sates. It should be remembered that, although this and other solubility procedures are not affected by the other most frequently occurring hemoglobins (HbC, HbD, HbE, and HbA2), other rare mutants could cause erroneous values. With regard to the presence of hemoglobin F, our calculations based on the data of Adachi et al. (9) show that for percentages of hemoglobin F up to 25%, there should be no effect on the solubility measurements. Because concentrations of hemoglobin F are high in samples from newborns, we do not recommend our procedure for such patients.

In conclusion: this technique has worked well in acute-care situations, and results compare well with those obtained by electrophoresis. We consider the method more precise than electrophoresis. No specialized instrumentation is needed. Standard curves are stable for at least three weeks for a given reagent lot, so emergency analyses can be performed by including two points along the standard curve along with each patient’s sample in duplicate. Current reagent cost is in the range of $3.00–$4.00. We believe that this method represents a rapid and simple alternative to electrophoresis during the evening hours, on weekends, or on days when the laboratory does not routinely provide hemoglobin electrophoresis.

References

Isoelectric Focusing in Agarose: Classification of Genetic Variants of α1-Antitrypsin
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Polyacrylamide has been the matrix of choice for isoelectric focusing owing to the virtual absence of electroendosmosis in this medium. Certain inherent limitations associated with polyacrylamide have prompted some investigators to use low-electroendosmosis agarose for isoelectric focusing, but with limited success thus far. We have developed a method for isoelectric focusing in agarose for the classification of α1-antitrypsin variants. Sera are applied directly to agarose gels containing a pH 4–5 ampholyte mixture, focused for less than 1 h, and directly immunofixed. Resolution of major bands is equivalent to polyacrylamide, and Pi M subtypes can be distinguished without the use of a separator. This application demonstrates the high resolution of isoelectric focusing in agarose, a more practical and convenient matrix than polyacrylamide.

Isoelectric focusing is a powerful tool for resolving proteins with only small differences in isoelectric point (1). Both convection from Joule heating and electroendosmosis contribute to gradient instability and thereby reduced resolution. Ultra-thin polyacrylamide gels (50–100 μm) provide an excellent medium for use in the technique, owing to the virtual absence of electroendosmosis and the more efficient heat exchange that is possible in ultra-thin gels (2). However, polyacrylamide has several disadvantages: high-molecular-mass proteins cannot be studied, the reagents used to prepare the gels are toxic, several hours are required to establish equilibrium, and direct immunofixation for accurate identification of bands is technically difficult.

With the introduction of electroendosmosis-free agarose several years ago, applications of isoelectric focusing in agarose can be found in the current literature (3). Nevertheless, most authors still maintain that the resolution attainable in polyacrylamide gel is superior to agarose, especially for applications where small differences in isoelectric point are expected.

The currently recommended method for the phenotypic classification of α1-antitrypsin is isoelectric focusing in polyacrylamide gel (4). The major variants, Pi M, Z, and S focus between pH 4.5 and 4.7 and exhibit substantial microheterogeneity, related to the mode of inheritance, differences in carbohydrate content, and amino acid substitutions (5). We describe an isoelectric focusing method for classification of α1-antitrypsin variants in agarose. Focusing requires less than 1 h, and the gels can be directly immunofixed, easily dried, and easily stored. Resolution is compara-

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Received Aug. 30, 1982; accepted Oct. 18, 1982.

328 CLINICAL CHEMISTRY, Vol. 29, No. 2, 1983
able with or better than results obtained in polyacrylamide with the same carrier ampholyte mixture. Isoelectric focusing in agarose is a convenient and versatile tool for high-resolution studies of proteins.

Materials and Methods

Apparatus

For isoelectric focusing we used a horizontal slab gel electrophoresis unit (Model HE900; Hoefer Scientific Instruments, San Francisco, CA 94107) and a high-voltage power supply (Model 2197; LKB, Rockville, MD 20852). The temperature of the slab surface was kept constant by using an LKB Multitemp circulating cold bath. Gels were cast in a glass mold consisting of two glass plates (17.7 × 13.8 × 0.2 cm), two polyvinyl chloride spacers (14 × 0.6 × 0.06 cm), and a Mylar sheet (GelBond film; FMC Corp., Marine Colloids Div., Rockland, ME 04841). The two plates were joined together on one side by filament tape as shown in Figure 1.

