Screening Test for \( \alpha_1 \)-Antitrypsin in Dried-Blood Specimens

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We describe a fluorescent spot test for detecting \( \alpha_1 \)-antitrypsin activity in dried-blood specimens. The eluate of a blood disc is mixed with carbobenzoxyl-L-arginine-7-amino-4-methylcoumarin amide and trypsin at the appropriate pH. In the absence of \( \alpha_1 \)-antitrypsin, amino-methylcoumarin, a strongly fluorescent compound, is released. The reaction mixture, when spotted on chromatography paper and viewed under ultraviolet light, exhibits a bright fluorescence only in the case of specimens with \( \alpha_1 \)-antitrypsin deficiency. \( \alpha_1 \)-Antitrypsin activity so estimated correlated well with quantitative assays of dried-blood spots and serum. The procedure is simple and inexpensive, and has the potential for use as a screening test.

Additional Keyphrases: \( \alpha_1 \)-antitrypsin deficiency - pulmonary emphysema - liver disease - mass screening - pediatric chemistry - fluorometry - enzymic methods - heritable disorders - occupational hazards

\( \alpha_1 \)-antitrypsin (\( \alpha_1 \)-AT), a major serum protease inhibitor, strongly inhibits trypsin, chymotrypsin, collagenase, and elastase. Genetically, it is determined by a series of co-dominant alleles, known as the Pi system. The absence of \( \alpha_1 \)-AT activity was first described as a clinical entity in 1953 by Laurell and Eriksson (1). Subsequently, its association with the onset of pulmonary emphysema in early middle life was confirmed (2), as was the association of liver disease in infants (3, 4) and adults (5). Some evidence has also been presented that suggests a possible correlation between the respiratory distress syndrome in neonates and \( \alpha_1 \)-AT concentration. Among the various electrophoretically defined Pi alleles, the homozygous PIMM type is the most commonly found in the normal population. The PIZZ type has markedly decreased inhibitor activity (<20% of value for normal adults) and is the type generally associated with clinical manifestations. Other Pi types—including SS, SZ, and MZ—show intermediate \( \alpha_1 \)-AT activities.

Several enzymic and immunological methods have been published for the quantitative determination of \( \alpha_1 \)-AT activity in plasma (7, 8). These relatively costly methods do not lend themselves well to mass-screening programs. In 1972, Laurell (9) described an \( \alpha_1 \)-AT screening test that was adapted to blood specimens dried on filter paper. This immunoelectrophoretic “rocket” technique was used in a pilot screening program of 108,000 newborns in Sweden. In 1978, O'Brien et al. (10) reported a pilot screening program in Oregon involving 107,000 newborns. The screening test they used was based on the hydrolysis of \( \alpha \)-N-benzoyl-arginine ethyl ester to ethanol by a known amount of trypsin, in a coupled kinetic reaction.

The following is a report of an improved \( \alpha_1 \)-AT screening test in which blood dried on filter paper is the specimen. This method is simpler, requires fewer reagents than either of the previous methods, and is more sensitive because of the highly fluorogenic substrate used.

The fluorogenic substrate carbobenzoxyl-L-arginine-7-amino-4-methylcoumarin amide (Cbz-L-arg-AMCA; coumarin 120) is normally converted to the highly fluorescent compound 7-amino-4-methylcoumarin (AMC) by a known amount of trypsin. In normal blood, the presence of \( \alpha_1 \)-AT inhibits this reaction. In the case of \( \alpha_1 \)-AT deficiency, there is no inhibition of the reaction, and the highly fluorescent AMC produced can be either observed qualitatively under ultraviolet light or measured quantitatively in a spectrofluorometer. This is the basis of the present test.

Materials and Methods

Apparatus

Filter paper (SS no. 903; Schleicher and Schuell, Keene, NH 03431).

Ultraviolet viewing cabinet, equipped with a long-wavelength lamp and a sharp-cut filter (passes light of wavelength longer than 415 nm) for fluorescence examination.

Plastic dimple trays (Flow Laboratories, Inc., McLean, VA 22102).

