Determination of Fecal $\alpha_1$-Antitrypsin Concentration by Radial Immunodiffusion: Two Systems Compared

Claire M. Wilson, Kathryn McGilligan, and Dan W. Thomas

We compared Helena "QUIPlates" and Calbiochem "LC-Partigen" radial immunodiffusion systems for their ability to measure fecal concentrations of $\alpha_1$-antitrypsin (FA1AT). Reference ranges for FA1AT concentrations in infants receiving various diets, in children, and in adults are given for each system. FA1AT values obtained with Calbiochem LC-Partigen plates averaged 30% greater than those obtained with Helena QUIPlates, but both systems distinguished between normal and high values. Studies involving variations of usual sample-handling procedures showed that storage at room temperature, repeated freezing and thawing, and long-term storage of frozen samples had no significant effect on measured FA1AT concentration. However, values obtained for lyophilized and non-lyophilized samples did not correlate well.

Additional Keyphrases: reference intervals • age-related effects • variation, source of • sample handling • intermethod comparison • protein-losing enteropathy • gastrointestinal disease

In diseases that damage the intestinal epithelium or impair lymphatic flow, serum proteins can be lost into the gut lumen. One such protein is $\alpha_1$-antitrypsin (also known as $\alpha_1$-proteinase inhibitor), which, as an antiprotease, is spared intraluminal degradation and can be measured in stool (1). To a molecular mass similar to that of albumin, the predominant serum protein, $\alpha_1$-antitrypsin is thought to leak into the intestinal lumen in a manner similar to that of albumin and thus can serve as a natural marker for protein-losing enteropathy. Measurement of $\alpha_1$-antitrypsin concentration in random (i.e., un timed) stool samples has been used both as a screening test for protein-losing enteropathy and to follow progression of disorders such as gluten-induced enteropathy (2) and Crohn's disease (3).

Fecal $\alpha_1$-antitrypsin (FA1AT) has been expressed in terms of concentration (J), clearance (4, 5), and 24-h excretion (6). Although our laboratory has experience with all three modalities, our primary focus remains the determination of FA1AT concentration in random (i.e., untimed) stool specimens.

FA1AT has most often been measured by radial immunodiffusion (RID) on plates designed to quantify concentrations of $\alpha_1$-antitrypsin in serum. Previously we used the "M-Partigen" plate system (Calbiochem Behring Diagnostics, La Jolla, CA), which is now unavailable. In the newer Calbiochem "Nor-Partigen" system, the antibody concentration of the plates has been increased to allow their use with undiluted serum; however, this change is unsuitable for measurement of FA1AT, because its concentration is far lower than that of serum $\alpha_1$-antitrypsin. Therefore, we now use the "QUIPlate" system (Helena Laboratories, Beaumont, TX) in our laboratory. Recently, Calbiochem has developed the LC-Partigen plate to measure low concentrations of $\alpha_1$-antitrypsin, but this plate has not yet been approved for clinical use.

In the study reported here we sought to: (a) compare measurement of FA1AT on Helena and Calbiochem LC-Partigen RID plates and determine for each system reference intervals for healthy persons, (b) assess the reliability of using non-lyophilized stool samples, and (c) assess the effect of variations in sample handling that might be encountered in assaying patients' samples, such as storage at room temperature, repeated freezing and thawing, and long-term frozen storage.

Samples

A group of 55 random stool specimens from healthy individuals and a group of 71 stool specimens from randomly selected patients were the source of all fecal specimens used in the experiments detailed below. The patients had various gastrointestinal disturbances known to be associated with protein-losing enteropathy.

Normal reference intervals for FA1AT concentrations were established from data on random stool specimens from the 55 healthy subjects, ages six months to 44 years. Because type of diet is known to influence FA1AT concentrations in infants (7), we divided the subjects as follows: 15 breast-fed infants, nine formula-fed infants, 11 cow's milk-fed infants, 10 children (ages 1.5-13 y), and 10 adults (ages 24-44 y). The mean age of the infant groups was 11 months. Subjects in the infant groups were also fed solids ad lib., but the milk they consumed was exclusively of the type indicated.

To compare FA1AT values obtained by using Helena and Calbiochem plates, we assayed samples from random stool specimens from the 55 healthy individuals and from 15 patients with various gastrointestinal illnesses expected to result in protein-losing enteropathy.

The samples were collected as follows. Stool specimens of at least 5 mL were obtained at home and placed in plastic specimen cups. These were then stored in a household freezer until delivery (preferably on ice) to the laboratory, where they were stored at -20°C until assayed.

Methods

FA1AT concentration was determined by RID assay, modified from Crossley and Elliott (1).

