Enzyme Immunoassay Screening of $\alpha_1$-Antitrypsin in Dried Blood Spots from 39 289 Newborns

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We present a new, simple, and inexpensive sandwich-type double-antibody enzyme immunoassay for $\alpha_1$-antitrypsin in dried blood collected on the fifth day post-partum. The method is very sensitive, having a detection limit of 2.84 fmol/well. Intra- and interassay CVs are 6.1% and 10.3%, respectively, for assay of 5-mm-diameter blood spots eluted into 7 mL of phosphate buffer. Since February 1984, we have used this method to systematically screen 39 289 consecutive births: 336 of these newborns (0.085%) showed values for $\alpha_1$-antitrypsin below the cutoff value of 800 mg/L (50th percentile, 1470 mg/L). Of these 336 we were able to obtain 0.5 mL of serum from 161 for further testing. Four presented with a ZZ phenotype and 15 with a SZ phenotype, which indicates a deficiency in $\alpha_1$-antitrypsin. Our data suggest a prevalence of 1.4% and 3.8% of Z and S alleles, respectively, in the French-speaking community of Belgium.

Additional Keyphrases: heritable disorders - population studies - enzyme-linked immunosorbent assay - peroxidase - protease inhibitors

$\alpha_1$-antitrypsin (A1AT), first isolated by Schultz et al. (1) in 1955 as an $\alpha_1$-glycoprotein, is a major protease inhibitor (Pi) in human plasma (2). Laurell and Eriksson (3) described the hereditary deficiency phenotype in 1963; its association with chronic obstructive pulmonary disease was first described by Eriksson in 1964 (4). The genetic polymorphism of the Pi system was studied by Fagerhol and Laurell in 1967 (5) by starch gel electrophoresis.

The detection of Pi alleles was further improved with the polyacrylamide gel isoelectrofocusing (IEF) method (6). The clinically most important alleles are M (normal), S, and Z. The SZ and ZZ phenotypes are mainly found in affected patients. The allele distribution in various countries has been established (7-10).

The association of Pi-deficient variants with liver disease in children was first reported by Sharp et al. (11). A semi-quantitative electroimmunoassay was developed (12) for A1AT on dried blood and used for systematic screening of more than 200 000 Swedish newborns (13). The follow-up of 120 children with the ZZ phenotype showed clinical liver disorders in 18%, the most frequent being a neonatal hepatitis syndrome; 13.6% of the ZZ patients presenting with clinical symptoms were dead at the age of eight years (14).

Clearly, A1AT deficiency is a severe disease, and systematic neonatal screening should help or prevent the development of symptoms. The use of enzyme-linked immunoassay (ELISA) for detecting A1AT deficiency offers at least two advantages: it is cheap and easy. Michalski et al. (15) used ELISA to quantify A1AT in various biological fluids; Kimpen et al. (16) used it to screen cord blood samples from 10 329 newborns. Jepsson and Sveger (17) analyzed dried blood by IEF, but their procedure had many inconveniences: costly reagents, the need for quick sample transport, and special storage conditions (-20 °C).

Here, we describe an easy, inexpensive method for quantifying A1AT in dried blood on blotting paper mailed to the laboratory. The method is suitable for systematic screening of newborns.

Materials and Methods

A1AT Determinations by ELISA

Solid phase: We used polyclonal rabbit antibodies against A1AT (A012; Dako, Copenhagen, Denmark). We isolated the IgG by precipitation with sodium sulfate, 180 g/L, and obtained the pure fraction by anion-exchange chromatography on diethylaminoethyl cellulose (DEAE-52; Fluka, Buchs, Switzerland). After checking its purity by immunoelectrophoresis, we immobilized the purified IgG on Titertek polynvinylchloride microplates (77-172-05; ICN Flow, Asse, Belgium) by physical adsorption. The IgG was coated at a 3000-fold dilution, corresponding to 1 µg/well, in the coating buffer (phosphate 50 mmol/L, pH 7.5) and incubated for 24 h at 4 °C.

After three washing steps with the same buffer containing 2 mL of Tween 20 per liter (cat. no. 822184; Merck-Schuchardt, Hohenbrunn, F.R.G.), we saturated the free sites on the plate surface by another incubation, this time with coating buffer containing bovine serum albumin, 20 g/L, for 30 min at room temperature. The microplates were washed three times again and used directly for the next step of the assay.