Fluorometer (Model 111; G. K. Turner Associates, Palo Alto, CA 94303) with the following filters: primary [excitation 365 nm, no. 110-811 (7-60)], secondary [emission, 460 nm, no. 110-831 (48), no. 110-816 (2A)], and a 10% neutral-density filter. Any fluorometer or spectrofluorometer of adequate sensitivity can be used.

Reagents

Tris buffer, 50 mmol/L. Dissolve 6.05 g of tris(hydroxymethyl)aminomethane (base) and 1.1 g of CaCl\(_2\) in distilled water. Adjust the pH to 8.0 with concentrated HCl and dilute to 1 L.

Trypsin (EC 3.4.1.4, stock solution. Dissolve 100 mg of salt-free crystalline pancreatic trypsin (activity: 3074 NF units/mg; Calbiochem-Behring Corp., San Diego, CA 92112) in 100 mL of 1 mol/L HCl. This solution is stable for several weeks in the freezer. Prepare a working solution (20 mg/L) by diluting 0.2 mL of the stock solution to 10 mL with Tris buffer. Prepare freshly each day, and keep at 0–4 °C.

Trypsin–albumin solution for controls. Mix 0.2 mL of trypsin stock solution and 0.15 mL of crystalline human or bovine albumin solution (40 g/L) and dilute to 10 mL with Tris buffer. Prepare freshly each day and keep at 0–4 °C.

Cbz-L-arg-AMCA, 20 mmol/L stock solution. Dissolve 20 mg of the Cbz-L-arg-AMCA substrate (Calbiochem-Behring) in 2 mL of dimethyl sulfoxide and keep at 0–4 °C. Prepare the working solution by mixing 15 μL of the stock solution with 10 mL of Tris buffer. This solution is stable for one week at 0–4 °C.

Reagents (Quantitative Assay)

Trypsin, 75 mg/L. Prepare by diluting 0.75 mL of the trypsin stock solution to 10 mL with Tris buffer.

Trypsin–albumin solution. Prepare by diluting 0.75 mL of the trypsin stock solution and 0.25 mL of albumin (40 g/L) to 10 mL with Tris buffer. The exact dilution required depends on the activity of the trypsin and should be such that the
fluorescence in the trypsin control tube under the assay conditions is about 75 arb. units. These trypsin reagents must be prepared freshly each day.

\[ \text{Cbz-L-arg-AMCA solution. Prepare by diluting } 40 \mu \text{L of the substrate stock solution to } 10 \text{ mL with Tris buffer.} \]

\[ \text{Trichloroacetic acid (TCA), } 100 \text{ g/L solution.} \]

**Blood Samples**

Blood specimens were collected on SS no. 903 filter paper. Capillary blood from a finger- or heel-prick was spotted and allowed to dry at room temperature, away from any source of heat. Care was taken to avoid double spotting. Specimens so prepared are suitable for mailing to a central laboratory for testing.

**Procedures**

**Screening test.** Punch a disc 3.2 mm ( \( \frac{1}{8} \text{ in.} \) ) in diameter from each dried-blood specimen and place it in the well of a dimple tray. Add exactly 0.1 mL of the trypsin working solution with an automatic pipet. Shake the sample occasionally to speed the elution of proteins. Also place two discs of plain filter paper into each tray and add 0.1 mL of the trypsin–albumin solution as controls. After a 30-min incubation at room temperature (23–25 °C), add 0.1 mL of the Cbz-L-arg-AMCA working solution to all wells and mix well by gently shaking the tray. After 15 min, take a drop from each well with a Pasteur pipet and spot it on Whatman no. 1 filter paper. Alternatively, an automatic spotter (spot diameter 10–15 mm) can be used. Examine the spots (wet or dry) for fluorescence under long-wavelength ultraviolet light.

