VOLUME ONE

GRADWOHL'S CLINICAL LABORATORY METHODS AND DIAGNOSIS

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MEASUREMENT OF PROTEINS IN BIOLOGIC FLUIDS

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In the years between the editions of this text, rapid progress has been made in methods for the quantitation of serum, CSF, and urinary proteins. No longer is it sufficient simply to determine the total mass per unit volume of biologic fluid, but rather the concentration of a distinct protein component. At the present time it is possible to quantitate 10 protein components using immunochemical nephelometric technics. The clinical significance of these measurements is growing rapidly, and in the near future their significance will be readily apparent. Hopefully, in the ensuing years, new technologic advances will be made that will increase the sensitivity of these technics and allow the quantitation of proteins found in very low concentration. Of special interest will be attempts at combining specific protein determinations with routine chemical analysis, such as enzyme determinations currently done in the chemistry laboratory. By choosing judiciously the specific proteins and enzyme tests it may be possible to establish a battery of determinations better able to assess accurately the biochemical state of the patient and detect pathologic conditions.

In the following description of various procedures for protein determinations, a brief discussion of the physiology and interpretation of the results is included as a convenience to allow for a rapid appraisal of the usefulness of the procedure. Also note in some cases there are several methods described for the same constituent, and generally 1 method may be included for historical interest along with the newer methods. Few of the procedures have been carried over from the previous edition due to their obsolescence. The Kjeldahl determination for total nitrogen has been retained for historical interest, but paper electrophoresis has been eliminated since its value is extremely limited.

Proteins, as a fundamental constituent of all protoplasm, have been termed "the essence of life process." They are involved as a basic part of the structure of the living cell and are also responsible for a major part of its function. For example, proteins serve as structural components of cells, enzymes, some hormones and hormone receptors, transport molecules, antibodies and clotting factors, plus additional functions too numerous to note here.

The "building blocks" of proteins are the 20 commonly occurring α-amino acids. These amino acids differ from each other in the structure of their side chain. In proteins these amino acids are linked through substitute amide or peptide bonds, which arise by the elimination of the elements of water from the carboxyl group of one amino acid and the α-amino group of the next.

Proteins differ from each other by the kinds of amino acids present, their arrangement, their quantity, molecular weight, surface changes, etc. As a result, there are probably an almost infinite number of proteins. Over the last 10 years many proteins have been isolated and physically characterized through use of such methods as ion-exchange chromatography, gel filtration, polyacrylamide electrophoresis, agarose electrophoresis, isoelectricfocusing, preparative ultracentrifugation, and analytical ultracentrifugal techniques. The development of radioimmunoassay techniques has lowered the limits of detection from the milligram and microgram range for some of the previously named technics to the nanogram or picogram range. Further technologic advances that would allow a rapid automated approach to radioimmunoassay are eagerly anticipated and currently under development.

Because of the size of the protein molecules, they cannot be absorbed through the intestinal mucosa. To obtain the necessary amino acids for protein synthesis, the ingested proteins must be degraded, absorbed, transported into the cell, and resynthesized into proteins.

It is in the form of amino acids that most of the necessary materials for protein synthesis are absorbed through the intestinal mucosa of the small intestine into the mesenteric venous blood. This does not exclude the possibility of larger molecules such as polypeptides or native protein be-

ing absorbed in small quantities. This is thought to be a contributing factor in causing certain food allergies. The fact that nitrogenous products and meat fibers are found in the colon and feces demonstrates that digestion and absorption are not always complete, especially in cases of diarrhea, pancreatic insufficiency, and common bile duct obstruction.

Amino acids entering the bloodstream are quickly taken up by the tissues so that there is usually only an insignificant rise in the blood amino acid level even after a protein-rich meal. Although the mechanics of this uptake of amino acids are not yet completely understood, it is generally thought that they are temporarily stored primarily in liver and muscle. Portions of these stored amino acids are later liberated for use by other tissue cells, and consequently little amino acid is excreted in the urine.

Excess amino acids are deaminated, and the ammonia formed, if not reused in the production of other amino acids or nucleic acid constituents, is converted in the liver to urea. The nonnitrogenous portion of the deaminated amino acid may be used as a source of energy in ways similar to that of carbohydrates and fats.

The dynamic state that exists between proteins and amino acids makes up part of what is termed "nitrogen balance." This means, in short, that a normal healthy individual takes in an amount of nitrogen that is equal to the amount excreted. Proteins in cell are constantly being broken down into their constituent amino acids, a portion of which is reutilized in the synthesis of new protein. The fact that the body is not a perfect machine results in the need of an exogenous source of amino acids, since some amino acids are deaminated in the liver and used for other purposes or are excreted directly in the urine. If the intake of nitrogen is below the level of that required for maintenance of destroyed protein, negative nitrogen balance results. Positive nitrogen balance, the opposite situation, exists in growing children, pregnant women, and recovery from negative balance.

Methods for the measurement of proteins in biologic fluids are separated in this chapter into manual and automated procedures. In addition, some reference methods are included, although we recognize that their application to routine analyses is limited.

The automated methods described use either the continuous-flow AutoAnalyzer system (Technicon Instruments Corp., Tarrytown, N.Y. 10591) or the centrifugal fast analyzer system. The latter was chosen as a representative of the discrete sample analyzers because of its flexibility in being able to make both end-point and kinetic measurements. In addition, it has been adapted to specific protein measurements, which constitutes an important part of this chapter. The centrifugal fast analyzer is based on the original concept of Dr. Norman Anderson, working at the Oak Ridge National Laboratories.[1] At the present time 3 companies manufacture this type of automated analyzer, and details of their instruments are available from company literature: Centrifichem (Union Carbide Corp., Tarrytown, N.Y. 10591), GeMSAEC (Electronucleonics, Inc., Fairfield, N.J. 07006), and Rotochem (American Instrument Co., Silver Spring, Md. 20910). We will discuss the Rotochem system, which has a 15-place rotor; however, these determinations may also be performed on the other centrifugal analyzers with minor modification of the sample and reagent volumes. Many other discrete sample analyzers also are capable of making both kinetic and end-point measurements. Thus the types of methods described in this chapter for the centrifugal fast analyzer are in most cases adaptable to other discrete sample analyzers.

TOTAL SERUM PROTEIN

Most clinical chemistry laboratories employ the biuret reaction for the measurement of total serum proteins and use the simple refractometric technic for confirmation of results. The biuret method is technically straightforward and precise, and is accurate with an appropriate means of standardization.