Enzymatic marker: We conjugated 5 mg of horseradish peroxidase (HRP, EC 1.11.1.7; Boehringer, Mannheim, F.R.G.) with 5 mg of pure IgG, isolated as described above, using the periodate method (18). We separated the conjugated IgG-HRP from the unreacted HRP by gel filtration on a 15 × 850 mm column of Sephacryl S200 (Pharmacia, Uppsala, Sweden), using a flow rate of 15 mL/h; the eluting buffer was phosphate buffer.
(50 mmol/L, pH 7.5). The chromatographic fractions containing the labeled enzyme were pooled and mixed with the same volume of glycerol, then stored frozen at 

-20 °C.

**Standards:** We used "nephelometric N" protein standard serum (OSAU 06/07; Behring, Marburg, F.R.G.) as calibrator for A1AT determination in serum. Just before use, we diluted it in assay buffer (phosphate 50 mmol/L, pH 7.5, containing 2 mL of Tween 20 and 2 g of bovine serum albumin per liter) 2000-, 4000-, ..., 256 000-fold, corresponding to 2×, 1×, ½×, ..., ¼×× the calibration value (g/L) for use with samples diluted 4000-fold.

To prepare dried blood standards, we mixed one volume of three-times-washed erythrocytes with one volume of OSAU 06/07 standard prediluted to various concentrations in buffer (phosphate 50 mmol/L, sodium chloride 150 mmol/L, pH 7.5) containing 40 g of human serum albumin per liter. We applied 30 µL of each dilution to filter paper (MN 818; Macheray-Nagels, Düren, F.R.G.), let them dry at room temperature for 2 h, and stored them desiccated at 4 °C in the dark until use.

**Substrate-chromogen solution:** The color development solution consists of, per liter, 33 mmol of citric acid, 66 mmol of Na₂HPO₄, 1.5 mmol of sodium perborate (pH 5.0), and 2 g of 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS; Boehringer). This solution is stable for one week at 4 °C.

**Procedure:** For assay of serum, predilute samples and standards 4000-fold in assay buffer. For dried blood samples, elute a spot of 5 mm diameter in 7 mL of assay buffer for 1 h at room temperature; alternatively, elute a ½-in. diameter spot in 280 µL of assay buffer. Incubate 20 µL of eluted or prediluted samples or standards with 200 µL of assay buffer in each well of the antibody-coated microplate for 2 h at room temperature. Wash the plate five times, then incubate it for another 2 h at room temperature with, per well, 200 µL of conjugated antibody- HRP appropriately diluted in assay buffer. Wash as before, then determine enzymatic activity by measuring the absorbance at 405 nm of each well after incubation with the substrate-chromogen solution for 1 h at room temperature.

### A1AT Phenotyping

For A1AT phenotyping, we used analytical thin-layer polyacrylamide gel IEF with an FBE 3000 system (Pharmacia) and a modification of the procedure of Jeppson and Franzen (19). Briefly, the gel plates, 1 mm thick, are prefocused for 30 min at 500 V. Then filter papers (3 MM; Whatman, Maidstone, U.K.) are soaked with 20 µL of pretreated serum and focused for 3 h in an electric field of 100 V/cm. After staining the plates with Coomasie Brilliant Blue R250 (Merck, Darmstadt, F.R.G.), we examined the A1AT bands under neon light for phenotyping.

### Results

**Analytical Variables**

The specificity of the immunologic system was assayed by testing dilutions of normal MM (2220 mg/L) and hereditary A1AT-deficient homozygous ZZ (170 mg/L) serum. The shift between the two dilution curves (Figure 1) corresponds to the ratio of A1AT concentrations of the two samples and indicates no cross-reactivity with other human proteins in this immunologic system. This result confirms the validity of using commercial calibrated sera to construct standard curves, by simple dilution in assay buffer.

We tested polyvinyl chloride and polystyrene as solid phase by assaying standard curves for each system. We chose polyvinyl chloride for its better precision profile (CV = 4.7% vs 6.4%) and its lower nonspecific binding (11.9% vs 14.4%).

Figure 2 displays a typical standard curve for serum and dried blood samples. The greater absorbance values for dried blood samples narrows the working dilution range. Moreover, as frequently observed in other immunologic systems, nonspecific binding is greater in dried blood samples than in serum; minimum/maximum absorbance ratio = 0.13 and 0.10, respectively.

Experiments designed to assess assay precision were conducted with sera from different phenotypes to obtain concentrations that spanned the useful range of the assay. For the MM and ZZ samples the CVs were <10% in serum and dried blood (Table 1). The detection limit, 2.84 fmol/well, corresponds in assay conditions to a sensitivity of 10 mg/L for serum and 3 mg/L for dried blood spots.