Lyophilization: After thawing the specimens at room temperature and stirring, we placed an approximate 1-g aliquot of each in a 12 × 75 mm polystyrene tube, which was then placed inside a 15-mL Corex tube (no. 844115; Corning, Medfield, MA) for lyophilization overnight.

Helena QUIPlate kit: Using a metal spatula, we ground dried stool in the polystyrene tube to a powder. We placed 50 mg of this in a disposable glass 12 × 75 mm tube and added...
1 mL of isotonic saline. After covering the mouth of the tube with Parafilm (American Can Co., Greenwich, CT), we shook it vigorously (Vortex Jr mixer with a test tube shaker head; American Scientific Products, McGaw Park, IL) for 20 min, transferred the contents to a clean 12 × 75 mm polystyrene tube, and centrifuged this at 4000 × g and 4 °C for 20 min. Using a 5-μL pipette, we transferred a 5-μL aliquot of the supernate to a well of the RID plate. We use 5-μL aliquots rather than the 4 μL suggested by the kit instruction sheet, for greater convenience. Any fat layer present should be avoided when the supernate is pipetted. We also assayed 5-μL aliquots of the three α1-antitrypsin standards included with the kit, as follows: the two highest concentrations (620 and 310 mg/L) were used undiluted, and the 150 mg/L standard was diluted fivefold with isotonic saline to 30 mg/L. To improve precision, we assayed the highest concentration standard in duplicate. Control serum (Helena’s "Kemtrol, Normal," containing 2203 mg of α1-antitrypsin per liter) was reconstituted with de-ionized water and then diluted 10-fold with isotonic saline before assay to give a concentration of 220.3 mg/L. We covered the plate with the lid provided and kept it level in a sealed moist chamber at room temperature (approximately 22 °C). After 24 h, we measured the diameter of the precipitin ring to the nearest 0.1 mm, under magnification with a calibrated viewer (Transidyne General Corp., Ann Arbor, MI) and plotted ring diameter squared vs α1-antitrypsin concentration of the standards (mg/L). Using this standard curve, we converted the diameter squared of a sample to concentration of α1-antitrypsin, and then expressed the result as milligrams of α1-antitrypsin per gram of dry stool by using the equation:

\[
\text{mg/L × } \frac{0.001 \text{ L}}{0.050 \text{ g dry stool}} = \text{mg/g dry stool}
\]

After the 5-μL samples were applied to the plates, the remaining solid stool and supernate were stored in sealed tubes at 4 °C overnight, in case re-assay was necessary because of off-scale results. Such samples were reincubated and the supernates were diluted with isotonic saline as judged appropriate on the basis of ring size and appearance. Another 5-μL aliquot was then plated and read in the usual manner.

Calbiochem LC-Partigen kits: Dried stool was ground to a powder as described above, and 50 mg was placed in a disposable 12 × 75 mm tube and mixed with 3 mL of isotonic saline. (To set normal ranges of FA1AT, we extracted samples from the healthy subjects with 2 mL of isotonic saline, this adjustment being necessary because of the narrow range of the Calbiochem plates.) The sample was shaken vigorously for 20 min and centrifuged as above. Using a 10–100 μL "Pipetman" pipette (Rainin Instrument Co., Woburn, MA), we applied 15 μL of supernate to a well of the Calbiochem LC-Partigen RID plate. The instruction sheet accompanying the kit recommends 20 μL, but we find 15 μL more convenient. To prepare standards we reconstituted lyophilized α1-antitrypsin (cat. no. 539510) to 120 mg/L with de-ionized water according to the manufacturer’s instructions. We further diluted this to 60 and 30 mg/L with isotonic saline, and pipetted 15 μL of each concentration as well as a Calbiochem serum control (cat. no. 512804) with an α1-antitrypsin concentration of 1600 mg/L, diluted 15-fold with isotonic saline to give a concentration of 106.7 mg/L, into wells of the RID plate. Rings were read and results calculated as for the Helena QUIPlates, substituting 0.003 L for 0.001 L in the above equation. In calculating the normal ranges for this paper, 0.002 L was used in the equation, corresponding to the method modification mentioned above.

Other Studies

Diarrheal stool samples from 25 patients were used to measure α1-antitrypsin concentration in both the lyophilized and nonlyophilized state. Helena QUIPlates were used. Lyophilized specimens were prepared as described above. In the nonlyophilized assay (8), 1.0 g of stool was homogenized with 1.5 mL of isotonic saline, centrifuged, and the supernate was assayed as above. Values obtained for lyophilized and nonlyophilized samples were compared by linear regression analysis.