The standard used in this determination may either be a human serum pool or a commercially available solution of crystalline bovine albumin. In both cases the concentration is usually determined by Kjeldahl analysis and expressed in terms of milligrams of nitrogen per milliliter of solution. In order to relate this value to mass of protein per unit volume, usually grams per deciliter, one must employ a factor that relates milligrams of nitrogen to milligrams of protein. This factor depends on the protein source. It has been recommended that a factor of 6.54 (i.e., 15.3% by weight) be used for human serum pools, and a factor of 6.41 (i.e., 15.6% by weight) be used for crystalline bovine albumin[2] (Armour Pharmaceutical Co., Phoenix, Ariz. 85077).

The automated procedures include the continuous-flow technique, which is probably used more than any other total protein method. The procedure described, or modifications such as those for adaptation to the continuous-flow SMA system, is precise and relatively trouble free. A discrete sample automated method is described using the centrifugal fast analyzer. Kinetic methods have been described for the centrifugal fast analyzer, but their use is to be discouraged, since precision is poor and accuracy is in question. The centrifugal fast analyzer method described here allows the biuret reaction to come to completion before a spectrophotometric measurement is made.

Manual methods for total serum proteins

Biuret reaction[3]

Principle. Serum proteins react with copper sulfate in sodium hydroxide to form a violet "biuret" complex. The intensity of the violet color is proportional to the concentration of protein.

tion (Ames Co., Division of Miles Laboratories, Inc., Elkhart, Ind. 46514).

Procedure

1. Check all urine specimens with Albustix and dilute all samples greater than "2+."
2. Into paired test tubes, pipet 2.0 ml urine or standard. While mixing add slowly 2.0 ml Tsuchiya's solution. Heat to 56 C for 15 min and then cool.
3. Centrifuge precipitate and wash twice with 1 ml ethanol.
4. To washed precipitate add 3-0 ml of 0.75N NaOH (30 g/L) and mix until precipitate dissolves.
5. To 1 test tube of each pair add 0.15 ml biuret blank solution, and to other tube add 0.15 ml biuret reagent. Mix by inversion and incubate at room temperature 20 min.
6. Zero the spectrophotometer at 540 nm with cuvette containing 3.0 ml of 0.75N sodium hydroxide plus 0.15 ml biuret blank reagent. Record absorbance of sample and standard blanks.
7. Zero the spectrophotometer with cuvette containing 0.15 ml biuret reagent and 3.0 ml 0.75N sodium hydroxide. Record absorbance of standard and sample.

Calculation

$$C_u = C_s \cdot \frac{A_u - A_{ub}}{A_s - A_{sb}}$$

where C_u = concentration of unknown; C_s = concentration of standard; A_u = absorbance of unknown; A_{ub} = absorbance of unknown blank; A_s = absorbance of standard; A_{sb} = absorbance of standard blank.

Remarks. Using a Bausch & Lomb Spectronic 20 spectrophotometer with cuvettes 1.27 cm in diameter, the linear range of this biuret assay is 0-2.0 g/L.

Automated immunochemical method[13]

An automated immunochemical method using the continuous-flow approach is described. An appropriately diluted urine sample is mixed automatically with antiserum to human serum proteins, followed by incubation at room temperature and quantitation by light-scattering in a fluoronephelometer.

Apparatus

1. An automatic pipet (e.g., MicroMedic Systems, Philadelphia, Pa. 19104) is used to prepare dilutions of urine samples.
2. A single-channel automated system is assembled from components of the Technicon AutoAnalyzer II. This system consists of a sampler, proportioning pump, modified analytical cartridge, a fluoronephelometer, and a chart recorder.

Reagents

1. Goat anti-whole human serum can be obtained from commercial sources (e.g., Technicon Instruments Corp., Tarrytown, N.Y. 10591). Antiserum is diluted 25-fold with physiologic saline (9 g NaCl/L) containing 40 g/L polyethylene glycol 6000 (Fisher Scientific, Fair Lawn, N.J. 07410).
2. Standard. Commercial reference serum (Technicon Instruments Corp., Tarrytown, N.Y. 10591) is used for standardization.
3. Albustix (Ames Co., Division of Miles Laboratories, Elkhart, Ind. 46514).

Procedure

1. All urine samples are screened for protein content with the Albustix and diluted appropriately in order to avoid antigen excess.

Albustix reading	*Dilution*
neg	1:10
trace	1:10
+	1:10 and 1:20
++	1:20, 1:40, and 1:100
+++	1:100 and 1:200
++++	1:200 and 1:500

2. After dilution samples, controls and standards are aspirated into the system, mixed with antiserum, and the light-scattering intensity of the antigen-antibody complexes is measured in the fluoronephelometer and recorded.

Calculations

1. Total protein concentration is determined for each sample using a minicomputer programmed to do linear interpolation between the points generating the standard curve.
2. If a minicomputer is not available, a standard curve may be plotted with sample and control values read from this curve.

Remarks

1. The useful range of the method is from 1.5-100 mg total protein/dl.
2. Precision studies show a coefficient of variation of about 3% for the method.
3. Extremely high samples must be diluted and repeated to ensure that measurement is made in the region of antibody excess.
4. This automated method is capable of analyzing 70 samples/h.
5. The standard curve is generated by making 7 solutions of the reference serum, covering the useful range of the method.
6. Blank readings are not required, since urine possesses low intrinsic light-scattering.

Measurement of serum albumin and globulins

Serum albumin can be measured directly in serum by dye-binding methods, or indirectly by estimation of total globulins. Fractionation of serum proteins by salt precipitation or electrophoresis also offers a means of estimating albumin.

Three manual methods are described, and the two that possess the greatest convenience are dye binding with bromocresol green and the measurement of total globulins. Both are technically straightforward but are subject to errors. The bromocresol green method has been shown to overestimate albumin due to nonspecific binding,[14] and the total globulin method occasionally gives aberrant results in some patients with dysproteinemia. Provided the limitations of the methods are recognized, these two approaches are recommended, since no other technically simple and fast method is widely available. Salt fractionation of albumin and globulins is described and gives excellent results when careful

technic is exercised, but unfortunately the method is too tedious for the modern busy laboratory.

