Optimizing Reference Values for the Measurement of Alpha$_1$-Antitrypsin in Serum: Comparison of Three Methods

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We studied three methods (rate nephelometry, radial immunodiffusion, and trypsin-inhibitory capacity) for their ability to detect those individuals with a deficiency of alpha$_1$-antitrypsin. The phenotype represented in 170 serum samples was determined by isoelectric focusing as the reference method. All three methods correctly identified Pi Z, Pi S, and Pi SZ phenotypes but varied in their ability to detect Pi MZ and Pi MS phenotypes. The rate-nephelometric method was the least sensitive in detecting Pi MZ and Pi MS variants because of the inappropriately low reference interval suggested by the manufacturer. We found that the three screening methods are comparable when the limiting values are properly selected. We suggest that the reference value for the rate-nephelometric method be increased from 0.85 g/L to 1.40 g/L to improve the sensitivity of the test.

Additional Keyphrases: rate nephelometry · radial immunodiffusion · trypsin-inhibitory capacity · heritable disorders

Normal reference intervals for clinically important analytes are generally established by measuring the substance in the serum of a sufficient number of normal subjects to obtain statistically representative values. This method may be satisfactory for compounds whose concentration becomes abnormal during a disease process and returns to normal upon recovery. However, when establishing reference intervals for substances related to the presence of a genetic variant, the usual method of identifying the apparently healthy population becomes less meaningful. Rather, it is necessary that the test detect the genetic variants underlying the disease state. A representative of this class of compounds is alpha$_1$-antitrypsin (AAT). In this case the protein concentration in serum is not used as a marker for clinical disease, but rather to screen for those individuals who have inherited a protein variant associated with a deficiency. Therefore, any method for measuring AAT in serum must be tested for its ability to detect those variants related to AAT deficiency, which in turn may predispose the individual to pulmonary and hepatic disease.

The biochemical investigation of suspected AAT deficiency is commonly approached in two stages. The first step, quantitative estimation of the AAT concentration in serum by immunoprecipitation or functional activity methods, is followed by confirmation of the suspected deficiency variant by use of isoelectric focusing to determine the phenotype. Clearly, the screening methods play an important role in sample pre-selection for phenotype determination. Manufacturers of the reagent kits used for the screening methods usually suggest a reference interval that has been established in a population of healthy subjects. Using these suggested reference intervals, we tested the ability of three currently used screening methods to detect AAT deficiency variants. The methods we studied were rate nephelometry (ICS), radial immunodiffusion (RID), and trypsin-inhibitory capacity assay (TIC).

Materials and Methods

Serum from patients' specimens was sent to us for phenotyping and measurement of AAT concentration or activity. They arrived frozen, and were kept at −20 °C until analyzed, usually within a week.

The Immunochemistry System (Beckman Instruments Inc., Fullerton, CA 92634) was used to measure AAT by rate nephelometry according to the procedure supplied by the manufacturer. Duplicate determinations were made on all specimens with values lower than 1 g/L. The suggested reference interval of 0.85 to 2.13 g/L was used for detection of deficiency states.

The "M-Partigen" kit (Calbiochem-Behring Corp., La Jolla, CA 92037) was used to measure AAT in serum by the RID endpoint technique, again according to the procedure supplied by the manufacturer. The reference interval of 2.0 to 4.0 g/L was used to classify the samples as normal or deficient.

Serum trypsin-inhibitory capacity was measured spectrophotometrically (1, 2). The reference interval of >0.7 g of

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1 Nonstandard abbreviations: AAT, alpha$_1$-antitrypsin; ICS, rate nephelometry method; RID, radial immunodiffusion; TIC, trypsin-inhibitory capacity assay.

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tryptic inhibited per liter of serum was established in our laboratory.

