs	400	174	86	39	18	9
5 Exp	—	398	199	100	50	25
Obs	795	449	236	101	48	22

Table 3. Analytical Recovery for the Proposed Pancreatic Amylase Assay (Protocol A)

Pancreatic amylase, μg/L			
Initially present	Added	Measured[a]	Recovery, %
132	100	85	85
	300	290	97
130	100	86	86
	300	264	88
98	100	79	79
	300	233	78
118	100	85	85
	300	265	88

[a] Corrected for that initially present.

Correlation with Other Methods

We compared results obtained with the present method (protocol A) with those by two established total amylase procedures, a pancreatic amylase procedure (immunoinhibition) and a lipase procedure (Table 4). As previously observed by others, the lipase procedure produced, in our hands, results with "negative" activity (six of 44 specimens). These specimens have been considered arbitrarily as having zero lipase activity during the correlations.

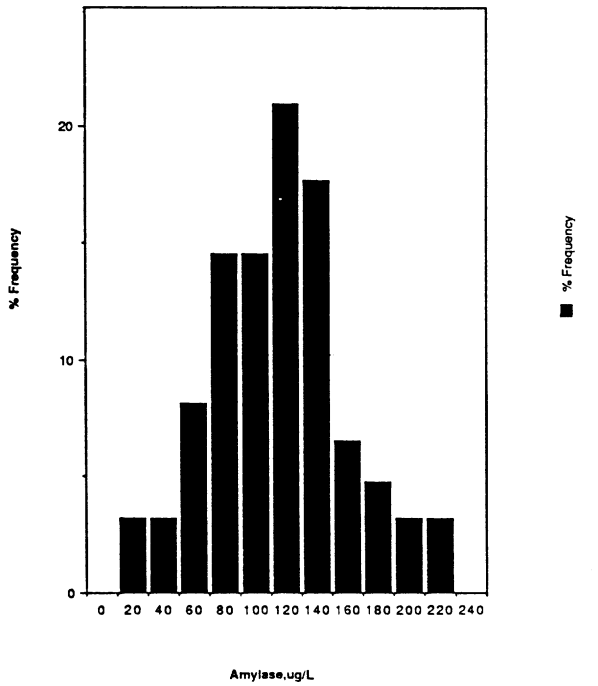

Fig. 3. Frequency distribution of pancreatic amylase values in the serum of normal individuals (n = 62)

Table 4. Linear-Regression Correlation of Amylase Results Obtained with the Proposed and Other Methods

Comparison methods (*x*)	Slope	Intercept	*r*	n
y = *Amylase FIA (μg/L)*				
Phadebas, total (U/L)	1.91	−286	0.97	62
BMC, total (U/L)	4.36	−229	0.98	62
BMC, pancreatic (U/L)	4.90	32	0.98	53
BMC, lipase (U/L)	1.72	91	0.87	44
y = *Phadebas, total (U/L)*				
BMC, total (U/L)	2.23	43	0.98	62
BMC, pancreatic (U/L)	2.43	187	0.96	53
BMC, lipase (U/L)	0.89	219	0.88	44
y = *BMC, total (U/L)*				
BMC, pancreatic (U/L)	1.10	68	0.98	53
BMC, lipase (U/L)	0.39	88	0.86	44

BMC, Boehringer Mannheim Corp.

Sequential Monitoring

We measured amylase and lipase activity and pancreatic amylase activity and mass in sequential specimens from two patients who showed marked changes in amylase values. Figure 4 summarizes the results. The patterns for all analytes used are similar in both of these cases.

Discussion

Although total amylase testing has been in use for decades for investigating pancreatic disorders, there is still interest in improving the methodology with more elaborate procedures. There is agreement in the literature that the quantification of pancreatic isoenzyme instead of total amylase activity should be preferred. However, practical isoenzyme methodologies have only recently become available. Specific monoclonal antibodies against the salivary or pancreatic isoenzymes have recently been produced. Some of these antibodies can cause inhibition in enzymatic activity, others do not. Thus, several immunoassay designs have been published that are based on either immunoinhibition or immunoextraction.

Instead of measuring enzyme or isoenzyme activity, it is also possible to measure enzyme mass by a methodology similar to a classical competitive or noncompetitive (e.g., immunoradiometric) technique. Assays of the mass of enzymes such as trypsin (*27, 28*), creatine kinase MB isoenzyme (*29*), elastase (*30*), amylase (*15, 21*), and phospholipase-A_2 (*31*) have recently been proposed. In some instances it has been shown that enzyme activity correlates well with immunoreactivity (*29*), but it must be kept in mind that immunoreactivity can be detected without enzyme activity if proenzyme forms or enzyme fragments are present in the sample. In the case of amylase, the preferred index in terms of clinical information has yet to be established.

The present assay is based on the principles of noncompetitive immunoassays, and the detection system we used has been thoroughly described before (*23*). To our knowledge, this is the first "two-site" immunoassay with monoclonal antibodies for pancreatic amylase. We have developed three different protocols having total incubation times of either 90, 45, or 25 min. Thus, with the 25-min assay, results can be generated rapidly while maintaining an acceptable turnaround time. The analytical characteristics

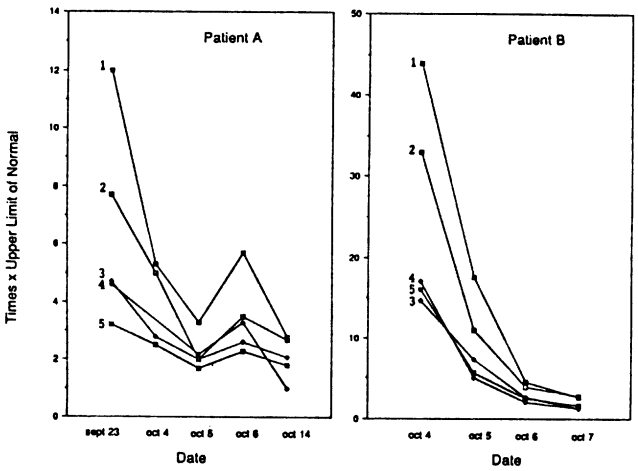

Fig. 4. Sequential monitoring of amylase, pancreatic isoamylase, and lipase in the serum of two patients

The *y*-axis represents ratios of observed concentration or activity and upper limit of normal for each methodology (stated in parentheses): *1*, Proposed method (214 μg/L); *2*, pancreatic amylase BMC (<64 U/L); *3*, Phadebas (<300 U/L); *4*, lipase (<190 U/L); and 5, BMC total (<120 U/L)

of the present assay are acceptable. A notable advantage in comparison to immunoinhibition assay (*20*) is that salivary amylase practically does not cross-react, but about 2–3% of the total activity remains even after salivary isoenzyme inhibition by two monoclonal antibodies (*20*).

Reports suggest that measurement of lipase instead of amylase in serum should be preferred, because lipase is more pancreato-specific. In our experience, the lipase assay is not satisfactory, because it gives negative activities in a high proportion of sera from normal individuals. Results by our assay were shown to correlate strongly with assays that measure pancreatic and total amylase activity in serum. However, our clinical evaluation should only be considered preliminary. We are now in the process of further studying the clinical information that this assay offers in comparison to immunoinhibition isoenzyme assays. Results of these studies, which will include patients with macroamylasemia and urinary testing, will soon be reported.

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