Automated methods for serum albumin and globulin are discussed using continuous-flow and centrifugal fast analyzer technics. Dye binding with bromocresol green offers the simplest approach to automation of serum albumin but is subject to the same limitations of accuracy as the manual method. Automated light-scattering immunochemical methods have been described for serum albumin[15] and appear to offer excellent specificity. However, the expense of the antiserum and the technical difficulties of obtaining consistent results preclude their use as a widespread routine method. Such light-scattering technics will be described later for other serum-specific proteins and can be applied with minor modifications to serum albumin.

Manual methods

Serum albumin by dye binding[16]

Principle. Serum albumin generally is thought to function as a carrier protein for a number of rather insoluble organic substances such as fatty acids and bilirubin, and hence it is endowed with unique binding properties. The binding of these substances to albumin is thought to be due to entropy factors of the solvent (water) and van der Waals interaction between aliphatic amino acid residues and the bound molecules. This binding is generally termed hydrophobic. A number of dyes have been shown to bind albumin hydrophobically and absorb light at a wavelength different from the unbound dye. Of the various dyes that have been used in serum determinations, bromocresol green is the most sensitive, specific, and relatively free from interference.

Reagents

1. Brij-35. Thirty-percent solution of Brij-35 in water (Fisher Scientific Co., Philadelphia, Pa. 19406).
2. Stock bromocresol green solution. Dissolve 419 mg bromocresol green (Sigma Chemical Co., St. Louis, Mo. 63118) in 10 ml of 0.1N sodium hydroxide, and dilute to 1 L with glass-distilled water.
3. Buffer. Dissolve 11.9 g citric acid monohydrate in 800 ml glass-distilled water. Adjust pH of solution to 4.2 with 10% sodium hydroxide (wt/vol) and dilute to 1000 ml with glass-distilled water.
4. Working bromocresol green solution. Mix 250 ml of stock bromocresol green solution with 750 ml citrate buffer and 4.0 ml of 30% Brij-35. NOTE: This solution is commercially available (Fisher Scientific Co., Philadelphia, Pa. 19406).
5. Standard. Weigh out 5 g human serum albumin fraction V (Sigma Chemical Co., St. Louis, Mo. 63118), and dissolve in 100 ml of 0.9% saline containing 0.20% sodium azide. Determine protein concentration of this solution by biuret procedure using bovine serum albumin for standard (Armour Pharmaceutical Co.).

Method

1. Pipet 0.020 ml serum into test tube, add 5.0 ml bromocresol green working solution, and mix.
2. Reagent blank. Pipet 0.020 ml of 0.9% sodium chloride into test tube and add 5.0 ml of bromocresol green reagent.
3. Measure absorbance at 628 nm in suitable spectrophotometer using reagent blank to zero instrument.

Calculations

$$C_u = C_{st} \cdot \frac{A_u}{A_{st}}$$

where C_u = concentration of unknown; C_{st} = concentration of standard; A_u = absorbance of unknown; A_{st} = absorbance of standard.

Remarks

1. The reaction between albumin and bromocresol green is extremely fast and goes to completion in only a few seconds.
2. The 1:250 dilution of serum generally eliminates interferences from hyperlipemia, hyperbilirubinemia, and hemolysis. Grossly lipemic sera can be blank corrected using 0.9% saline as a reagent blank method.
3. The method is linear from 0-7 g/dl.
4. The human albumin standard should be divided into aliquots and stored at 4 C. This standard is stable up to 1 mo.

Total globulin in serum[17]

Principle. In the presence of strong acids, glyoxylic acid reacts with tryptophan residues of proteins to form a purple color. Copper sulfate is added to enhance the color formation. Since human globulins are known to contain 2-3% tryptophan, it is possible to derive an empirical factor relating the concentration of *N*-acetyltryptophan to serum globulin levels.

Reagent

1. Globulin reagent. This reagent consists of 800 mg glyoxylic acid, 600 mg copper sulfate, 15 mole acetic acid, and 2.2 mole sulfuric acid per liter of solution and is available under the trade name Diagnostest (Dow Chemical Co., Indianapolis, Ind. 46206).
2. Standardization. *N*-acetyl-DL-tryptophan 220 mg/dl equivalent to 4.0 g/dl globulin (Sigma Chemical Co., St. Louis, Mo. 63118).

Procedure

1. Label three 13 × 100 mm test tubes as "standard," "control," and "test." Using volumetric pipet, transfer 4 ml globulin reagent into each tube.
2. Transfer 20 µl standard into tube marked "standard," cover, and mix thoroughly. Treat control and test samples in a similar manner.
3. Cover test tubes with aluminum foil and heat 5 min at 100 C.
4. Cool 3 min in tap water and remix test tubes.
5. Adjust wavelength of spectrophotometer to 550 nm. Set zero with unused portion of globulin reagent and measure absorbance of all tubes.

Calculations

$$C_u = C_s \cdot \frac{A_u}{A_s}$$

where C_u = concentration of unknown; C_s = concentration of standard; A_u = absorbance of unknown; A_s = absorbance of standard.

Remarks

1. The method is linear to 7.5 g globulin/dl.
2. The *N*-acetyltryptophan is recommended as

the standard of choice, since it can be purchased in a highly pure form and is soluble in a dilute alkaline solution.[3] The empirical factor relating globulin and *N*-acetyltryptophan was based on 28 sera containing normal amounts of albumin, thus the contribution of albumin to the colored product is included in the factor.

3. Bilirubin up to 20 mg/dl gives less than a 5% interference, and moderate hemolysis and lipemia contribute no appreciable interference.

Albumin by difference[18]

Principle. The serum albumin concentration can be calculated by subtracting the total globulin concentration from the total protein concentration.

Reagent and method. See biuret total protein method and total globulin method.

Remarks

1. This method is relatively free of interference from moderate lipemia, icterus, and hemolysis.
2. Significant errors in serum albumin determinations by this method can arise in patients with multiple myeloma where the tryptophan content of the globulins can vary markedly.

Serum albumin by salt fractionation[19]

Principle. The accepted reference method for the determination of albumin is the salt fractionation method of Wolfson et al., which uses sulfite to precipitate globulins from serum. The precipitated globulins are removed by shaking with diethyl ether followed by centrifugation. Albumin remains in the aqueous phase and is quantitated by the biuret procedure.

Reagents

1. Sodium sulfite 28.3% (wt/vol).
2. Ether reagent. 1% triton X-100 in absolute diethyl ether.
3. Working biuret reagent. Prepare as described under total protein method.
4. Standard. Crystalline bovine albumin, approximately 6.4 g/dl (Armour Pharmaceutical Co.).