Sample-application masks were obtained from Pharmacia Fine Chemicals, Piscataway, NJ 08854.

Reagents

**Agarose gel, 10 g/L.** Dissolve 1 g of dry agarose powder (IsoGel™; FMC Corp.) per 100 mL of boiling de-ionized water. While maintaining the temperature at 100 °C, stir the mixture vigorously for 45 min in a refluxing vessel, to prevent significant evaporation. Divide the solution into aliquots and store in sealed tubes at 4 °C. Gels can be stored for as long as three months.

**Servalyt AG 4–5** (we used lots 14040, 18120, and 18052; Serva Fine Biochemicals, Garden City Park, NY 11040).

**Electrode solutions: Phosphoric acid and sodium hydroxide, 1 mol/L each.** Anode: dilute 5.6 mL of an 850 g/L solution of analytical-grade H₃PO₄ to 100 mL with de-ionized water. Cathode: add 4.0 g of NaOH to 100 mL of de-ionized water.

**Dithiothreitol, 1.0 mol/L.** Add 1.54 g of dithiothreitol to 5 mL of de-ionized water, dissolve, then dilute to 10 mL.

**Dilute ampholyte pH 5–8, 4 mol/L.** Dilute Ampholine pH 5–8 (LKB) with de-ionized water to give a 4 mol/L solution.

**Staining solution.** Dissolve 2.5 g of Coomassie Brilliant Blue R-250 (Sigma Chemical Co., St. Louis, MO 63178) in 400 mL of ethanol and 100 mL of glacial acetic acid. Stir for 1 h, then add 500 mL of water and continue stirring for 1 h. Filter the suspension and store in a sealed container.

**Destaining solution.** Mix ethanol/glacial acetic acid/de-ionized water (30/10/60 by vol) and store in a sealed container.

**Antibody dilution.** Dilute anti-human α₁-antitrypsin (Atlantic Antibodies, Scarborough, ME 04074) twofold with phosphate-buffered saline (per liter, 10 mmol of PO₄ and 150 mmol of NaCl).

**Technical grade kerosene.**

Procedures

**Sample storage and preparation.** On the day of receipt, all sera were analyzed for α₁-antitrypsin concentration by rate nephelometry (ICS; Beckman Instruments, Brea, CA 92621) and either electrofocused the same day or stored at −70 °C. Before focusing we mixed 10 µL of serum with 2 µL of dithiothreitol and diluted to an α₁-antitrypsin concentration of approximately 500–600 mg/L with dilute ampholyte pH 5–8. The diluted sera were left to stand at least 15 min at ambient temperature before they were applied to the plate.

**Casting the gel.** The agarose gel is molded between two glass surfaces separated by the polyvinyl chloride spacers (Figure 1). The two glass plates are joined on one long edge by filament tape, such that they can be opened and closed like the covers of a book. The lower glass plate is covered with a Mylar sheet of the same size as the glass plate. Use a thin layer of water to get the Mylar to adhere to the glass during this procedure. The polyvinyl chloride spacers are coated with a nonwettable lubricant to ensure a good seal between the Mylar and the spacers. The mold should now open from the top edge opposite the tape, thus allowing the agarose to be poured onto the Mylar surface. Place the mold on a metal leveling table, and heat the leveling table and glass mold at approximately 70 °C, for 10 min. During this time, cool 14 mL of liquified IsoGel to 56 °C, then mix it with 1 mL of Servalyt AG 4–5; avoid introducing air bubbles into the mixture. Open the premolded jar, pour the agarose quickly onto the lower plate, and carefully lower the top flap of the mold onto the spacers. Allow the gel to solidify at ambient temperature, then wrap the form with suitable covering to prevent evaporation at the edges. The gels can be stored at 4 °C for one to 10 days before use. For best results, the gel should be allowed to age for at least 24 h before use.