For isoelectric focusing we used polyacrylamide gels containing 291 g of acrylamide and 9 g of bisacrylamide per liter, the Bio-Rad apparatus (Bio-Rad Laboratories, Richmond, CA 94804), and a Haake-F4 water circulator to maintain cooling at 4 °C during the focusing. After pre-focusing the gel for 1 h, samples were applied 2 cm from the cathode end. Several control sera, including one containing a known MMz variant, were placed on every third or fourth lane. The electrode solutions were 1 mol/L NaOH (cathode) and 1 mol/L H₂PO₄ (anode). The focusing was at 3 W for 5 h. The gel was stained with Coomassie Brilliant Blue R in ethanol/acetic acid/water (180/40/180 by vol) at 60 °C for 15 min, then destained overnight at room temperature in an ethanol/acetic acid/water mixture (250/100/650 by vol).

Results

We measured the AAT concentrations and determined the AAT phenotype in 170 serum samples. Of the 116 normal subjects (M and M-subtype phenotypes), the ICS method correctly identified 115 as normal and misclassified one as deficient. The RID method correctly identified 105 as normal, and the TIC method 99. The TIC method identified the two Pi IM and the one Pi GM specimens as deficient; however, these sera contained normal AAT concentrations as measured by the immunochemical methods. We classified these three phenotypes and the one Pi MR, a rare variant that had normal values as measured by all three methods, with the normal population because no disease state had previously been associated with these phenotypes. Of the 24 Pi MZ heterozygotes, the ICS identified 13 as deficient, the RID 21, and the TIC 23. The recommended ICS referent value of 0.85 g/L cuts through the middle of the Pi MZ population (Figure 1).

The Pi MS phenotypes were classified as follows: ICS identified none of the 14 Pi MS variants as deficient, whereas RID classified only two of 14 and TIC six of 14 as deficient. All methods correctly identified as deficient the one Pi SZ, the 10 Pi Z, and the one Pi S phenotypes. These phenotypes have less than 60% of the normal AAT concentration (3). We calculated sensitivities and specificities for the screening methods, using the manufacturer's recommended reference intervals and assuming that the Pi S, Z, SZ, MZ, and MS are clinically important phenotypes. The ICS, RID, and TIC methods showed sensitivities of 50%, 70%, and 82%, respectively, and specificities of 99%, 91%, and 83%, respectively. As expected, the three methods show widely differing sensitivities when recommended reference intervals are used.

Discussion

The diagnostic accuracy of any test used to identify or confirm the presence of a disease state depends on the limiting or normal reference value established by the investigator. This dividing line between health and disease may be set so that only obvious or severe disease is identified (the result being a test with high specificity and low sensitivity). Or it may be set at a value selected to detect all patients with the disease, even those with mild or sub-clinical diseases (low specificity and high sensitivity). Arguably, the latter approach is appropriate for a screening test, whereas a more rigorous criterion must be established for a confirmatory test. Accurate classification of subjects by diagnosis for the purposes of test evaluation is often difficult, especially when based on less than accurate clinical assessment (4, 5). With the measurement of AAT, however, we are attempting to diagnose a deficiency state associated with one of several genetically determined phenotypes, each of which can be accurately identified by the reference method, isoelectric focusing. Certain of these deficiency phenotypes are known to be associated with a high incidence of pulmonary embolism and hepatic cirrhosis (5).

There is little doubt that, whatever the screening test used, it should be capable of identifying the severely deficient phenotypes such as Pi Z, Pi S, and Pi SZ. With use of the recommended reference intervals all three of the methods we studied easily detected the severe AAT deficiency states. There is less agreement regarding the clinical importance of detecting the heterozygotes that carry a normal and a deficient allele, such as Pi MS or Pi MZ. These phenotypes are rarely associated with overt pulmonary embolism (6, 7), but the Pi MZ pattern has been found in adults with chronic active hepatitis and cryptogenic cirrhosis (8–11). Detection of these heterozygotes may also be desirable for the purposes of genetic counseling.