Procedure

1. Into a dry ground-glass stoppered tube, place in order:
   a. 4.0 ml of 28.3% sodium sulfite using a class A volumetric pipet.
   b. 0.2 ml serum dispensed volumetrically.
   c. 4.0 ml ether reagent dispensed with serologic pipet.
2. Stopper tube and invert gently 8 times.
3. Centrifuge at 2000 rpm for 5 min. If aqueous phase is not perfectly clear, add an additional milliliter of ether reagent and recentrifuge for 5 min. After centrifugation, globulin precipitate forms a pellet between the 2 phases.
4. Carefully tip centrifuge tube and insert 2 ml class A volumetric pipet into aqueous phase, keeping top of pipet covered with index finger to prevent ether reagent from entering pipet. Remove exactly 2.0 ml aqueous phase, wipe off pipet tip, and transfer contents to test tube marked "albumin test."
5. Add 2.0 ml of 28.4% sodium sulfite to another test tube labeled "albumin blank."
6. To test tube labeled "standard," add 0.1 ml bovine serum albumin standard and 1.9 ml of 28.3% sodium sulfite.
7. Add 4.0 ml working biuret reagent to all tubes.
8. Mix and allow to stand 20 min.
9. Measure absorbances at 555 nm of all solutions in appropriate spectrophotometer.

Calculations

$$C_u = C_s \times \frac{A_u}{A_s}$$

where C_u = concentration of unknown; C_s = concentration of standard; A_u = absorbance of unknown; A_s = absorbance of standard.

Remarks

1. If serum blanks are required for lipemic samples, follow the above procedure and obtain the absorbance of all the unknowns. Then add 1 mg potassium cyanide to the albumin standard as well as all the serum samples. This step will destroy the absorbance due to the copper-protein complex leaving only the absorbance due to interfering substances. Re-read all the unknowns. Calculate as follows:

$$C_U = C_S \times \frac{(A_U - A_{UKCN})}{(A_S - A_{SKCN})}$$

where A_S = absorbance of standard; A_{SKCN} = absorbance of standard after addition of potassium cyanide; A_U = absorbance of unknown; A_{UKCN} = absorbance of unknown after addition of potassium cyanide; C_S = concentration of standard; C_U = concentration of unknown.

2. Vigorous mixing of the samples after the addition of the ether reagent may result in the denaturation and precipitation of albumin, resulting in lower albumin values.

Automated methods

The direct spectrophotometric determination of globulin in serum by the method of Goldenberg and Drewes[17] adapted to the continuous-flow system of automation[18] is described. A dual-channel system also can be employed for the simultaneous determination of total protein by the modified biuret method. This combination of determinations allowed the calculation of serum albumin by subtracting the globulin value from the total protein. Total globulin is measured by reaction of the tryptophan residues of the globulin molecules with glyoxylic acid in a strongly acidic medium. The colored product of the reaction absorbs strongly at 550 nm.

Reagents

1. Globulin reagent. See manual method.
2. Standard. See manual method.

Apparatus. The method requires the following continuous-flow equipment: samples, proportioning pump, manifold, heating bath, colorimeter with 550 nm

interference filters, and chart recorder. A flow diagram is described in the original publication.[18]

Remarks
1. The automated globulin method is linear to 6 g/dl.
2. Recovery of gamma globulin added to serum is essentially quantitative.
3. The day-to-day precision of the method has been found to be 4.5%.
4. The method can be standardized with *N*-acetyltryptophan.

Serum albumin by continuous-flow analysis[20]

The continuous-flow albumin method is based on the bromocresol green dye-binding method of Doumas. This method measures the increase in absorbance at 630 nm due to the albumin bromocresol green complex formed at pH 4.2. The need for a blank channel is eliminated due to the high dilution of the sample, approximately 1:441.

Reagents
1. Bromocresol green solution. See manual method.
2. Diluent that consists of 1.0 ml of 30% Brij-35 per liter distilled water.

Apparatus. This method requires a sampler; 60/h cam, 9:1; proportioning pump, manifold no. 170-A002-01; single-channel colorimeter with 630 nm interference filter; and chart recorder. Further information is provided in Technicon method no. SE4-0030FD4.

Remarks
1. The method is linear to 6 g/dl.
2. Quantitative recoveries are obtained.
3. Studies show a precision of the method (95% limits) is approximately ±3%. Interferences due to hyperlipemia and hyperbilirubinemia are minimized because of the initial high dilution of the sample.

Serum albumin by centrifugal fast analysis[21]

The bromocresol green method of Doumas has been adapted to the centrifugal fast analyzer.

Reagents
1. Bromocresol green working solution. See manual bromocresol green albumin method modified with antifoam (see remarks).
2. Standard. See manual bromocresol green albumin method.

Apparatus. The method is described for the Aminco Rotochem II system. With minor modification of volume it may be used with other centrifugal analyzers.

Procedure
1. Pipet 600 μl distilled water into reference position of transfer disk.
2. Pipet 5 μl distilled water, standard or sample, 90 μl saline, and 400 μl bromocresol green reagent into appropriate position of transfer disk.
3. Load transfer disk into analyzer and initiate program.
4. Take absorbance reading 30 s after initiation of reaction.
5. Calculation of sample concentrations is made automatically by computer using absorbance measurements of standard and samples taken 30 s after acceleration of rotor.

Remarks
1. The method is linear to 7 g/dl of albumin.
2. Day-to-day precision (95% limits) is about ±3%.
3. Before using commercially available working bromocresol green solution, add 2 ml Dow antifoam B per liter of working solution. This process prevents intercuvette transfer of samples during vacuum mix cycle.
4. The method is free of interference from icterus and hemolysis.

Fractionation of proteins by electrophoresis

Only 2 serum protein measurements have been described up to this point, total protein and albumin. These are the 2 most common protein measurements made in clinical chemistry laboratories, and serum albumin is of considerable value to the clinician. Total-protein measurements in serum are of limited value, since there are so many individual specific protein constituents that change to different degees in disease states. For example, in acute inflammation some serum proteins become elevated (haptoglobin, orosomucoid, α_1-antitrypsin), while others are decreased (albumin, transferrin). Thus fractionation of serum proteins has become an extremely important laboratory test.

Electrophoresis has been the most common means of fractionating serum proteins, and traditionally quantitation of the protein fractions has been accomplished by densitometric scanning. In recent years, high-resolution technics using agarose gels have been proposed with qualitative visual examination being preferred to quantitative scanning. The proteins visualized on agarose gel electrophoresis are shown in Fig. 13-1.