Stability of α1-antitrypsin at room temperature was examined by use of Helena plates. Aliquots of 12 patients’ stool samples were lyophilized and assayed for α1-antitrypsin, then the original specimens were stored in tightly covered plastic cups at approximately 22 °C. Aliquots were removed, lyophilized and assayed at one, two, three, and seven days.

A serum sample from a single blood donor was used in assessing the effect on measured α1-antitrypsin concentration of freezing and thawing. This situation applies to the handling of standard and control α1-antitrypsin solutions used with the RID kits. The serum sample was divided into seven aliquots, diluted threefold with isotonic saline, and 5 μL of each aliquot was applied to a Helena plate. The original seven aliquots were then frozen and thawed twice, and re-plated. Freezing was at −20 °C for one week.

Aliquots of seven patients’ stool samples were assayed as above with the Helena system. After freezing and thawing three times, they were re-assayed. Freezing was at −70 °C overnight.

We examined the effect of long-term storage on α1-antitrypsin concentration, using stool samples from 37 of the normal subjects, assayed on Helena plates. The original samples were stored as whole stool in sealed plastic containers at −70 °C for six months, and re-assayed.

Aliquots of five of the patients’ samples were assayed on Helena plates, in duplicate. The lyophilized samples were stored at −70 °C for a year, then re-assayed in duplicate.

Precision and Variation

To evaluate assay precision, we sampled 10 standards on both the Helena and Calbiochem systems. The concentration of the standard used on the Helena plate was 310 mg of α1-antitrypsin per liter; 90 mg/L was used on the Calbiochem plate.

Within-run variation for the QUIPlate kit was evaluated by using four stool samples from subjects known to have low FA1AT concentrations (mean 1.0 mg per gram of dry stool, range 0.7–1.2) and four samples known to have high values (mean 8.6 mg per gram of dry stool, range 6.0–12.1). Eight aliquots of supernate from each sample were assayed.

We assessed variation due to differences in extraction from dried stool, using four stool samples of low α1-antitrypsin concentration (mean 1.9 mg per gram of dry stool, range 1.3–2.7) and four samples with a high value (mean 9.4 per gram of dry stool, range 5.1–14.8). Four aliquots of each specimen were lyophilized separately, and plated in triplicate (Helena system).

Between-run or day-to-day variation was determined by using four fecal samples of lower α1-antitrypsin (0.8–4.0 mg/L)
mg/g) and four samples in the high range (5.4–12.4 mg/g). In each of eight separate runs, an aliquot of each sample was plated with a set of standards. Results of each run were then determined, with use of its own standard curve.

Statistics

We compared diet-related differences in mean FA1AT concentrations within each RID system by using analysis of variance and Scheffe testing.

FA1AT concentrations obtained with Helena and Calbiochem RID plates were compared by the paired two-tailed t-test and by linear regression.

Results

Normal reference intervals for FA1AT: The mean FA1AT concentration for each group of healthy individuals is given in Table 1 for the Helena and Calbiochem systems. In the Helena system, the range of FA1AT values was 1.5 to 5.3 mg/g for breast-fed subjects, 0.7–2.3 for formula-fed, and 0.0–2.0 for the others. For the Calbiochem system the corresponding ranges were 2.0–10, 1.0–3.3, and 0.0–2.8 mg/g.

Breast-fed infants had a significantly higher mean value than did all other groups with the Helena (comparison a and c, Table 1) but not the Calbiochem system (comparison d, not significant). Formula-fed infants had significantly higher mean values with both systems than did the group comprised of adults, children, and infants fed cow’s milk, designated as “others” (comparisons b and e). There was no significant difference in mean FA1AT concentration among adults, children, and infants fed cow’s milk in either RID system (analysis of variance, P > 0.2).

Comparison of FA1AT values obtained with Helena and Calbiochem plates: FA1AT concentrations from 70 sample pairs (55 healthy subjects and 15 patients) were compared by linear regression and are shown in Figure 1. The correlation coefficient (r) was 0.98. When compared by paired two-tailed t-test, the values obtained with the two systems differed significantly (P < 0.001). Values obtained with the Calbiochem system averaged 30% greater than those obtained with the QUIPlates.