The correlation coefficient was excellent (r = 0.91, P <0.001) between ELISA (x) and the amidolytic method (y) for proteinase inhibitors, the latter method being based on measurement with N-benzoyl-D,L-arginine p-nitroanilide of the residual activity of serum preincubated with porcine trypsin. To cover a large scale of values, we assayed by both methods 44 samples with different known phenotypes. The calculated regression line equation is y = 329 + 0.971x.

We compiled the concentrations of serum A1AT in rare Pi classes ZZ, SZ, and SS over six years and compared these values with the results for a sample of each of the most common phenotypes—MZ, MS, and MM—obtained during the same time. The statistical
analysis of the data is summarized in Table 2. Results are in good agreement with those found earlier (19, 21, 22) and confirm the accuracy of the ELISA.

The storage of dried blood did not reveal any in vitro destruction at room temperature over a period of one month. Three samples, Pi MM, Pi MZ, and Pi ZZ, were assayed bi-weekly for four weeks and showed no significant decrease of A1AT. For longer storage, samples must be kept in dried sealed plastic bags at 4 °C in the dark. Otherwise, A1AT, as with other proteins, may sometimes not be eluted from the dried blood spot. Thus, the elution step must be done just before the ELISA because the diluted solution of A1AT in buffers has a short half-life.

IEF Phenotyping

The stained polyacrylamide gels after IEF showed (Figure 3) the major 4, 6, 7, 8 bands of the Pi M normal phenotype. Bands 4 and 6, which are the most intense in Pi S and Pi Z, were easily visible. Because our study was not intended to discriminate the M1, M2, M3, or M4 subtypes, we optimized the IEF to show clearly only single 4 and 6 bands in normal MM patterns.

Neonatal Screening

Blood samples were collected at the fifth day after birth on a card of blotting paper that was also used for neonatal detection of phenylketonuria, congenital hypothyroidism, mucoviscidosis, or surrenal hyperplasia. Cards were sent by mail to the laboratory from 12 obstetric departments in the French-speaking region of Belgium. In this screening study, from February 1984 to December 1989, 39 289 babies were tested. During mail transport, which took one to three days, the temperature conditions were not controlled; however, Belgian climate shows neither high temperatures in summer nor low temperatures in winter.

Analyses were performed once a week. The distribution of A1AT values in our neonatal population had an average of 1612 (SD 539) mg/L; the median was 1470 mg/L and the mode 1400 mg/L, indicating a positive skewness of the distribution (Figure 4 illustrates the distribution for the first year of screening). For neonatal screening, we chose a cutoff value of 800 mg/L as normal, representing 54% of the activity of the median value for the population. In our study, 336 (0.88%) newborns had A1AT below the cutoff value. We systematically requested from each of them 500 μL of blood for A1AT phenotyping; 203 samples were received from these subjects between the third and twelfth weeks postpartum, but 47 were available only as dried blood. Phenotyping of the remaining 156 (46%) of the original 336 newborns revealed 31 with severe deficiencies (ZZ, SZ, SS), 64 heterozygotes (MZ, MS), and 61 normal MM (Table 3).

Analysis of variance performed on the first series,

![Figure 2](image2)

**Table 1. Precision of the ELISA for A1AT**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Mean, mg/L</th>
<th>SD, mg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>2140</td>
<td>2120</td>
<td>200</td>
</tr>
<tr>
<td>ZZ</td>
<td>1230</td>
<td>1230</td>
<td>120</td>
</tr>
<tr>
<td>MZ</td>
<td>170</td>
<td>170</td>
<td>15</td>
</tr>
</tbody>
</table>

**Table 2. ELISA Values of A1AT in Serum for Different Phenotypes**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>MM</th>
<th>MS</th>
<th>MZ</th>
<th>SS</th>
<th>SZ</th>
<th>ZZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>199</td>
<td>176</td>
<td>397</td>
<td>196</td>
<td>100</td>
<td>133</td>
</tr>
<tr>
<td>Mean A1AT, mg/L</td>
<td>2189</td>
<td>1859</td>
<td>1493</td>
<td>1371</td>
<td>969</td>
<td>582</td>
</tr>
<tr>
<td>SD, mg/L</td>
<td>307</td>
<td>421</td>
<td>317</td>
<td>338</td>
<td>228</td>
<td>268</td>
</tr>
<tr>
<td>Mean / 2 SD, mg/L</td>
<td>1575</td>
<td>1016</td>
<td>819</td>
<td>694</td>
<td>513</td>
<td>46</td>
</tr>
<tr>
<td>Mean + 2 SD, mg/L</td>
<td>2803</td>
<td>2701</td>
<td>2127</td>
<td>2047</td>
<td>1425</td>
<td>1114</td>
</tr>
<tr>
<td>% of MM mean</td>
<td>100</td>
<td>84.9</td>
<td>66.2</td>
<td>62.6</td>
<td>44.3</td>
<td>26.8</td>
</tr>
</tbody>
</table>

