Rapid Two-Dimensional Immunoelectrophoresis of Human Serum Proteins

G. L. Wright, Jr.,¹ Louis Pollack, and D. B. Roberts

We describe two versions of a two-dimensional immunoelectrophoretic system and illustrate their use for separating and estimating human serum proteins. Each is sensitive, rapid, and simple to perform, and requires no special apparatus and only 0.1–0.6 ml of antiserum. Both procedures appear to be potentially useful in the clinical laboratory. Time required to obtain a stained pattern varies from 4.5 to 7 h, depending on the intended purpose and sensitivity (number of possible precipitin peaks) desired. With the faster method ("rapid screening") 25 to 35 precipitin peaks are detected. With the slower but more sensitive method 40 to >50 peaks are detected. The sensitivity of either version is a function of the titer of the polyvalent antiserum to whole human serum. Of the precipitin peaks thus far detected, 35 have been identified.

IEP² is a highly sensitive technique for study of proteins in both normal and pathologic sera and other biological fluids and has been successfully applied to monitoring serum profiles of patients (1). Yet, in spite of its prognostic value, IEP has fallen short of expectations as a practical diagnostic tool, primarily owing to difficulties in interpreting IEP patterns and quantitating the arcs, as well as to the inconsistent resolution of abnormal proteins characteristic of disease processes.

Development of two-dimensional or “cross” electrophoresis by Laurell (2) has increased the potential use of IEP in the clinical laboratory. This method provides greater sensitivity and facilitates interpretation and quantitation of the precipitin peaks obtained. Within the last four years, numerous modifications have been proposed in an attempt to improve the clarity of such electrophoretic patterns, to identify the precipitin peaks, and to decrease the time required to perform the test.

Clarke and Freeman (3), Raisys and Arvan (4), Kröll (5, 6), and Weeke (7) have used “macro” techniques, which necessitate large amounts of antiserum (2–5 ml) and a 17–24 h electrophoretic separation in the second direction. They could quantitate many of the serum proteins by measuring either the area or height of the precipitin peaks, which are proportional to their antigen concentration. However, such methods were not economically practical, and further modifications were needed to facilitate clinical application.

Accordingly, Firestone and Aronson (8), Davies et al. (9), and Crowle (10) have developed “micro” techniques that require much smaller amounts of antiserum (125–300 µl) and much shorter running times (3–3.5 h). Individual serum proteins could be estimated, and some 30–40 precipitin peaks were consistently observed. Yet the performance of these procedures is somewhat awkward and requires specially constructed apparatus.

This report describes two further modifications: a “micro” 2D-IEP procedure and a “rapid screening” 2D-IEP procedure. These techniques were developed for clinical applications: first, only economical but practicable amounts of serum and antiserum are required; second, less than 8 h is required to produce a completely stained electrophoretic pattern; third, the serum protein pattern is easily identified and quantitated; and fourth, the procedure is uncomplicated, readily mastered, and requires no specially-constructed apparatus.

