An Immunochemical Procedure for Determination of Mitochondrial Aspartate Aminotransferase in Human Serum

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An immunochemical procedure is described for quantitation of mitochondrial aspartate aminotransferase (m-AspAT; EC 2.6.1.1) activity in human serum specimens. Antibodies directed against purified soluble aspartate aminotransferase (s-AspAT) from human erythrocytes were produced in rabbits and partly purified. Antibody sufficient for analyses of >6000 specimens could be obtained from 15 mL of rabbit antiserum; contaminant AspAT activity of the antibody preparation was <0.4 U/L. Addition of antibody directly to purified AspAT isoenzymes resulted in inhibition of s-AspAT but had no measurable effect upon m-AspAT. Antibody is incubated with serum in the presence of polyethylene glycol for 60 min at room temperature, then 60 min at 4 °C, and centrifuged (7000 × g, 4 °C, 15 min). No detectable s-AspAT activity remains in the supernatant fluid; thus m-AspAT activity can be measured directly. Precision, both within-day and day-to-day, was <1 U/L, or 3.0% of residual m-AspAT activity. The method completely removed 1200 U of purified s-AspAT activity per liter; addition of s-AspAT to serum in increasing concentrations to about 500 U/L had no effect upon the measurement of residual m-AspAT activity. Results of the procedure described showed excellent correlation with those by an alternative procedure involving antibodies directed against m-AspAT. Addition of both anti-s- and anti-m-AspAT antibodies resulted in complete removal of serum AspAT activity. Univalent Fab fragments prepared from anti-s-AspAT antibodies were capable of directly inhibiting s-AspAT activity without precipitation. Although a homogeneous immunoinhibition assay was possible, the greater precision of the precipitation assay made it preferable.

Additional Keyphrases: heart disease • isoenzymes

Measurement of the isoenzymes of aspartate aminotransferase (AspAT; L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) as a diagnostic tool was suggested in the mid-1960s by Schmidt et al. (1, 2). This measurement has since been recommended by several other workers for diagnosis and estimation of severity of both heart and liver disease (3–8). Despite the possible improved diagnostic information resulting from the measurement of AspAT isoenzymes, this test has not been widely accepted. One reason for this lack of acceptance may be that only cumbersome chromatographic or semiquantitative electrophoretic techniques have been available. The advantages and shortcomings of the available techniques have recently been reviewed (9).

Recent publications from this laboratory (10, 11) have described the use of antibodies directed against the human mitochondrial isoenzyme (m-AspAT), allowing quantitation of residual soluble isoenzyme (s-AspAT). This report describes a convenient assay technique using antibodies directed against human s-AspAT, which allows direct measurement of residual m-AspAT activity.

Materials and Methods

L-Aspatic acid, 2-oxoglutaric acid, pyridoxal phosphate, β-NADH (Grade III), p-hydroxymercurobenzoate (sodium salt), and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Chemical Co., St. Louis, MO 63178. Porcine-heart dehydrogenase (EC 1.1.1.37), supplied in glycerol, and porcine-heart s-AspAT were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN 46250. Papan (EC 2.4.22.2), twice crystallized, was obtained from Worthington Biochemicals Co., Freehold, NJ 07728; polyethylene glycol 6000 (av M, 6000–7500) from J. T. Baker Chemical Co., Phillipsburg, NJ 08865; and Ethidin (6,9-diamino-2-ethoxyacidine lactate; Rivanol) from Winthrop Laboratories, New York, NY 10016. Sephadex ion exchangers were obtained from Pharmacia, Piscataway, NJ 08854, and prepared as directed. All other chemicals were of reagent grade or higher purity. Distilled, de-ionized water with a resistivity >15 MΩ-cm at 25 °C was used throughout. Wherever possible, reagents were passed through filters of 0.22-μm pore diameter (Millipore Corp., Bedford, MA 01730) before use.