Electrophoresis is a technic for the separation of charged molecules, which is dependent on differences in the migration velocities of the molecules in a buffer solution through which an electrical current is passed. Differences between the rates of migration of various molecular species in an electrical field are the result of inequalities in their ionic charges at a given pH. Although electrophoretic fractionations may be performed in a liquid buffered medium (i.e., free, Tiselius, or moving boundary electrophoresis), it is usually advantageous to minimize the diffusion of the separated molecules by conducting the electrophoretic migration in an inert stabilizing medium that is saturated with buffer solution. Examples of the stabilizing media used for electrophoresis include filter paper, cellulose acetate, sucrose gradient starch gel, acrylamide gel, agarose, dextran gel, and glass beads. A number of synonyms for electrophoresis have been used such as ionophoresis, ionography, and electrochromatography. The common applications of electrophoresis in clinical laboratories include fractionations of proteins, lipoproteins, glycoproteins, isoenzymes, hemoglobins, amino acids, and catecholamine metabolites. In the

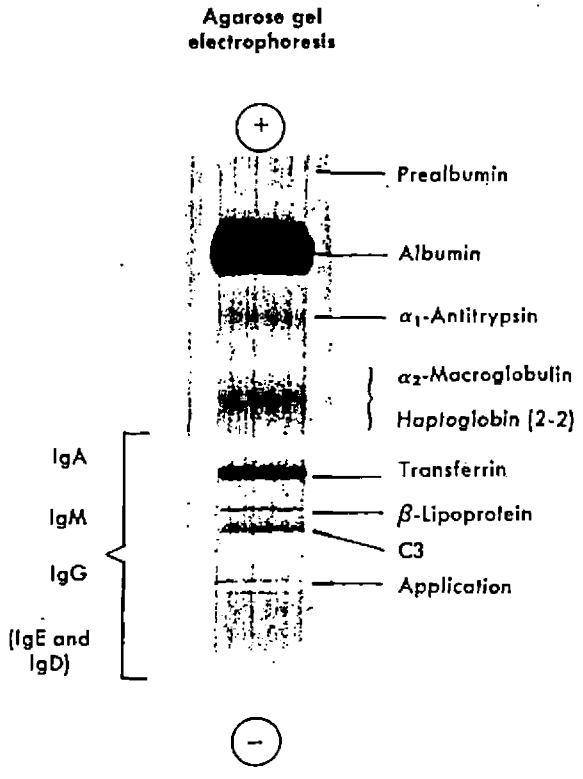

Fig. 13-1. Electrophoretic separation of serum proteins on agarose gel. (Courtesy L. M. Killingsworth, University of North Carolina, Chapel Hill, N.C.)

present discussion, attention will be focused on 2 electrophoretic methods currently employed in clinical laboratories for the separation and quantitative determination of serum protein fractions.

Cellulose acetate electrophoresis[22]

Principle. Cellulose acetate membrane is used as an inert supporting medium for the separation of serum proteins. This technic separates the serum into 5 principal regions, which are then detected with ponceau S stain. Proteins generally are quantitated by scanning the cleared, destained membrane. Results are reported as a percentage of the total, or if the total protein is known, the protein concentration per region may be calculated.

Apparatus

1. Many different cellulose acetate electrophoresis systems are commercially available. The Microzone system will be discussed in detail (Beckman Instruments, Fullerton, Calif. 94302).
2. The Microzone system consists of an electrophoresis cell, a regulated power supply, and a densitometer with scanning attachment.
3. Cellulose acetate membranes are supplied with the Microzone system.

Reagents

1. Barbital buffer. Dissolve 15.40 g sodium diethyl barbiturate and 2.76 g barbituric acid in 1 L water. This produces a solution of pH 8.6 and ionic strength of 0.075.
2. Fixative-stain solution. Dissolve 2 g ponceau S in 30 g trichloroacetic acid and make up to 1 L volume with water.
3. Rinse solution. 5% (vol/vol) acetic acid in water. Dilute 100 ml glacial acetic acid to 2 L.
4. Alcohol rinse. Absolute methanol, A.C.S. reagent grade.
5. Cleaning solution. Mix 20 ml glacial acetic acid with 80 ml methanol.

Procedure

1. Follow manufacturer's instruction for preparing electrophoresis cell.
2. Place small amount of buffer in shallow dish and carefully float membrane on surface. Membrane will slowly absorb buffer and turn uniform gray in color. If air is entrapped in membrane, uniform gray color will not be obtained and membrane should not be used.
3. Remove membrane from buffer, blot gently with filter paper, and place membrane in electrophoresis cell.
4. Apply approximately 0.25 μl serum to membrane with serum applicator supplied with Microzone system.
5. Connect power leads and turn on power supply. Samples are subjected to electrophoresis for 20 min at 240 V.
6. Following electrophoresis step, remove membrane and float it serum side up in shallow dish containing 100 ml ponceau S stain. As soon as bands become stained, immerse membrane in dye solution for additional 7 min.
7. Remove membrane from stain and rinse in 5% acetic acid until background is white.
8. Dehydrate membrane by immersion in absolute methanol.
9. After dehydration, position membrane on glass plate and immerse for 45 s in methanol–acetic acid clearing solution. Air-dry membrane 2-3 min and heat 20 min in oven at 70-80 C.
10. Transfer cleared, dry membrane gently from glass plate to plastic storage envelope and label.
11. Mount plastic envelope with membrane in carriage densitometer and scan at 520 nm.

Calculations

1. Using areas of each protein region derived from densitometer scan, calculate total area, and percentage of total area of each given region then may be calculated.
2. Protein concentration of each region may be determined by multiplying percentage of total area for that region times serum total protein concentration.

Remarks

1. Cellulose acetate electrophoresis resolves serum proteins into 5 multicomponent regions.
2. Some errors arise in quantitation due to variation in dye-building affinities of the various protein components.
3. The resolving capability of the system is less than agarose gel electrophoresis.

Agarose gel electrophoresis[23]

Apparatus

1. Electrophoresis cell. Water-cooled electrophoresis cell large enough to accomodate glass plate

20.5 × 11.0 cm is used (Water-cooled Agarose Electrophoresis Cell—Laurell type, MRA Corp., Boston, Mass. 02121). This size of cell is large enough to permit simultaneous electrophoresis of 9 samples.
2. Water cooling. Electrophoresis cells are cooled by a refrigerated liquid circulator utilizing diluted ethylene glycol at 4 C, which will provide sufficient cooling to dissipate heat generated during electrophoresis (Forma, Scientific, Marietta, Ohio 45740).
3. Power supply. Any well-regulated power supply capable of producing 0-400 V and 0-150 mA may be used (Model SP-17A, Health Co., Benton Harbor, Mich. 49022).