Materials and Methods

Antisera. Serum (NHS) was pooled from 102 normal, healthy individuals, and stored at −20 °C in small aliquots until required. Antiserum was prepared against the NHS pool in rabbits according to the method described by Hirschfield (11). Booster regimens were used when the initial test-bleeding revealed a low-titered antiserum (i.e., few precipitin curves). Commercial polyvalent and monovalent antihuman antisera were obtained from Behring Diagnostic, Somerville, N. J. 08876; Kallestad Laboratories, Chaska, Minn. 55318; Hyland, Division Travenol Laboratories, Inc., Costa Mesa, Calif. 92626; Pentex, Miles Laboratories, Inc., Kankakee, Ill. 60901;
Two-dimensional immuno-electrophoresis: Method I. The "micro" 2D-IEP procedure is a modification of a "macro" technique described by Roberts et al. (12). A 5 × 7.5 cm slide was placed on a level surface and coated with 6 ml of hot (90 °C) agarose ("Sea-Kem"; Bausch & Lomb, Rochester, N. Y. 14602), dissolved in the barbital gel buffer (well buffer) described below, diluted with an equal volume of distilled water containing 0.1 g of merthiolate per liter (final concentration of agarose, 10 g/liter). An antigen well (2 mm diameter) was punched with its center 1 cm from both the bottom and the cathodic sides of the slide, and filled with 4 μl of an equal mixture of NHS and a saturated aqueous solution of Evans Blue dye. Four hundred-fifty milliliters of 0.06 ionic strength barbital well buffer, pH 8.2, was placed in each side of a Colab (Chicago Heights, Ill. 60411) electrophoresis chamber. Cellulose sponges, which serve as supports and as buffer wicks, were placed in the buffer and covered with filter paper to prevent scarring the agarose surface. The agarose-coated slides were placed, agarose-surface down, across the sponge wicks so that the direction of the first electrophoresis was carried out at 10 mA per slide (9 V/cm) at 4 °C until the blue albumin front had reached a point 0.8 cm from the anodic edge of the slide (1.5-2 h). After this first electrophoretic separation, the agarose above a line parallel to and 1.5 cm from the bottom edge of the slide was removed and replaced with 4.2 ml of a warm (55 °C) mixture consisting of 2.1 ml of a 20 g/liter solution of agarose in gel buffer and 2.1 ml of an appropriate mixture of antiserum and gel buffer. The amount of antiserum used per slide varied between 0.2 ml and 0.8 ml, depending on its titer. After the agarose-antiserum mixture solidified, the slides were inverted and returned to the electrophoresis chamber so that the direction of electrophoresis was parallel to the 5-cm edge of the slide. Electrophoresis in the second direction was carried out at 9 V/cm (4 °C) until the dye front reached a point 0.5 cm from the anodic edge of the slide (1-1.5 h). After this second electrophoresis, the slides were washed in agitating saline (9 g/liter) that was replaced at 30-min intervals for 2 h. The slides were then dried under bilubul paper, stained with a 5 g/liter solution of Coomassie Brilliant Blue in ethanol:glacial acetic acid:distilled water (4:5:1:4.5, by vol) (13) for 7 min, destained in solvent, allowed to dry, and photographed. The total time required to perform the test was 6.5-7 h. Diagrammatic representations of the precipitin patterns were produced by placing the stained slide in a photographic enlarger and tracing the projected image on graph paper.

Two-dimensional immuno-electrophoresis: Method II. The "rapid screening" 2D-IEP procedure was a further modification of the "micro" 2D-IEP procedure described above. Slides 5 × 7.5 cm were coated with 6 ml of a 10 g/liter hot agarose solution in gel buffer. The agarose was then cut and the designated portions removed as illustrated in Figure 1. An equal volume of agarose (20 g/liter) and antiserum3 in gel buffer was mixed at 55 °C and 1.2 ml of the mixture was poured into each of the three antiserum-agarose matrix troughs and allowed to solidify. Antigen wells (diameter, 2 mm) were punched with their centers 1 cm from the cathodic edge of the slide and 0.5 cm from each edge of the antigen strip and filled with 4 μl of serum: Evans blue dye mixture (1:3, by vol).

The first electrophoretic separation was run parallel to the 5 cm edge of the slides at a current of 10 mA per slide (9 V/cm) at 4 °C until the blue albumin front had reached a point 0.8 cm from the anodic edge of the slides (1 h). The slides were then turned 90°, and the second electrophoresis was carried out at 20 mA per slide (7 V/cm) at 4 °C until the blue albumin front had reached the bottom edge of the next anodic serum strip (about 45-60 min). The slides were then washed, stained, destained, and dried as previously described. The total time required to perform the test was 4.5-5 h.

Results

Representative two-dimensional immunoelectrophorograms of normal human serum obtained by Method I are presented in Figure 2. The patterns obtained when the same serum sample was analyzed on several slides run at the same time or on different days were very reproducible if the same polyvalent antiserum was used. The patterns obtained in Figure 2 were obtained with 400 μl of the same antiserum. Although quantitative differences were observed when different normal serum samples were run against the same antiserum, the number and position of the precipitin curves were consistent. It is, however, important to point out that not all individual or pooled polyvalent antisera prepared in rabbits against the same NHS pool were consistently able to precipitate the same serum protein antigens. For example, in Figure 2 the thyroxine-binding prealbumin is readily observed in the two-dimensional patterns representing three different serum samples. At first glance, this antiserum appears to be of the quality

---

3 The amount of antiserum required depended upon its titer.
and high titer necessary to achieve maximum sensitivity and economy (i.e., lesser quantities of antiserum) for this IEP technique. However, identification of some of the precipitin curves and analysis of different batches of antisera revealed that this antiserum (Figure 2) failed to precipitate the α1-lipoprotein.