AspAT activity was measured in the presence of exogenous pyridoxal phosphate by coupling oxalacetate production with NADH in reaction with malate dehydrogenase. Absorbance change was monitored at 339 nm; all units (U) are μmol·min⁻¹ at 30 °C. Assay concentrations, in millimoles per liter, were: L-aspartate, 180; 2-oxoglutarate, 15; NADH, 0.18; pyridoxal phosphate, 0.11; and Tris buffer, 89. The pH was 7.8, and malate dehydrogenase activity was ≥ 1.0 U/mL at 25 °C. Volume fraction of specimen was 0.083 (1:12). Reactions were initiated by adding 2-oxoglutarate after the specimen had been incubated for 10 min with an otherwise complete reaction mixture. Aspartate-free blanks and reagents blanks were performed as specified in the Results and Discussion sections. Activities were calculated by using an absorptivity of 6.22 × 10³ L·mol⁻¹·cm⁻¹ for NADH at 339 nm.

Patient sera containing a wide range of AspAT activities were selected from a hospitalized population. The sera were frozen at < −60 °C within 24 h of collection and used within eight weeks.

Rates of absorbance change were monitored and recorded with a Cary 219 spectrophotometer (Varian Associates, Palo Alto, CA 94303) and LKB 8600 and 2086 Reaction Rate Analyzers (Clinicon/BMC, Indianapolis, IN 45387). Reaction mixtures were maintained at 30 ± 0.1 °C by using fluid circulator baths with external cooling (Haake Instruments, Rockville, MD 20852). The LKB instruments have temperature-control units, but an LKB 8620 cooling stage was used to ensure accuracy. Temperatures within the reaction cuvettes were verified with a Model 45 CU cuvette thermometer and gallium cell (Yellow Springs Instruments, Yellow Springs, OH 45387). Centrifugations were performed in a Model RC-5 refrigerated centrifuge (DuPont Sorval, Newtown, CT 06470). Double immunodiffusion (12) was carried out on agar gel
plates (Hyland, Division of Travenol Laboratories, Costa Mesa, CA 92626) for 24–48 h at room temperature and in a moist chamber. The plates were then washed well with distilled water and photographed directly against a black background.

Electrophoresis was performed with cellulose acetate media and equipment from Helena Laboratories, Beaumont, TX 77704, and protein was made visible by using Ponceau S dye (Beckman Instruments Corp., Fullerton, CA 92634). The indium slide method of Gieaver (13) was used to estimate concentrations of antibody in rabbit antisera. (I am grateful to Dr. Ivar Gieaver, Schenectady, NY, for performing these measurements.) Total protein was determined by either the biuret (14) or Folin–phenol (15) technique.

Antibody Fab fragments were prepared by adjusting purified IgG fractions (prepared by Ethidion precipitation, see below) to a protein concentration of about 5 mg/mL in acetic acid buffer, 60 mmol/L, pH 5.5, and EDTA and cysteine, both at 2.5 mmol/L. Papain was added at 0.9 U/mg of IgG and incubated at 37 °C for 8.5 h. Digestions were stopped by adding 0.01 M hydroxymercuribenzoate to 2 mmol/L, and samples were dialyzed against acetic acid buffer (10 mmol/L, pH 5.5) at 4 °C, with frequent changes. Fab fragments were isolated by chromatography on CM-Sephadex C-50 equilibrated with acetic acid buffer, 10 mmol/L, pH 5.5. Whole IgG could not be detected in this preparation by double immunodiffusion against human s-AspAT.

Enzyme preparation. Human s-AspAT was prepared from erythrocytes by the procedure of Rej et al. (16). The purification scheme was modified to include pyridoxal phosphate (50 μmol/L) in all buffers and solutions. The enzyme was eluted from the final DEAE-Sephadex chromatography column by a linear gradient rather than by the stepwise elution originally described. The preparation was homogeneous by polyacrylamide gel electrophoresis under both dissociating (17) and nondissociating (18) conditions, with only s-AspAT detected by cellulose acetate electrophoresis (9). Specific activity was >150 U/mg at 30 °C.

Production of antisera. The purified s-AspAT was adjusted to 130 μg of protein per milliliter (about 20 U/mL) in Tris-acetate buffer, pH 7. This solution was thoroughly homogenized with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI 48232) and used to prepare antisera in five- to six-month-old female rabbits. Six subcutaneous injections of 2 mL each (130 μg of enzyme protein) were administered at one-week intervals. After completion of the course of immunization, about 35 mL of blood was drawn from each animal at two-week intervals. The serum was separated and stored at −20 °C. Sera from each animal were individually pooled as required and antibodies were purified either by adding Ethidin (19), as described below, or by (NH₄)₂SO₄ fractionation (10).