Reagents

1. Buffer. Dissolve 105.15 g sodium barbital, 16.5 g barbital, and 6.9 g calcium lactate in 2 L hot deionized water and dilute to 8 L. All chemicals are reagent grade.
2. Agarose. Dissolve 1 g agarose in 100 ml barbital buffer by heating solution to boiling. Agarose will remain in liquid state at 50 C (Sea Kem Brand Marine Collidssher, Springfield, N.J. 07081).
3. Fixative. Fixative is a solution composed of 1000 ml deionized water, 1000 ml methanol, and 200 ml glacial acetic acid.
4. Stain. Dissolve 1 g naphthol blue blank dye in 100 ml fixative solution. Filter once before use (Sigma Chemical Co., St. Louis, Mo. 63118).
5. Tracking dye. Dissolve 20 mg bromophenol blue in 100 ml barbital buffer (Sigma Chemical Co., St. Louis, Mo. 63118).

Procedure

1. Due to variability between electrophoresis cells commercially available, no attempt will be made to discuss assembly of cell. Please see manufacturer's instructions.
2. Coat glass plates (20.2 × 10.8 cm) with 0.5% solution of agarose in barbital buffer (8 ml of 0.5% agarose per plate) and allow to dry completely. This procedure ensures binding of separation gel to plate.
3. To pour separation gel, make a sandwich of a precoated plate, a $\frac{1}{32}$-inch single-chamber plastic frame, and a slot-forming plate. (Glass plate #92-2; plastic frame, 92-34; slot-forming plates, #92-3D; MRA Corp., Boston, Mass. 02121.)
4. Place assembly in oven at 60-70 C (for about 10 min).
5. Remove assembly from oven and fill hollow space with hot 1% agarose as rapidly as possible.
6. Place filled assembly in refrigerator to gel. During gelling process the agarose may shrink, and if this happens, add additional hot 1% agarose.
7. When plate has gelled completely, remove clamp, place plate on a bench, and carefully pry up and remove slot-forming plate. Sample wells will hold approximately 10 μl of liquid.
8. Sprinkle a few drops of distilled water on bottom of electrophoresis cell and place plate in cell. Press down on plate to remove air bubbles. (NOTE: Do not substitute buffer for distilled water.) The water ensures good heat transfer from plate to cooling liquid pumped through bottom of cell.
9. Mix 2 parts serum or other fluid with 1 part bromophenol blue. Blot sample wells in plate and fill each well with 10 μl sample containing bromophenol blue.
10. Connect plate with cell by means of Whatman 3 mm filter paper wicks with buffer. Cover chamber with glass plate.
11. Subject plate to electrophoresis at 250 V (approximately 100 mA). After 15 min turn power off, moisten wicks, fill sample wells with buffer, and continue electrophoresis for another 55 min.
12. When electrophoresis is finished, turn off power, remove plate from cell, and submerge it in fixing solution 15 min. At end of this period transfer plate to another tray and place under stream of gentle running water for 30 min to remove buffer salts from gel. Dry plate.
13. Stain plate for 5 min in 1% naphthol blue black. Destain in fix solution until background is clear and then dry. Plate is now ready for clinical interpretation.

Remarks

1. Agar may not be substituted for agarose. Agar contains highly charged polysaccharides that may interact with protein components of serum.
2. Thin and regular application wells are extremely important for high-resolution electrophoretic patterns. The slot-forming plate produces consistently well-defined application wells, and it is superior to other methods.
3. After fixation of the proteins, plates should be washed with water to remove buffer salts. The plates are then dried and after staining may be stored indefinitely without deterioration of the stained bands.
4. The high-resolution properties of the system are demonstrated in Fig. 13-1.

Fractionation of urine and CSF proteins by electrophoresis

The electrophoretic technics for serum protein fractionation are applicable to urine and CSF analysis, provided the sample undergoes a concentration step. Concentration of CSF and urine samples can be accomplished simply and rapidly by employing the Amincon macrosolute concentrator (Amincon Corp., Lexington, Mass. 02173). This disposable device consists of an 8-chambered plastic case, with each chamber backed by a 15,000 mol wt cutoff membrane bonded to a filter paper absorbent. The chambers hold a maximum of 5.0 ml, and the sample can be concentrated 100-fold to a final volume of 50 μl. This system is technically simple to use and well suited for use in the routine clinical laboratory.

Determination of specific proteins in serum CSF and urine

There is probably little doubt that the clinical chemistry laboratory of the future will emphasize the measurement of specific proteins and rely on electrophoresis as a means of detecting "M" components in plasma cell dyscrasias and cathodal bonding as seen in lupus erythematosus.[24,25] The gel-diffusion methods for specific protein measurements are usually slow, expensive, imprecise, and have limited applications in the modern laboratory. A more rapid and precise technic involves light-scattering or nephelometric measurements of antigen-antibody

complexes. This approach allows specific protein measurements to be made in a matter of 1 or 2 minutes with a precision of better then 5% versus the gel-diffusion methods, which take several hours with poor precision. In addition, the light-scattering technic can be automated and adapted to both continuous-flow and centrifugal fast analyzer systems.

In the light-scattering technic the reagent used is a specific antiserum (antibody) to the human serum protein (antigen). On mixing diluted serum with antiserum the antigen reacts with the antibody, producing large antigen-antibody complexes that will scatter light. Most commonly the light-scattering is measured at 90° in a fluorometer with the spectrophotometric system adjusted so that the wavelengths of the incident and emitted light are identical. Mostly 360 nm is used, although higher wavelengths can be employed if slightly reduced sensitivity is acceptable. The angle of light-scattering measurements can be varied, and increased sensitivity can be achieved at angles other than 90°. However, for most routine measurements 90° is most convenient, since conventional fluorometers allow only this angle for measurements. Recently an adaptation of a commercial centrifugal fast analyzer has allowed near front surface light-scattering measurements to be made.

In recent years polyethylene glycol 6000 (PEG) has been incorporated into the reaction mixture and has a profound effect on the rate of formation of antigen-antibody complexes. Whereas in the absence of PEG the antigen-antibody reaction takes 15 minutes to 2 hours to come to completion, the presence of PEG achieves equilibrium in 2-4 minutes. The PEG does not appear to be part of the complexes and probably acts by steric exclusion of macromolecules for the domain of the polymer.