A second polyvalent antiserum prepared in rabbits against the same NHS pool (Figure 2B) not only permitted the detection of the α1-lipoprotein, but revealed the genetic polymorphism (13) of this serum protein as indicated by the triphasic shape of the precipitin curve in the NHS pool pattern (Figure 3A) and the diphasic pattern of the α1-lipoprotein as observed in an individual serum pattern (Figure 3B). The location, identity, and polymorphism of the α1-lipoprotein were confirmed by the use of a monospecific antiserum (Figure 3C). Although the polyvalent antiserum used to obtain the patterns in Figure 3A and B contained a wide spectrum of high titer antibodies for the α and β globulins, it failed to precipitate the prealbumin.

It is most important to have a polyvalent antiserum containing a high titer of antibodies directed against a diverse spectrum of serum protein antigenic determinants, to achieve maximum sensitivity of the "micro" 2D-IEP methods described herein. Because of the potential application of the rapid 2D-IEP systems in the clinical laboratory, several commercial rabbit polyvalent antisera to whole human serum were evaluated by Method I.

The difference in 2D-IEP patterns observed after analysis of the same serum sample with four commercial antisera is shown in Figure 4. The concentration of antiserum was the same (600 μl) in all electrophoretic slides. Based on the largest number of precipitin peaks, the Behring antiserum with 38 precipitin peaks was by far the best quality antiserum tested and compared well with the best polyvalent antiserum produced in this laboratory (41 peaks). Next best was Kallestad (29 peaks), followed by Hyland (25 peaks), and Pentex (18 peaks). Antiserum obtained by Meloy (not shown in Figure 4) gave a similar pattern to that of Hyland. The number of precipitin peaks depended on the titer of the
antiserum. The number of precipitin curves that could be detected by a poor or low-titered antiserum could be increased by increasing the concentration of antiserum. But then the “micro” 2D-IEP system was no longer economically performed.

The number of serum proteins detected in NHS by Method I with antisera produced in this laboratory was 42. The 35 serum proteins identified to date are indicated in Figure 5. These proteins were identified by monospecific antisera and specific staining reactions (7).

The patterns obtained by Method II or the “rapid screening” 2D-IEP modification are presented in Figure 6. The patterns were similar to the larger version (Method I), although fewer precipitin peaks (25–35 peaks) were observed. The patterns were reproducible within a given run and from day to day as long as the same concentration of the same antiserum was used. Again, it was important to have a high-titered polyvalent antiserum to achieve maximum sensitivity (25–35 peaks) while keeping the required antiserum volume to a minimum (100 μl). Although the number of precipitin peaks could be increased by increasing the concentration of the antiserum, it was necessary to keep the quantity of antiserum low, otherwise the background would be so intensely stained as to mask many of the precipitin peaks.

Figure 7 shows some electrophoretic (Method I) patterns of sera from patients with pathologic disorders, analyzed by the use of antisera against NHS. As expected, a marked elevation in the immunoglobulins (IgG, IgM, IgA) was observed in the alcoholic cirrhosis serum pattern (Figure 6B). Aside from the expected elevations of the α- and β-globulins in the immunoelectrophoretograms of sera from patients with renal disorders (Figure 6A and D), the total number of precipitin peaks increased from the 42 detected in NHS to more than 50 peaks, with the same polyvalent antiserum that was used to obtain the normal serum patterns in Figure 3A and B. Such observations were consistently found, yet their significance remains to be determined.

The pattern obtained with serum from a patient with macroglobulinemia (Waldenström) showed a marked decrease in albumin, and in the α- and β-globulins. Characteristic of this disorder was the finding of an elevated diphasic IgM precipitin curve. The shape of the IgM curve agrees with the usual “seagull-winged” IgM precipitin line observed by conventional IEP, which is indicative of an IgM monoclonal gammapathy.

Discussion

The 2D-IEP modifications described in this report are potentially applicable in the clinical laboratory,
In that the procedures are simple, rapid, economical, and require no special-purpose apparatus.