**Quantitative assay.** The quantitative assay for \( \alpha_1 \)-AT in dried-blood specimens is based on the same principle as the qualitative screening test. Punch a disc 6.3 mm ( \( \frac{1}{4} \text{ in.} \) ) in diameter from the dried-blood specimen and place it into a 12×75 mm test tube containing exactly 0.2 mL of the trypsin solution. Let stand for 30 min at 23–25 °C, shaking occasionally to ensure complete elution of the components. Set up two controls with exactly 0.2 mL of the trypsin–albumin solution in each and a blank with 0.2 mL of Tris buffer. Add 0.1 mL of the Cbz-L-arg-AMCA substrate to each tube, mix, and start timing. Exactly 5 min later, add 0.1 mL of TCA solution to stop the reaction. Centrifuge at 3500–4000 rpm for 3–4 min, and transfer 0.1 mL of the supernate to fluorometric tubes (10×75 mm, Pyrex no. 9820) containing 2 mL of Tris buffer. Measure the fluorescence in a fluorometer or spectrophotometer that has been set on zero with the blank.

To calculate the activity of \( \alpha_1 \)-AT, a standard curve of fluorescence intensity vs AMC concentration should be made under the same conditions. The difference (\( \Delta F \)) between the fluorescence of the sample and that of the trypsin–albumin control is converted into nanomoles of AMC per liter (\( C_{AMC}/5 \)) and the inhibition activity (I.A.) is given by the equation: I.A. = (\( C_{AMC}/5 \)) × (1/0.001), where \( C_{AMC} \) is the concentration of AMC in nanomoles; 5 is the assay time in minutes; and 0.011 is the approximate volume (in milliliters) of blood per 6.3-mm disc. The \( \alpha_1 \)-AT activity in the serum is expressed in nmol/min per milliliter of blood.

**Results**

On using the qualitative screening test, we found that normal whole-blood specimens appeared dark when examined under ultraviolet light, showing no visible fluorescence. In contrast, specimens from patients with \( \alpha_1 \)-AT deficiency showed a bright-blue fluorescence. As a control, discs were also punched from plain filter paper and from dried spots of hemolysates (which contain no plasma) from the same patients and treated as described above. Both of these controls showed bright fluorescence under ultraviolet light.

The very small amount of hemoglobin, which is concentrated mostly in a ring at the periphery of the test spot, has a negligible effect on the visible fluorescence. The \( \alpha_1 \)-AT is quite stable in dried-blood spots. We determined this by daily testing of dried-blood specimens from normal individuals, left at room temperature (23–25 °C) for various periods of time, using the quantitative assay. No substantial change in inhibitory activity was found until after one week. This interval suffices for mailing specimens to a central laboratory for screening without appreciable loss of activity. The activity was much more stable when the specimens were kept refrigerated or frozen.

The inhibition of trypsin by human blood or plasma was demonstrated by assaying one to four 3.2-mm ( \( \frac{1}{8} \text{ in.} \) ) blood-containing discs from a single sample with an inhibitory activity of 80 nmol/min per milliliter.

The difference in the fluorescence of trypsin controls as well as the inhibition activity was found to be linear and proportional to the number of discs (Table 1). Specimens with an activity of 62.0 or less were considered abnormal in this quantitative assay. ZZ homozygotes have values ranging from 13.5 to 23.0 nmol/min per milliliter, and MZ heterozygotes have intermediate values, ranging from 23.0 to 62.0 nmol/min per milliliter.

To evaluate the accuracy of the test, we collected 16 heparinized venous-blood specimens. Aliquots were spotted on filter paper and the remainder of the sample was centrifuged to obtain the plasma. The plasma specimens were assayed by the quantitative method described by Dietz et al. (11). The corresponding dried-blood specimens were tested by both our qualitative screening method and the quantitative blood-spot assay. The results were then compared with those from the plasma assays.

All specimens except one showed an absence of fluorescence with the screening test, indicating normal \( \alpha_1 \)-AT activity. The corresponding quantitative values in dried-blood specimens ranged from 71 to 106 nmol/min per milliliter of whole blood. In plasma, by the Dietz method, they ranged from 2.1 to 3.0 \( \mu \text{mol/min per milliliter.} \) The screening test specimen that was considered abnormal showed faint fluorescence and was considered likely to be a PiMZ heterozygote. The results from both of the quantitative assays also placed this specimen in the heterozygous range.