Anti-human-m-AspAT and anti-porcine-s-AspAT antisera were prepared as described previously (9–11).

Immunoprecipitation Assay of Aspartate Aminotransferase Isoenzymes

The following procedure was found to be convenient for routine laboratory determination of AspAT isoenzyme activity. Validation of these conditions is given under Results.

Principle. Serum specimens are incubated with anti-s-AspAT antibodies and centrifuged, and the supernatant fluid is assayed for residual m-AspAT activity. Total AspAT activity is determined in the usual manner; s-AspAT activity may be estimated by difference.

Reagents:

PEG solution (200 g of polyethylene glycol per liter, in Tris buffer, 50 mmol/L, pH 7.0). To about 800 mL of de-ionized water, add 6.05 g of Tris base and 200 g of polyethylene glycol 6000. Adjust the pH to 7.0 ± 0.05 at room temperature, and bring the volume to exactly 1 L. Store at 4 °C. This solution will be stable for at least six months.

Anti-s-AspAT antibodies. To each 100 mL of rabbit antisera (prepared as above), add 350 mL of a solution containing 4 g per L of Ethidin (Rivanol). Allow the mixture to stand for 30 min at room temperature. Remove denatured protein by centrifugation at 5000 X g for 30 min. The supernate contains the IgG serum fraction and Ethidin (19). Dialyze extensively at 4 °C against de-ionized water, followed by Tris buffer, 10 mmol/L, pH 7.5, until no yellow precipitates. Dispense in 5.0-mL aliquots and lyophilize; store at 4 °C. If lyophilization is not feasible, store at ≤−40 °C in 5-mL aliquots.

Reconstitute (or thaw) one vial of antibody preparation before assay. One 5-mL aliquot suffices for about 500 analyses. Lyophilized or frozen antibody is stable for longer than six months. Liquid preparations are stable for at least two weeks at 4 °C.

Procedure. Prepare a small centrifuge tube for each serum specimen to be analyzed, and pipette 500 μL of each specimen into its tube. Add 10 μL of antibody solution, followed by 20 μL of PEG solution. Mix the contents of each tube gently but thoroughly, and allow the tube to stand at room temperature with gentle shaking for 1 h. Place at 4 °C for 1–16 h, and centrifuge (7000 g, 4 °C, 15 min).

Determine the AspAT activity in the supernate as described above; this is the m-AspAT activity of the specimen. Addition of the antibody preparation and PEG solution results in a slight dilution of specimen. Correct for this dilution by a factor of 1.15, in addition to the usual factors for calculating enzyme activity from absorbance changes.

Results

Ouchterlony double immunodiffusion of rabbit anti-human-s-AspAT with purified human isoenzymes and porcine s-AspAT revealed precipitin lines with both human and porcine s-AspAT and partial identity between these two soluble isoenzymes (Figure 1). We could see no cross reactivity between anti-human-s-AspAT antibodies from four experimental animals and m-AspAT. Addition to the human s-AspAT of purified Fab fragments, prepared from the anti-human-s-AspAT, blocked formation of precipitin lines between complete antibody and the enzyme (Figure 1).

The amount of rabbit serum IgG directed against s-AspAT was estimated by the indium slide assay (13) to be about 2 g/L for each of the immunized rabbits; total protein ranged from 65 to 70 g/L. Electrophoretic examination of the antibodies prepared by Ethidin treatment showed predominantly γ-globulin with some contamination by β-globulins and a trace of albumin. Its total protein content was typically 2.4 g/L; if the dilution of serum by the Ethidin treatment is taken into account, this is equivalent to 15% of the total protein of the complete serum. Average contaminant AspAT activity of this preparation from four animals was <0.2 U/L.

Addition of antibody preparation to the complete AspAT assay mixture resulted in a progressive inhibition of enzyme activity with time if pure s-AspAT was used as a sample (Figure 2). Enzyme activity was decreased by about 97% after 30 min. If m-AspAT was the sole source of enzyme activity, no decrease in activity was observed. Addition of Fab fragments to the reaction mixture resulted in only slightly less efficient inhibition of s-AspAT than was achieved with whole antibodies (Figure 2). Comparable rates of inhibition were found in the absence of 2-oxoglutarate. Increasing the amount of s-AspAT added to about 500 U/L resulted in similar inhibition, except that 7% of the original activity remained after 30 min.