We experienced none of the procedural difficulties with the methods of Clarke and Freeman (3) and Davies et al. (9), as evaluated by Fuller and Keyser (14). These included difficulties in maintaining the proper gel pH and electrolyte conditions during the electrophoretic run, the appearance of distortions in the gel caused by current abnormalities, the necessity for excessive manipulation of apparatus, and difficulty in removing the unprecipitated proteins from the gel.

The optimal electrophoretic conditions were maintained in the present procedures by reversing the electrodes after every second electrophoresis and by changing the buffer in the electrophoretic chamber after four complete (both directions) electrophoretic runs. The only problem encountered in either of the 2D-IEP modifications was the presence of a staining artifact as seen in the upper left corner of the patterns in Figures 2 and 7. This was due to protein contamination remaining in the sponge wicks from a previous run. This could easily be eliminated by rinsing the sponge wicks with water after each run or protecting the sponge wicks with a sheet of cellulose membrane cut from dialysis tubing.

The first 2D-IEP procedure (Method I) provides optimal conditions where maximum sensitivity (i.e., greatest number of precipitin peaks) is desired. After the second electrophoresis the slides do not require an independent incubation step for development of the precipitin arcs since the slides are washed and incubated simultaneously in agitating isotonic saline. Forty-two precipitin peaks can be obtained with NHS in reaction with at least as 400 µl of high-titered polyclonal antiserum. Over 50 precipitin peaks were observed with sera from patients with renal abnormalities, analyzed with 400 µl of the same polyclonal antiserum to whole NHS. Thirty-five of the serum antigens have been identified and many confirm the identifications of Weeke (7). The entire procedure, from preparing the agarose slide to obtaining a dried and stained slide, requires about 6.5-7 h.

If speed is desired rather than maximum sensitivity, then the second modification (Method II) may be more appropriate as a quick screening procedure for determining qualitative alterations in the serum proteins. All of the procedural manipulations, including coating the slide with agarose, punching out the antigen well, and applying the agarose-antiserum mixture, are performed at the start and require only about 30 min. Once the slide is placed in the electrophoresis chamber, the only additional step is to turn the slide 90°, after the first electrophoretic separation is completed, to allow electrophoresis of the antigen into the agarose-antiserum matrix. The entire procedure takes about 5 h, so that a trained laboratory technician, with two electrophoresis chambers, could run 48 individual serum samples in an 8-h working day. The vertical alignment of the developed patterns also allows individual serum components from a number of samples to be compared.

The most important parameter for success in obtaining maximum sensitivity and reproducibility is having a polyclonal antiserum containing high titer of antibodies to a great diversity of serum protein antigenic determinants. Since the quality (i.e., number of antibody specificities) and titer of antiserum varies from batch to batch, the optimal amount to be used cannot be assigned to either of the methods described in this report. The various commercially prepared antisera gave no more consistent immuno-electrophoregrams than did antiserum prepared in our laboratory. Each antiserum, therefore, must be tested against a standard serum pool and the antiserum concentration adjusted to give optimal 2D-IEP patterns. Although increasing the concentration of a
poor titer antiserum increases the number of precipitin peaks, the test is no longer economical. With
good quality antiserum, as little as 0.1 ml can result
in as many as 35 precipitin curves if Method II is
used. This procedure is then not only fast but very
economical.

Most of the emphasis in developing or modifying
existing 2D-IEP systems has been on obtaining a
rapid procedure (8, 9, 10, 15), designing special ap-
paratus (4, 8, 9, 15), identifying the precipitin curves
(3, 5–7, 16), and quantitating the serum protein an-
tigens (1, 3, 17–19). Our major emphasis in this
study was on the development of a rapid 2D-IEP
procedure by using existing and simple apparatus,
and on the identification of the protein antigens for
the purpose of being able to determine the reproduc-
ibility and quality of the antisera.

Now that rapid, reproducible, and economical pro-
cedures have been developed in our laboratory and
most of the serum proteins identified, we are cur-
rently attempting to establish a quantitative normal
serum profile with respect to sex and age for each of
the serum antigens. After a normal serum profile is
obtained with reference antiserum and quantitated,
normal serum standards are established, it may be
possible to quantitate 20 or more of the biologically
important serum proteins simultaneously on the
same slide and thereby eliminate the present need
for different radial immunodiffusion plates to quan-
titate each serum protein. This would not only save
time but would be considerably less expensive, mak-
ing the rapid 2D-IEP systems increasingly valuable
to the clinical laboratory for detecting both qualita-
tive and quantitative differences in patients’ sera.