The failure of the ICS method to identify half of the Pi MZ and all of the Pi MS phenotypes as abnormal (Figure 1) rests with the choice of an inappropriately low reference value of 0.85 g/L. If the decision point is raised to 1.25 g/L, the sensitivity increases from 50% to 72%, while the specificity remains unchanged at 98%. If the reference value is raised to 1.40 g/L, the sensitivity becomes 78%, but specificity declines to 90%.

![Graph](image-url)
The performance of the three screening methods is very similar, regardless of whether the Pi MS phenotype is classified as normal or abnormal. This suggests that the discriminating power of all three methods is very much the same if the limiting value is carefully selected. The specificity of all three screening tests improves dramatically when the Pi MS heterozygote is included in the normal population. This of course is expected, because the Pi MS phenotype gives values for the screening tests that cluster near the lower range of AAT concentration for the normal phenotypes (Pi M and M subtypes).

Assignment of an appropriate reference value for each of the three methods depends on the way in which each screening test is to be used. The overall prevalence of deficiency variants of AAT varies widely in different populations, ranging from 1 to 7% (3). However, AAT screening is not performed randomly on individuals from the general population, but instead is used to detect a deficiency in patients who are preselected on the basis of other clinical data. Under these circumstances a false-positive rate of 10–20% is quite acceptable, because a definitive test for detection of the genetic basis of the deficiency may then be performed for confirmation.

We find the three methods that we have studied here to be equally suitable for AAT screening. We recommend, however, that the reference value for the Beckman ICS method be increased from 0.85 g/L to 1.40 g/L, so that all three methods will show comparable discriminative power.

References


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Liquid-Chromatographic Determination of Alpha- and Gamma-Tocopherols in Erythrocytes, with Fluorescence Detection

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We have adapted to erythrocytes a method for the determination of alpha- and gamma-tocopherol in plasma and platelets. Erythrocytes (50 μL) were extracted with methanol containing tocol (internal standard) and pyrogallol. Tocopherols were partitioned into chloroform, washed, and injected in methanol onto a reversed-phase (C18) “high-performance” liquid-chromatographic column. The mobile phase was methanol/water (99/1 by vol) at a flow rate of 2 mL/min and detection was with a “high-performance” spectrophotofluorometer. The limit of detection for either tocopherol is 0.10 μg/mL of packed cells. Analytical recoveries ranged from 93 to 104%. Some values for tocopherols in human erythrocytes are presented.

Additional Keyphrases: fluorometry · diet-related effects

Primarily because of methodologic difficulties, there are few studies on the influences of dietary and physiological factors on tocopherol concentrations in erythrocytes (1, 2). We recently described (3) an improved, direct method for determining alpha- and gamma-tocopherol in plasma and platelets by liquid chromatography with fluorescence detection. With minor modifications to this procedure, one can determine tocopherols in erythrocytes. Only 50 μL of packed cells is required, and the lower limit of detection for either tocopherol is 0.10 μg/mL of packed cells.

Materials and Methods

The extraction procedure is similar to that described for platelets (3) except that pyrogallol is added as an antioxidant. Tocopherols are separated on a Zorbax ODS 4.6 mm × 25 cm reversed-phase column (1) (Fisher Scientific Co., Silver Spring, MD 20910) with a mobile phase of methanol/water (99/1 by vol), at a flow rate of 2 mL/min. Except for the analytical column, the instrumentation is the same as that described previously (3).

Internal standard: Prepare a calibration standard mixture of tocol (Pierce Chemical Co., Rockford, IL 61105) in methanol (diluted to give a recorder response of 60–80% full scale) and alpha- and gamma-tocopherol (0.2 and 0.06 μg/mL, respectively). Inject 75 μL of tocol onto the column and determine the relative area response of tocol to each tocopherol by electronic integration (we used a Model 3390A reporting integrator from Hewlett-Packard, Avondale, PA 19311). Check the relative area responses by injecting a calibration

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