The methods described in this chapter include manual, automated continuous-flow, and centrifugal fast analyzer technics. The manual methods described do not use PEG, since its incorporation into the reaction requires very accurate timing, otherwise flocculation of the precipitated antigen-antibody complexes occurs. Both the manual and continuous-flow technics allow the antigen-antibody reaction to achieve equilibrium before light-scattering measurements are made. However, in the centrifugal fast analyzer approach the rate of formation of the complexes is measured by fixed-time kinetics. Here the change in light-scattering between 2 fixed times (e.g., 15 seconds and 90 seconds) is measured and related to a standard curve.

In all of these light-scattering methods the standard curve is nonlinear and follows the characteristic precipitin curve (Fig. 13-2), which is divided into zones of antibody excess (region 1), equivalence (region 2), and antigen excess (region 3). All of the methods described operate in the antibody excess zone. Examination of Fig. 13-2 reveals that one can obtain identical light-scattering intensity for widely variable antigen concentrations depending on whether the concentrations fall in antibody or antigen excess. Thus some means of detecting antigen excess samples is a necessary part of any procedure. In the continuous-flow technic a bimodal recorder peak readily identifies antigen excess samples and alerts the analyst to make a dilution of the sample. In the manual and centrifugal fast analyzer methods a second measurement with a 1:1 dilution of the sample is needed to detect antigen excess. In practice, however, serum protein electrophoresis can be used in screening for antigen excess, and the conditions described in this text are adjusted so that only patients with monoclonal gammopathies can be expected to achieve antigen excess.

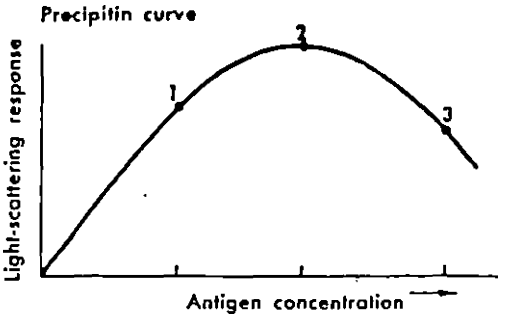

Fig. 13-2. Precipitin curve of antigen-antibody reactions followed by light scattering. (Courtesy L. M. Killingsworth, University of North Carolina, Chapel Hill, N.C.)

Since CSF-specific proteins can be measured using identical methods to serum with different dilutions, the method description includes procedures for both serum and CSF.

Manual nephelometric methods for the determination of α_1-antitrypsin, IgG, IgA, and IgM

Principle. Serum proteins are quantitated using immunochemical reactions with specific commercial antisera. A fluorometer modified for nephelometry is employed to make light-scattering measurements of the antibody-antigen complexes formed during the immunochemical reaction, which is carried out in the region of antibody excess. A multipoint standard curve is required since the relationship between light-scattering and protein concentration is nonlinear.

Apparatus

1. Light-scattering measurements can be made in any suitable fluorometer, and the instrument utilized in the initial development of this method was the Turner Model III Fluorometer (G.K. Turner Assoc., Palo Alto, Calif. 94303). A recorder may be connected to the fluorometer; however, it is possible to take readings from the readout dial on the fluorometer. The fluorometer is equipped with a narrow bandpass primary filter, peak transmission at 360 nm (Turner no. 110-811). No

secondary filter is used. The fluorometer is also equipped with a quartz microscale flow-through cell (no. 176F-QS, Hellma Cells, Inc., Jamaica, N.Y. 11431). The light source is a general purpose lamp that provides peak emission at 350 nm (Turner no. 110-850).
2. Serum may be diluted by an automatic pipet (Model 2500; MicroMedic Systems, Philadelphia, Pa. 10104).

Reagents
1. Saline. Nine grams of reagent grade sodium chloride and 15 drops of triton X-100 are dissolved in 1 L deionized water. The solution is then filtered through a 0.45 μm Millipore filter (Millipore, Bedford, Mass. 01730) to remove particulate matter. The filtered saline is used to make all dilutions of antiserum and standards.
2. Antiserum. Goat antisera specific for α_1-antitrypsin, IgG, IgM, and IgA are commercially available (Technicon Instruments Corp., Tarrytown, N.Y. 10591; Atlantic Antibodies, Westbrook, Me. 04092; Meloy Laboratories, Falls Church, Va. 22046).
3. Standard. Commercial reference serum (Technicon Instruments Corp., Tarrytown, N.Y. 10591) is used to provide standardization for these proteins. Reference serum has been standardized versus WHO standard pool. Standard curve is generated by dilutions of this reference serum.

Procedure

α_1-Antitrypsin[27]
1. Prepare working standards by making 4:5, 2:3, 1:2, 1:5, and 1:10 dilutions of reference serum.
2. Antiserum. Dilute goat antiserum specific for α_1-antitrypsin 1:25 with saline.
3. Using a 5 μl syringe, add 1.5 μl of standard or unknown sample to 1.0 ml diluted antiserum in 10 × 75 mm test tube. To second tube add 0.75 μl of sample to 1.0 ml diluted antiserum as check for antigen excess. Cover all tubes, mix 10 times by gentle inversion, and incubate at room temperature for 45 min.
4. Prepare blanks by addition of 1.5 μl serum to 1.0 ml saline. Blank readings greater than zero are only encountered with grossly lipemic samples.
5. Samples, standards, or blanks are injected into flow cell with 3 ml syringe by injection into inlet port. Flow cell and syringe are washed with saline between injections. Fluorometer is adjusted to zero with saline for blank measurements and with dilute antiserum for measurements of samples or standards.
6. Blank values are subtracted from reaction values. Nonlinear standard curve is constructed by plotting blank-corrected relative intensity of light-scattering versus concentration of α_1-antitrypsin calculated from dilutions of reference serum. All unknowns are calculated by interpolation from standard curve.
7. Standard curve measurements assume antibody excess, and if determination is carried out as directed, antibody excess is achieved up to 300 mg/dl.
8. Concentrations of α_1-antitrypsin greater than 300 mg/dl can be detected by the fact that the reaction containing one-half the amount of sample will not show a corresponding decrease in light-scattering. If this is observed, then appropriate dilution must be made and the determination repeated.