This study was supported in part by a grant from the John A.
Hartford Foundation, Inc., New York, N. Y.

References

Serum proteins changes in Still’s disease, rheumatoid arthritis

Biochem. 10, 358 (1965).

3. Clarke, H. G. M., and Freeman, T., Quantitative immuno-

4. Raijys, V. A., and Arvan, D. A., Determination of proteins in
biological fluids by electroimmunodiffusion and two-dimensional

5. Kröll, J., On the immunoelctrophoretical identification and
79 (1968).

6. Kröll, J., Immunochemical identification of specific precipitin
lines in quantitative immunoelectrophoresis patterns. Scand. J.

7. Weeke, B., Serum protein identification by means of the Lau-

8. Firestone, H. J., and Aronson, S. B., Thin gel quantitation
and identification of the protein antigens for
the purpose of being able to determine the reproduc-
ibility and quality of the antisera.

Now that rapid, reproducible, and economical pro-
cedures have been developed in our laboratory and
most of the serum proteins identified, we are cur-
rently attempting to establish a quantitative normal
serum profile with respect to sex and age for each of
the serum antigens. After a normal serum profile is
obtained with reference antiserum and quantitated,
normal serum standards are established, it may be
possible to quantitate 20 or more of the biologically
important serum proteins simultaneously on the
same slide and thereby eliminate the present need
for different radial immunodiffusion plates to quan-
titate each serum protein. This would not only save
time but would be considerably less expensive, mak-
ing the rapid 2D-IEP systems increasingly valuable
to the clinical laboratory for detecting both qualita-
tive and quantitative differences in patients’ sera.

This study was supported in part by a grant from the John A.
Hartford Foundation, Inc., New York, N. Y.

References

Serum proteins changes in Still’s disease, rheumatoid arthritis

Biochem. 10, 358 (1965).

3. Clarke, H. G. M., and Freeman, T., Quantitative immuno-

4. Raijys, V. A., and Arvan, D. A., Determination of proteins in
biological fluids by electroimmunodiffusion and two-dimensional

5. Kröll, J., On the immunoelctrophoretical identification and
79 (1968).

6. Kröll, J., Immunochemical identification of specific precipitin
lines in quantitative immunoelectrophoresis patterns. Scand. J.

7. Weeke, B., Serum protein identification by means of the Lau-

8. Firestone, H. J., and Aronson, S. B., Thin gel quantitation
Pathol. 52, 615 (1969).

9. Davies, D. R., Spurr, E. D., and Versey, J. B., Modification to

10. Crowle, A. J., Preparatory electroimmunodiffusion for making
precipitins to selected native antigens. Immunol. Commun. 1, 325
(1972).

11. Hirschfield, J., Immunoelctrophoretic procedure and appli-
cation of the study of group specific variations in sera. Sci. Tools
7, 18 (1960).

12. Roberts, D. B., Wright, G. L., Affronti, L. F., and Reich, M.,
Characterization and comparison of mycobacterial antigens by
two-dimensional immunoelectrophoresis. Infec. Immunity 6, 564
(1972).

13. Schultz, H. E., and Hermanns, J. F., Molecular Biology of

14. Fuller, J. M., and Keyser, J. W., Some technical aspects of
two-dimensional immunoelectrophoresis of human serum and cere-

15. Pizzolato, M. A., Two-dimensional immunoelectrophoresis on
cellulose acetate: improved method for routine protein estima-

16. Weeke, B., Carbamylated transferrin used as the reference in
the Laurell cross-electrophoresis. Scand. J. Clin. Lab. Invest. 25,

17. Kröll, J., Changes in the $\beta_C$-$\beta_A$-globulin during the coagu-
lation process demonstrated from means of a quantitative immu-
noelectrophoretic method. In Proteides of Biological Fluids, 17th
Colloquium. H. Peters, Ed. Pergamon Press, Elmsford, N. Y. 10523,
1969, p 529.

18. Lyngbye, J., and Kroll, J., Quantitative immunoelctrophore-

19. Stephan, V. W., and Frrahm, U., Quantitative simultan immu-