Dried-blood specimens from two known PiMZ heterozygotes and one PiZZ homozygote were obtained and tested blindly with two normal blood specimens. The results are shown in Table 2. Specimens from more than 850 normal newborns were also tested, along with a heterozygote control. No positive screening result was observed. Seven specimens that showed a very faint fluorescence on the screening test were assayed with the quantitative procedure and found to have \( \alpha_1 \)-AT activity in the low-normal range. In routine screening, however, these specimens would not have been considered presumptive positives. The proportion of repeat

<table>
<thead>
<tr>
<th>Blood discs, no. X mm diameter</th>
<th>( \Delta F )</th>
<th>Activity, nmol/min per mL</th>
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<tbody>
<tr>
<td>1 X 3.2</td>
<td>15.5</td>
<td>23.5</td>
</tr>
<tr>
<td>2 X 3.2</td>
<td>28.5</td>
<td>43.6</td>
</tr>
<tr>
<td>3 X 3.2</td>
<td>40.0</td>
<td>62.5</td>
</tr>
<tr>
<td>4 X 3.2</td>
<td>51.5</td>
<td>79.1</td>
</tr>
<tr>
<td>1 X 6.3*</td>
<td>53.0</td>
<td>81.5</td>
</tr>
</tbody>
</table>

* The 6.3-mm diameter disc contains approximately the same volume of blood as four 3.2-mm discs.
Table 2. Comparison of Screening-Test Results with Quantitative Assays for α₁-Antitrypsin (Five Specimens)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Fluorescence</th>
<th>Activity, nmol/min per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MZ</td>
<td>+</td>
<td>62</td>
</tr>
<tr>
<td>MZ</td>
<td>+</td>
<td>51</td>
</tr>
<tr>
<td>MM</td>
<td></td>
<td>85</td>
</tr>
<tr>
<td>ZZ</td>
<td>+++</td>
<td>17</td>
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</table>

specimens that would need to be requested in routine use of the test is therefore expected to be quite low.

Discussion

The screening test described above is simple and inexpensive, and can detect diminished activities of α₁-AT as reliably as other quantitative screening methods. Its repeatability and accuracy have been demonstrated. The test is both convenient and sensitive. Because 3.2-mm blood discs can be punched directly into dimple trays with a quadratic punch-index machine, the test can be used in screening laboratories that are carrying out multiple testing procedures. The stability of the α₁-AT has been adequately demonstrated; thus, deterioration in transit is not anticipated.

The only disadvantage of this method is that it estimates the total antiproteic activity of the blood, not just the α₁-AT. Therefore, it is sometimes difficult to diagnose the MZ heterozygotes by these procedures. In those cases where doubt exists, an antigen-antibody crossed electrophoresis is recommended. ZZ homozygotes evidently can always be correctly recognized by both assays.

The prevalence of α₁-AT deficiency has been estimated at between 1/1500 and 1/5000 (10, 12), comparable to cystic fibrosis (1/2000) and congenital hypothyroidism (1/4000).

For a screening test to be acceptable, the seriousness of the disorder must be sufficient to justify screening, and effective therapy should be available. In the case of α₁-AT deficiency, the association with chronic pulmonary emphysema is strong, but not all affected individuals develop overt respiratory symptoms. Instead, it has been suggested that smoking may be a critical determinant in whether the pulmonary problems develop (12). In addition, from 28 to 50% of deficient individuals develop some symptoms or evidence of liver disease in childhood (10, 14). The prognosis for these children with liver problems is generally considered to be relatively good. The long-term prognosis for the high risk of chronic pulmonary emphysema is more guarded. Early detection, combined with appropriate counseling against cigarette smoking or unusual exposure to environmental respiratory irritants, may prevent the development of symptomatic pulmonary disease in a relatively large proportion of affected individuals (15). Early identification of individuals with α₁-AT deficiency also identifies families at risk and can potentially reduce the birth of additional affected sibs through the use of genetic counseling.

Specific high-risk groups of individuals should also be routinely screened for α₁-AT deficiency. Such groups might include patients with neonatal hepatitis, infantile cirrhosis, and obstructive lung disease. In addition, adults seeking employment in industries where they would be exposed to irritating fumes, dusts, or excessive smog should be routinely screened.

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References