Addition of antibody to s-AspAT in the presence of poly-
ethylene glycol, followed by centrifugation, resulted in complete removal of s-AspAT activity. The effect of increasing amounts of anti-s-AspAT antibody upon supernatant s-AspAT activity is presented in Figure 3. No activity was detectable at antibody volume fractions ≥ 0.025, and the volume fraction of 0.043 was selected for the routine procedure described in Materials and Methods. The effects of temperature and length of incubation of specimen with antibody are shown in Table 1. If treated specimens were centrifuged as described, the conditions selected for the routine assay (60 min at room temperature followed by 60 min at 4 °C) sufficed to precipitate all s-AspAT activity. Without centrifugation, the minimum residual s-AspAT activity was 1–2%.

The procedure described requires incubation of serum at a volume fraction of 0.87 in the presence of polyethylene glycol and Tris buffer (incubation concentrations, 17 g/L and 1.5 mmol/L, respectively). To ensure that the decrease in measured AspAT activity is due solely to the presence of anti-s-AspAT antibody, the activities of 30 patients’ specimens and of 10 control specimens containing purified human m- or s-AspAT, or both, were determined without antibody. In this experiment, antibody was replaced by Tris buffer (10 mmol/L, pH 7.5). Incubation was for 60 min at room temperature, followed by 18 h at 4 °C. The activities of these specimens were

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**Fig. 1.** Ouchterlony double-diffusion patterns of rabbit antisera against AspAT isoenzymes

A. Center well contained 5 μL of anti-s-AspAT antiserum. Outer wells contained 0.06 U of human s-AspAT (1,3), 1.0 U of porcine s-AspAT (2,4), or 0.08 U of human m-AspAT (5).

B. Center well contained 0.06 U of human s-AspAT. Wells 1–4 contained 5 μL each of anti-human-s-AspAT antiserum obtained from four immunized animals; well 5 contained 5 μL of anti-porcine-s-AspAT.

C. Center well contained 5 μL of anti-human-s-AspAT. Each outer well contained 0.06 U of human s-AspAT. Purified Fab fragments were added to wells 2 and 4.

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**Fig. 2.** Inhibition of human AspAT isoenzymes by anti-human-s-AspAT antibodies as a function of time

- (a) Purified human s-AspAT was the source of enzyme (100% = 124 U/L), and purified rabbit anti-human-s-AspAT antibodies were added at a volume fraction of 0.077 to the assay mixture. (b) Purified human m-AspAT was the source of enzyme (100% = 48 U/L), and purified rabbit anti-human-s-AspAT antibodies were added at a volume fraction of 0.077. Substrate was exhausted after 25 min.

- (c) Purified human s-AspAT was the source of enzyme (100% = 124 U/L), and purified Fab fragments of rabbit anti-human-s-AspAT antibodies were added at a volume fraction of 0.077. Reactions were initiated by simultaneously adding 2-oxoglutarate and either antibody or Fab fragments. Other conditions were identical to the assay for AspAT activity specified in Materials and Methods; temperature was 30 °C. Change in absorbance was constantly recorded and activities calculated from tangents to the curve at times shown.

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**Fig. 3.** Effect of increasing volume fraction of anti-human-s-AspAT antibody preparation on activity of human AspAT isoenzymes

Sources for enzyme activity were: (a) m-AspAT (100% = 90 U/L) and (c) s-AspAT (100%–148 U/L). Anti-s-AspAT antibodies were added as shown; differences in volume were compensated by adding Tris buffer, 10 mmol/L, pH 7.5; polyethylene glycol was added to a final concentration of 17 g/L. The solutions were incubated at room temperature for 80 min, cooled to 4 °C for 60 min, and centrifuged (7000 × g, 4 °C, 15 min). Residual activity in the supernate was determined by the routine assay for AspAT activity. Arrow shows the volume fraction (0.043) selected for the direct assay of m-AspAT.
also determined by the routine assay for AspAT activity with no specimen dilution or incubation. No significant difference in total AspAT activities as determined by these two procedures could be shown by paired t-test (p < 0.4).