*Significantly different from values for phenotype to the immediate right at *P* < 0.001, **P* = 0.05.
blood spots from day 5 postpartum (n = 336), did not reveal any significant difference between each phenotype class except for SZ and ZZ. By contrast, the same test on the repeat samples showed a highly significant difference (n = 156; P <0.0001). Comparison of the screening and the retested values in each phenotype class gave a sensitivity of 100% for the ZZ, 80% for SZ, 50% for SS, and 48.9% for MZ. Specificity of the test was 99.7% in the overall screened population and 90% in the retested MM samples with initial low A1AT concentrations.

The number of false-negative results was assessed by

Table 3. A1AT Concentration in 336 Newborns with A1AT ≤800 mg/L.

<table>
<thead>
<tr>
<th></th>
<th>Median at birth</th>
<th>Median at repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>mg/L</td>
</tr>
<tr>
<td>Unknown</td>
<td>128</td>
<td>740</td>
</tr>
<tr>
<td>Unknown</td>
<td>47</td>
<td>740</td>
</tr>
<tr>
<td>PI MM</td>
<td>61</td>
<td>730</td>
</tr>
<tr>
<td>PI MS</td>
<td>19</td>
<td>720</td>
</tr>
<tr>
<td>PI MZ</td>
<td>45</td>
<td>710</td>
</tr>
<tr>
<td>PI SS</td>
<td>15</td>
<td>720</td>
</tr>
<tr>
<td>PI SZ</td>
<td>15</td>
<td>520</td>
</tr>
<tr>
<td>PI ZZ</td>
<td>4</td>
<td>430</td>
</tr>
<tr>
<td>No visible bands</td>
<td>3</td>
<td>850</td>
</tr>
<tr>
<td>PI IM</td>
<td>2</td>
<td>680</td>
</tr>
<tr>
<td>PI MP</td>
<td>1</td>
<td>570</td>
</tr>
</tbody>
</table>

*a* See text for discussion of samples and choice of cutoff value.

*b* Kruskal-Wallis test on repeat samples: χ² = 59.2, P =0.0001.

*c* Unknown = newborns with low A1AT at birth. Repeat not received.

*d* Unknown = newborns with low A1AT at birth. Repeat by ELISA; no phenotype available.

We chose ELISA for quantifying A1AT in dried blood because of the simplicity and low cost of the assay, in addition to its high sensitivity. Amidolytic or nephelometric methods cannot be used because of hemoglobin contamination of the eluted proteins from the dried blood spot. Both radial immunodiffusion and rocket immunoelectrophoresis are difficult to use with large series of samples. In contrast, the ELISA can be performed automatically without any limitation on the number of samples and is less time consuming. Cost is another important consideration: IEF seems the best tool for phenotyping protein, but the high cost of reagents (e.g., ampholines and silver buffers to stain the gels) for assaying dried blood samples makes it unsuitable for mass screening. Occasionally we used IEF as a control to check a low concentration of A1AT when blood was still available on the card. IEF protein bands obtained by this method were more difficult to interpret than those found by the more usual serum method. We use immunoblotting to enhance the results of phenotyping.

We estimated the Z and S allele frequencies in the French-speaking region of Belgium on the basis of the ELISA results. According to the Hardy-Weinberg principle and taking into account the percentage of cases that could not be retested (unknown b), we calculated the frequency of the Z allele as 1.4% (SD 0.5%). From the number of SS and SZ patients we observed, the frequency of the S allele is 3.6% (SD 1.2%). These results are in agreement with those found in neighboring areas such as the Netherlands (23), French Normandy (24), or southern Great Britain (25), suggesting that the technique is indeed reliable.

Our series of neonatal screening samples lacks large numbers of samples with low A1AT content. To correct this situation, which is unrelated to the laboratory analysis, we suggest that a second biochemical analyte be measured to enhance sensitivity and specificity. In 1985, Carlson and Eriksson (26) used monoclonal antibody monospecific to allele Z. Using this antibody as the
second antibody in a sandwich ELISA would allow specific quantification of A1AT in a dried blood sample of Pi Z. In that case, presumably the Z A1AT/total A1AT ratio could efficiently discriminate between phenotypes ZZ, SZ, and the rest of the population. All the samples falsely positive for MM, those with moderately low values for A1AT at birth related to hepatic immaturity, should then be eliminated. A test involving such a monoclonal antibody is currently in preparation in our laboratory.

References