**Electrofocusing conditions and immunofixation.** The isoelectric focusing should be carried out at 4–8 °C. Carefully remove the gel from the mold and trim irregular edges with a sharp blade. Apply a few drops of kerosene to the surface of the electrofocusing unit to act as an insulating fluid before laying the agarose gel on top of the unit. Carefully remove air bubbles to ensure efficient heat conduction from the gel surface. The agarose gel is surrounded by—but not in contact with—adsorbent wick material approximately 0.5–1 cm wide, to collect water that condenses during the focusing period. Saturate the electrode strips in electrolyte solution, blot to remove excess fluid, and then place them in their appropriate positions on the surface of the agarose, approximately 0.4–1 cm from the long edge of the plate. Place the sample mask on the agarose surface just inside the cathode strip, and apply 2 µL of diluted sample directly to the gel. Seal the apparatus, connect the electrodes, and continue sample application for 10 min at a setting of 1 W. Then remove the sample template and increase the power setting to 20 W, at a maximum of 20 mA and 1500 V. Continue the focusing until the amperage has decreased from the maximum of 20 mA to a consistent value of approximately 5 mA (total focusing time is 45 min). Then remove the gel from the...
apparatus, blot it, and spread 1 mL of diluted anti-human α₁-antitrypsin directly on the plate, using a glass rod to ensure uniform coating of the surface. Cover the gel with plastic sheeting (Saran Wrap) and allow immunofixation to proceed for 1 h at ambient temperature. Then press the gel with Whatman No. 40 paper to remove excess antiserum and soluble serum proteins, wash the gel in isotonic saline for several hours or preferably overnight, press again, dry completely, and stain for 10 min with the dye. Destain the gel until the background clears, then dry the plate and interpret the results.

**Results**

Current-time measurement on several different days established the time required to approach equilibrium (Figure 2). An initial plateau was reached in approximately 30 min. There was little or no decrease in current during the next 15–20 min. Subsequently, we focused gels for 35 min in addition to the 10-min sample-application period.

To test the reproducibility of the pH profile, we cut a single gel into three separate sections and stored them at 4 °C. Figure 3 shows the results of three experiments on the gel sections, done on different days. The pH was measured at ambient temperature in degassed, de-ionized water after equilibration with uniform sections of the gel. We noted no significant variation in slope in the region between pH 4.5 and 4.8.

Known α₁-antitrypsin phenotypes and patients' samples focused under the conditions described are shown in Figures 4 and 5. A pH profile is shown in Figure 4 to indicate the relative position of each pattern in the gradient. The resolution of M subtypes can be seen in Figure 5. We compared the results in agarose with those obtained in polyacrylamide with the procedure of Jeppsson and Franzen (4) (Figure 6). The results shown for the polyacrylamide gel method are an example of direct protein staining with no immunofixation.
polyacrylamide gel with 3% crosslinking. In addition, shorter focusing times in agarose are an advantage when labile proteins are being studied.

In the application demonstrated here we achieved good resolution of common α₁-antitrypsin variants. In addition, M subtypes can be identified without the inclusion of a separator species in the gel (Figure 5) (6, 7). Focusing time is reduced from 4–5 h in polyacrylamide to only 45 min, and direct immunofixation for positive band identification is easy. The results shown depend highly on the characteristics of the carrier ampholyte. The use of two other batches (lots 18120 and 18052) of Servalyt AG 4–5 resulted in a modestly steeper slope between pH 4.4 and 4.8, thereby compressing the patterns. Use of glutamic acid, pH 3.5, as the ampholyte did not flatten the pH gradient between pH 4.4 and 4.8, and therefore did not improve the result obtained with lots 18120 and 18052. Other commercial ampholytes with a specified range pH 4–5 were not available for direct comparison. We also evaluated LKB and Serva pre-cast polyacrylamide gel plates for α₁-antitrypsin variant classification, but the results were not comparable with those for either agarose or acrylamide plates cast in our laboratory. An additional disadvantage of the precast acrylamide plates is their high cost.

Discussion

Isolelectric focusing in agarose has several advantages over polyacrylamide if resolution in the two media is comparable. The most significant advantages are that direct immunofixation of proteins is technically simple and high-molecular-mass proteins can be studied. Spherical proteins of molecular mass ≤60 × 10⁶ daltons will enter a 10 g/L agarose gel, compared with ≤2.5 × 10⁶ daltons for a 50 g/L polyacrylamide gel with 3% crosslinking.

Fig. 6. Patterns obtained in polyacrylamide with Servalyt pH 4-5 according to the method of Jeppsson and Franzen (4)

Direct protein stain used to make bands visible

One can readily appreciate that, with use of the same carrier ampholyte, equivalent or superior resolution is possible in agarose.

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