The ability of the procedure to completely remove various amounts of s-AspAT added to a patient's serum pool is shown in Figure 4A. No residual s-AspAT activity could be demonstrated in the supernate; mean m-AspAT activity was estimated in this series as 2.2 (SD 0.7) U/L. In a similar study in which we used a different serum pool to which human m-AspAT had been added (Figure 4B); residual m-AspAT activity was estimated as 49.1 (SD 1.2) U/L. Neither case showed a significant correlation between residual m-AspAT activity and the amount of soluble enzyme added over the range 0-450 U/L.

Within-day precision was estimated from data on eight replicate analyses of patients' sera or patients' sera to which purified human iso-enzymes had been added. Day-to-day precision was estimated from data on 20 replicate analyses done during five days by two different analysts. For the specimen with the lowest m-AspAT activity (mean m-AspAT activity, 7.21 U/L) the within-day SD was 0.71 U/L (CV, 9.8%), and the day-to-day SD was 0.83 U/L (CV, 11.5%). For the specimen with the highest m-AspAT activity (mean, 138.2 U/L) the within-day SD was 3.6 U/L (CV = 2.6%), and the day-to-day SD was 3.9 U/L (CV = 2.8%). Limiting precision for the series was either 0.7 U/L or 2.5%. Location and dispersion parameters were verified by use of a robust calculation procedure (20).

The present procedure was compared with the immunoprecipitation assay with use of anti-m-AspAT antibodies (10, 11), as shown in Figure 5. Individual patients' sera were selected to provide representative m-AspAT activities ranging from 2.8 to 73 U/L; total AspAT activities ranged from 23 to 560 U/L. Correlation between the two methods was good; the slope was near unity and the intercept < 1 U/L (Figure 5). Measured m-AspAT activities of a dilute homogenate of human liver and of a control specimen containing human m- and s-AspAT in a human albumin matrix were identical by both methods (Figure 5).

Discussion

The described procedure reliably and conveniently determines m-AspAT in human serum or tissue homogenates. Antibodies, prepared as described, react immunologically with both human and porcine s-AspAT but do not inhibit or show

- **Table 1. Effect of Incubation Conditions and Centrifugation on Residual s-AspAT Activity**

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<th>Incubation 1</th>
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<th>Activity, U/L (%)</th>
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* Residual s-AspAT. Specimen contained only s-AspAT activity in an albumin (10 g/L) matrix.
* Centrifugation for 15 min at 7000 × g and 4 °C.
* Control assay without antibody.
* Room temp. = 20 ± 1 °C.

- **Fig. 4. Effectiveness of immunoprecipitation assay with specimens containing high s-AspAT activities**

- **Fig. 5. Comparison of two immunoprecipitation assays for measurement of m-AspAT**

Patients' specimens (⊗) selected to represent a large range of m-AspAT activities were assayed by the procedure described in Materials and Methods (ordinate) and by the immunoprecipitation assay (10, 11) using anti-m-AspAT antibodies (abscissa). Robust correlation methods (20) gave a first-order regression equation of $y = 1.021x - 0.75; r^2 = 0.982$. Specimens containing dilute human liver homogenate (△) and an albumin-based specimen with purified human s- and m-AspAT iso-enzymes (O) were also assayed by both procedures but not used in regression calculations. Total AspAT activities of these patients' sera ranged from 23 to 560 U/L.
precipitin lines with human m-AspAT (Figure 1). This is consistent with several reports of immunologic, kinetic, and primary structural similarities among homologous AspAT isoenzymes of different species and with reports of differences (by the same criteria) between the soluble and mitochondrial isoenzymes (9, 21-23).

We used four anti-human-s-AspAT antibody preparations and one anti-porcine-s-AspAT antiserum to demonstrate partial identity between porcine and human s-AspAT by Ouchterlony double diffusion (Figure 1). Two partially fused precipitin lines were occasionally observed between the antibody and human s-AspAT. These were not attributed to contamination of the s-AspAT preparation used, because only slightly decreased amounts of s-AspAT showed single precipitin lines. Moreover, addition to the antisem of porcine heart s-AspAT, which is unlikely to contain contaminants common to our human s-AspAT preparation, prevented the formation of precipitin lines. The secondary lines might have formed in reaction to monomeric s-AspAT subunits, which can form at the dilutions used (24), or they may be merely line duplication, owing to an imbalance between the amount of enzyme and antibody applied to the agar gel (12).