Faint halos occasionally appear around precipitin rings on Helena plates, usually after two to three days. They are seldom seen with the naked eye and are always too pale to be mistaken for the actual precipitin rings. The same sample assayed on another plate often does not exhibit these “ghost” rings; they are not seen with serum, serum standards, or serum controls, and have not been observed on plates from subsequent lots. Buffalo and Shulman (9) report that FA1AT determined by crossed immunoelectrophoresis can be either in its native form, complexed with a protease, or both. To determine whether a “ghost” ring represents a slight reaction between complex and Helena antibody, we examined stool supernate with crossed immunoelectrophoresis (Dako antibody; Accurate Chemical, Westbury, NY) to see if two peaks would appear. Of eight samples having halos when plated, only one produced two peaks. To examine the possibility of a reaction between a non-α1-antitrypsin component of the stool supernate and a component of goat serum used in the plates, we assayed halo-producing samples on plates that contained goat serum with no antibodies to α1-antitrypsin (kindly supplied by Helena). No rings appeared.

α1-Antitrypsin concentration in nonlyophilized stool: The mean water content of 25 unselected stool specimens was 88% (SD 7%). The correlation coefficient between values for α1-antitrypsin obtained for dried vs non-dried stool was 0.75 (Figure 2).

Stability at room temperature: The mean FA1AT value at initial sampling was 5.3 mg per gram of dry stool, with range 0.4 to 19 mg/g (n = 12). Re-assay mean values after storage at 22 °C for one, two, three, and seven days ranged from 5.1 to 5.4 mg/g, showing no significant change (analysis of variance, P = 0.999). Comparison of the initial FA1AT values with the values at seven days (paired two-tailed t-test) showed no significant difference (P = 0.63).

Effect of freezing and thawing: At initial assay, the α1-antitrypsin concentration in serum was 1010 mg/L. Remeasured after freezing and thawing, it was 1030 mg/L—not significantly different by Student’s t-test (P = 0.32). Before freezing, stool samples had a mean α1-antitrypsin concentration of 6.3 mg/g. After freezing and thawing, the mean α1-antitrypsin concentration was 6.4 mg/g. By paired two-tailed t-test, these values are not significantly different (P = 0.52).

Effect of storage: After storage of 37 samples from normal subjects for six months, the mean FA1AT concentration was not significantly altered (P = 0.20, paired two-tailed t-test):

![Graph showing linear regression comparing FA1AT concentrations (mg/g dry stool) obtained with Helena QUIPlates and Calbiochem LC-Partigen RID systems: y = 1.45x – 0.12](image)

Table 1. FA1AT (mg/g dry stool): Reference Values for Healthy Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>( \bar{x} )</th>
<th>SD</th>
<th>( \bar{x} )</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast-fed infants</td>
<td>15</td>
<td>2.6&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>0.9</td>
<td>3.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.2</td>
</tr>
<tr>
<td>Formula-fed infants</td>
<td>9</td>
<td>1.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.6</td>
<td>2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.7</td>
</tr>
<tr>
<td>Others</td>
<td>31</td>
<td>0.9&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.4</td>
<td>1.2&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Mean concentrations with the same letter superscript differ significantly at the given P value. *P < 0.05. **P < 0.01. ***P < 0.005. ****P < 0.005. *****P < 0.01.
before storage 1.0 mg/g; after storage 1.0 mg/g. Five patients' samples stored for a year had an initial mean α1-antitrypsin of 7.0 mg/g. After storage, the mean was 6.9 mg/g, showing no significant change (P = 0.6, paired two-tailed t-test).

Precision and variation: The CV for serum standards assayed with the Helena system was 2.4%, with Calbiochem plates, 5.0%.

Determination of within-run variation for fecal samples showed that the mean α1-antitrypsin for the higher-concentration stools was 8.6 mg/g, with an average difference from the mean of 4%. For the lower-concentration stools these values were 1.0 mg/g and 7.4%.

Assessment of variation due to extraction showed that the mean α1-antitrypsin was 9.4 mg/g for the higher-concentration group, with an average difference from the mean of 7.0%, and 1.9 mg/g for the lower-concentration group, with an average difference from the mean of 3.1%.

Between-run or day-to-day variation (CV) was 9.5% for the samples of lower α1-antitrypsin concentration, 6.4% for those in the high range.

Discussion

In our laboratory a normal FA1AT concentration is less than the mean value for the appropriate diet group plus 2 SD (Table 1). The Calbiochem LC-Partigen plates detect α1-antitrypsin concentrations in the range 0–120 mg/L, which corresponds to 0–7.2 mg per gram of dry stool. Helena plates have a greater range, 0–620 mg/L, corresponding to 0–12.4 mg per gram of dry stool, so fewer off-scale samples (7.5% vs 18%, based on our recent experience with patients' samples) would have to be diluted and re-assayed.