The inhibition of activity by antibody preparations added to enzymes, as shown for s-AspAT in Figure 2, may be caused by inhibiting antibodies, by gradual precipitation of the active enzyme from solution, or by formation of large aggregates which limit substrate diffusion. Inhibition of s-AspAT by univalent Fab fragments (Figure 2) indicates that the antibodies produced are directed to determinants near the catalytic site or that they bind in such a manner that enzyme activity is markedly disrupted. Thus removal of enzyme activity in the procedure described is a result of enzyme inhibition as well as precipitation. This probably explains the broad nadir of enzyme activity at widely varying ratios of antibody to antigen (Figure 3).

Because inhibiting antibodies are produced, a homogeneous immunoinhibition assay is possible, which would obviate the need for centrifugation. As shown in Figure 2, antibody at a volume fraction of 0.077 in the AspAT assay reagent can inhibit s-AspAT activity within about 30 min to about 3% of that originally determined. Similar observations are shown in Table 1 for preincubation of specimen directly with antibody at a volume fraction of 0.043 without centrifugation. We estimated the minimum residual s-AspAT activity in homogeneous immunoinhibition assays, using our antibodies, to be 1 to 2% of the s-AspAT added.

Similar low residual activities have been tolerated for other immunoinhibition assays (10), most notably for creatine kinase (CK; EC 2.7.3.2) B-subunit activity (25). In fact, there are considerable similarities between the measurement of the CK and AspAT isoenzymes. Like m-AspAT (4, 10, 26, 27), CK-MB or B-subunit activity (25, 28) is usually present in serum at quite low activities, often near the capability of some routine clinical analyzers, even when the serum total enzyme activity is greatly elevated.

We have estimated that the contribution of m-AspAT to total AspAT activity in patients' sera ranges from 1 to 40% and represents 10-12% (about 1.5 U/L) of the enzyme in normal sera. Thus even 2% residual s-AspAT activity may be equivalent to the total true m-AspAT activity. Even with a systematic correction for this uninhibited s-AspAT, as has been suggested for other immunoinhibition techniques (10, 11, 25), this residual activity sharply limits the precision of m-AspAT activity measurement. In addition, variations in the percentage of residual s-AspAT argue against the use of a correction factor. Furthermore, the clinical utility of m-AspAT measurements, unlike that of CK B-subunit activity, is still at the investigational stage (9-11), and only robust methods will help clarify its true significance.

Despite the appealing nature of a homogeneous immunoinhibition assay, the greater precision and accuracy of the precipitation assay make it preferable. The single centrifugation step is not labor-intensive and increases analysis time by only about 20 min. I judge this to be minimal, especially for batch analysis.

The duration of incubation of specimen with antibody is not critical. For most specimens 30 min at 37 °C or 45 min at room temperature, followed by 15 min at 4 °C and centrifugation as described, suffices to completely inhibit s-AspAT. The incubation (specified under "Procedure") for 1 h at room temperature followed by at least 1 h at 4 °C inhibited even large excess amounts of s-AspAT activity. The duration of the incubation at 4 °C is not critical; specimens can be left overnight at this stage without significantly altering the results. The 60-min incubation at room temperature can be replaced by 40 min at 37 °C with identical results (Table 1). The data given in Table 1 were for s-AspAT added to an albumin (10 g/L) matrix. Precipitation occurs more readily in serum-based specimens, and the incubation intervals required for sera are slightly briefer than those presented in Table 1.

For this procedure, we found antibodies to human s-AspAT to be superior to antibodies directed against the soluble enzyme of porcine origin. Although we (9) and others (29) have used antiporcine antibodies with success, precipitation was complete at lower volume fractions and after shorter incubation with the anti-human antibodies.

Only small amounts of antibody preparation are required, ordinarily 10 μL for each specimen. This assumes that the measurement of AspAT activity requires 200 μL or less. If 100 μL or less is required (as is typical for most analytical systems), duplicate measurements can be made of m-AspAT activity from a single prepared specimen. Care must be taken not to disturb the bottom of the centrifuge tube, because vigorous shaking of centrifuged specimens produced residual s-AspAT activities comparable to those obtained without centrifugation. A single bleeding of an immunized rabbit yielded ≥15 mL of antiserum; this is equivalent to >65 mL of the purified antibody preparation, which suffices for more than 6000 analyses.

Contaminant AspAT activity in the antibody preparations never exceeded 0.4 U/L, which is equivalent to <0.02 U/L within the assay and can be ignored. Dilution of the specimen by antibody preparation and PEG solution to a volume fraction of 0.87 results in only a slight loss in sensitivity as compared to assay of the undiluted specimen. This is significantly better than the twofold dilution of specimen required for the immunoprecipitation assay using anti-m-AspAT antibody (10). Dilution of the specimen necessary for the preincubation with antibody led to no measurable loss in precision. The correction factor for this dilution is exactly 1.15.

In the new procedure, s-AspAT added to >450 U/L (Figure 4) was completely precipitated. Additional experiments with purified s-AspAT demonstrated complete removal of activity to at least 1200 U/L (about 40 times the upper limit of the reference interval for this isoenzyme in our laboratory). We and others (16, 30) have shown that the properties of the purified s-AspAT closely resemble those of the isoenzyme in serum. Further evidence that the procedure removes all endogenous s-AspAT activity in human serum is its excellent correlation with the assay involving anti-m-AspAT antibodies (Figure 5). If it had failed to precipitate all endogenous serum s-AspAT, a significant bias would have been observed.

We performed additional experiments using antibodies to both m- and s-AspAT in a single precipitation test to show that all serum AspAT is precipitable by these antibodies. None of the 30 sera tested (maximum total AspAT activity, 560 U/L) contained such activity, and residual activities were <1.0 U/L after deduction of any specimen blank activity. This
finding supports previous observations from our laboratory with use of anti-porcine-s-AspAT (10, 11, 31).

Although the two immunoprecipitation procedures correlated well (Figure 5), precision was greater with antibodies to s-AspAT than with those to m-AspAT. Because it appears that measurement of m-AspAT provides significantly more information than s-AspAT (see below), a major advantage of the new procedure is that it measures this isoenzyme directly, while it must be calculated by difference in the assay involving antibodies directed against the mitochondrial isoenzyme. This advantage is particularly significant in the measurement of low m-AspAT activities in the presence of greatly increased s-AspAT activity, as is often the case for human serum specimens.

The combined day-to-day and within-day precision of the procedure was within 1 U/L, or 3.0%, a precision approaching that achievable for measurement of equivalent activities of total AspAT. Indeed, the adaptation of the new procedure to a variety of analytical systems gave precision data typical of the measurement of total AspAT activity in each system. We therefore concluded that the largest source of variation was at the stage of measurement of enzyme activity, rather than in the immunoprecipitation step.

Measurement of very low m-AspAT activities (<1.0 U/L) is impeded by the presence of any reagent blank activity in the activity measurement step. This apparent AspAT activity may be caused by contaminant AspAT of the malate dehydrogenase (32) or by nonspecific lability of NADH in the reaction cuvette. Blank activity for all cases was <1 U/L in all experiments described here and averaged 0.4 U/L over the course of this investigation. All activities were corrected for the blank activity determined within 2 h of the analysis, although estimation of reagent blank activity once each day gave equivalent results. We have, however, encountered significantly higher reagent blank activities—in particular, in commercial kit preparations—that would severely hamper precise estimation of residual m-AspAT activity. We find reagent blank activities ≤2.5 U/L to be acceptable; greater blank activities were found to be variable over the course of the day, further complicating correction.

The m-AspAT activity can be reported directly or as a percentage of total AspAT activity. The analogy with CK is again appropriate, as there are equally determined proponents for clinical decisions based upon the independent catalytic activity of the B-subunit (33) or its proportion to total CK activity (34). Preliminary data obtained here suggest that direct expression of m-AspAT activity is the more stable indicator of tissue damage.

Although the clinical utility of m-AspAT measurements remains at the investigational stage, several recent reports (3, 4, 6, 8, 35–37) show that significant clinical information, especially on the extent of tissue damage, is gained by determinations of m-AspAT activity. The recent communication by Kamei et al. (35) on the value of both apo- and holo-m-AspAT in clinical assessment of hepatic diseases is of particular interest.

Preliminary studies of the new procedure with patients’ specimens support our previous observations that m-AspAT activity does not merely follow the course of total AspAT activity in serial specimens from individual patients, and therefore provides new clinical information (10, 11, 31). Evaluation of this test with patients entering the cardiac care unit is now in progress.

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References


24. Melander, W. R., Effect of aggregation on the kinetic properties

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