The estimated time required to assay 12 stool samples is 3.5 h, not including time needed to re-assay any off-scale samples. The current cost per sample for the Helena system is $2.00, and $2.63 for Calbiochem plates. The QUIPlate kits include standard α1-antitrypsin concentrations, but they must be purchased separately for the Calbiochem system, and the cost figure above is derived assuming purchase of one vial of standard serum per five plates. Helena control serum currently costs $46 for 10 vials of lyophilized serum, each of which is reconstituted to 2 mL. Calbiochem control serum currently costs $21 for three vials of lyophilized serum, each of which is reconstituted to 0.5 mL. Control serum values in our laboratory remain constant for over a month with reconstituted serum stored at 4 °C. Diets et al. have found that lyophilized control serum can be safely stored for five years (10). To ensure consistency during a long-term study, a single lot of control serum and QUIPlates can be manufactured specifically for a laboratory and shipped in batches through arrangement with Helena Laboratories.

Rings on the Calbiochem plates are pale at 24 h and become sharper and easier to read at three days, the manufacturer's recommended time. Rings on Helena plates are sharp within 24 h, and the manufacturer states that they can be accurately read at 18 h when 4-μL aliquots are used. Precipitin rings with maximum diameters of ≤10 mm at 24 h correspond to α1-antitrypsin concentrations falling within the range of the standard curves for the kits. Rings do not usually increase in size after 24 h, but this should be checked with each new plate lot.

A sample with an extremely high α1-antitrypsin concentration (e.g., 50 mg/g) may not produce an obvious ring at 24 h, making a false-negative result most likely for the most strongly positive samples. Such samples can produce a hazy ring on Helena plates but no ring with the Calbiochem system. Therefore, we recommend that samples producing ill-defined (Helena) or no rings (Calbiochem) at 24 h be diluted 10-fold and re-plated. If no ring appears after dilution, the α1-antitrypsin concentration in the sample is 0 mg/g.

Comparison of Helena and Calbiochem plates in the analysis of stool samples showed a good correlation (r = 0.98) regardless of any "ghost" rings on Helena plates. When samples with such rings are re-plated and the halo does not appear, the precipitin ring size is the same as it was originally. The halo frequently appears after the precipitin ring has completely developed. It appears that the halos seen on RID plates do not represent α1-antitrypsin–protease complexes. We believe that these "ghost" rings have no effect on assay reliability of the Helena system, and indeed have not been seen on subsequent plate lots.

In summary, Calbiochem's LC-Partigen plates require a longer developing time than do Helena plates (three days vs 18 h), have a much narrower range, and are slightly more expensive. However, either RID system can be used to distinguish between normal and abnormal values for FA1AT. At present, Calbiochem LC-Partigen kits are not approved for clinical use.

Although we request that fecal specimens for α1-antitrypsin analysis be maintained frozen and delivered frozen to the laboratory, it is not uncommon for a sample to thaw during transport and be refrozen on arrival. Our data show that neither storage at room temperature, repeated freezing and thawing, nor long-term storage in the frozen state results in any significant differences in α1-antitrypsin values.

Despite the fact that α1-antitrypsin in serum is an acute-phase reactant, often considerably increased in disease states, previous studies have shown that there is no significant correlation between serum and fecal α1-antitrypsin (2).

Catassi et al. (8) have stated that non-lyophilized stool samples can be accurately assayed for α1-antitrypsin, but our study showed a weak correlation between values obtained with and without lyophilization. Water content of the samples assayed by Catassi et al. was closer to that of

---

**Fig. 2.** Linear regression comparing FA1AT concentrations obtained for lyophilized and nonlyophilized stool specimens: $y = 0.074x + 0.0003$
normal stool than was the water content of our diarrheal samples. Because clinical use of the FA1AT assay commonly involves diarrheal samples, we cannot recommend omission of the lyophilization step in this context.

Previous studies in our laboratory (7) have shown that while FA1AT concentration varies with the type of milk in an infant diet, 24-h excretion of \( \alpha_1 \)-antitrypsin does not. Higher concentrations were seen in breast-fed infants because their total stool output is less. Similarly, formula-fed infants had higher values than those fed cow's milk. Exclusively breast-fed infants also had higher values than those consuming breast milk plus solids. It is therefore important to establish the particular infant's diet before clinical interpretation of values. The effect of low-bulk diets such as "Vivonex" on FA1AT concentration in children and adults is unknown. Theoretically this could result in increased value in the absence of protein-losing enteropathy. As a related phenomenon, high fecal fat content may result in a depressed FA1AT value.

We thank Drs. Allen Lipsey of the Clinical Laboratory, and Russell J. Merritt and Philip Rosenthal of the Division of Gastroenterology and Nutrition, Children's Hospital of Los Angeles, for their suggestions regarding study methods and statistical analyses.

References