IgG[28]
1. Dilute serum 700-fold by mixing 20 μl serum with 14 ml saline in 16 × 125 mm plastic screw-top tubes (Falcon Plastics, Oxnard, Calif. 93090).
2. Pipet 1.0 ml diluted serum sample into 10 × 100 mm disposable test tubes for reaction and blank mixtures. At this time 0.5 ml diluted serum is pipetted into test tube followed by 0.5 ml saline resulting in 2-fold dilution, which is used to screen for antigen excess.
3. Make 1:25 dilution of goat antiserum specific for human IgG, i.e., 1 part antiserum to 24 parts saline.
4. Add 1.0 ml antiserum to each reaction mixture. Likewise, 1 ml saline is added to each blank.
5. Cover tubes and mix by gentle inversion.
6. Standardization is accomplished by making appropriate dilutions of reference serum (Technicon Instruments Corp., Tarrytown, N.Y. 10591) such that IgG concentrations are approximately 1400, 700, and 350 mg/dl. Standards are analyzed as described above.
7. Allow reaction tubes to incubate at room temperature 40 min. During this period, blanks may be measured.
8. Adjust fluorometer to zero for blank measurement with saline in flow cell. Blanks are then introduced into cell with 3 ml syringe, reading is taken, and flow cell is washed with saline.
9. At end of 40 min incubation, fluorometer is zeroed with solution consisting of 1 ml diluted antiserum and 1 ml diluted antiserum plus 1 ml saline. Reactions are then measured. Blank values are subtracted from reaction values for both standards as well as unknown samples.
10. A nonlinear standard curve is generated and unknowns, blank corrected, are read from this curve.

IgA[28]
1. Since concentration of IgA is lower than IgG, initial dilution of only 150-fold is made, i.e., 0.10 ml serum diluted with 15.0 ml saline.
2. Procedure for measuring IgA is identical to that of IgG. Standard curve is generated from dilution of reference serum such that IgA concentrations are 180, 90, and 45 mg/dl.

IgM[28]
1. Initial serum dilution for determination of IgM is 100-fold, i.e., 0.10 ml serum diluted with 10.0 ml serum diluted with 10.0 ml saline.
2. Procedure for measuring IgM is identical to that of IgG. Standard curve is generated from dilution of reference serum such that IgM concentrations are 220, 110, and 55 mg/dl.

Remarks
1. Stock and diluted antiserum should be stored at 2-5 C.
2. Diluted antiserum is stable for 2 wk when stored as directed.
3. Precipitin curves should be constructed for each new batch of antiserum in order to ensure that measurements are in zone of antibody excess.

Automated immunochemical determination of serum proteins

Automated immunochemical technics have been developed that allow the quantitation of several serum proteins such as orosomucoid, α_1-antitrypsin, α_2-macroglobulin, haptoglobin, transferrin, C3, C4, IgG, IgA, and IgM.

Continuous-flow methods[29]

The automated specific protein determination most widely used today is based on the continuous-flow concept coupled with nephelometry for quantitation of the immunogobulin complex. The basic system is simple and involves (1) mixing of the diluted serum sample with diluted monospecific antiserum, (2) incubation at room temperature in a delay coil, (3) measurement of light-scattering in a flow cell mounted in a nephelometer with a recorder tracing of the measurements, (4) measurement of blanks in order to correct for the intrinsic light-scattering of serum samples, and (5) construction of the nonlinear standard curves from appropriate dilutions of the reference serum. The immunochemical reaction is allowed to come to completion before light-scattering measurements are made.

Apparatus

1. An automatic pipet (MicroMedic Systems, Philadelphia, Pa. 19104) is used to make a 100-fold dilution of samples, controls, and standards.
2. A 3-channel continuous-flow system is constructed from parts of the AutoAnalyzer II (Technicon Instruments Corp., Tarrytown, N.Y. 10591). This system is composed of 1 sampler, 1 proportioning pump, 3 modified analytical cartridges, 3 fluoronephelometers, 1 dual-pen recorder, and 1 single-pen recorder. The system is designed for 100 analyses per hour with a 1:1 sample-to-wash ratio.
3. More detailed assembly instructions are available from Technicon Instruments Corp., Tarrytown, N.Y. 10591.

Reagents

1. Monospecific antisera to orosomucoid, α_1-antitrypsin, α_2-macroglobulin, haptoglobin, transferrin, C3, C4, IgG, IgA, and IgM are available commercially (Technicon Instruments Corp., Tarrytown, N.Y. 10591; Atlantic Antibodies, Westbrook, Me. 04092).
2. Antisera are diluted appropriately before use with physiologic saline (9 g/L) containing 40 g/L polyethylene glycol 6000 (Fisher Scientific Co., Fair Lawn, N.Y. 07410).
3. Standards. Appropriate dilutions of reference serum (Technicon Instruments Corp., Tarrytown, N.Y. 10591) are used to standardize system.

Procedure

1. Dilute all standards, samples, and controls 1:100 with physiologic saline (9 g/L) using automatic pipet.
2. Determine blank values by aspirating samples into continuous-flow system using physiologic saline containing polyethylene glycol 6000 as reagent.
3. Transfer 3 antiserum lines to appropriate antisera solutions and aspirate samples. Reaction values are recorded. In this manner proteins may be quantitated in groups of 3.

Calculations. Data reduction can be carried out by a minicomputer or programmable calculator programmed to do linear interpolation between points making up the standard curve, subtract blank values, and print concentration of controls as well as unknown. These operations may be carried out manually.

Remarks

1. The system utilizes only 580 µl of the 1:100 dilution of the serum sample for the blank as well as for the 9 serum protein determinations.
2. Precision (95% limit) for the 9 serum proteins ranges from 2.5-12% with 9 of the 10 proteins less than 5%.
3. The polyethylene glycol 6000 is added to enhance the rate of reaction.[30,31]
4. Antigen excess samples produce a doublet instead of a symmetrical peak. This doublet is a consequence of the continuous-flow technic with its unique dilutions and mixing systems and makes the identification of antigen excess samples simple.

Centrifugal fast analyzer methods[26,32]

The centrifugal fast analyzer can be modified for near front surface light-scattering using a helium-neon laser light source. The laser is mounted in place of the conventional light source and a quartz disk with a dark-field is placed behind the cuvette assembly. The laser beam passes through the cuvette scattering light from antigen-antibody complexes, and the incident beam then strikes the dark-field, which prevents its detection by the photomultiplier tube. The scattered light is detected through the quartz disk around the dark-field.

The methods described in this chapter are kinetic and obviate the use of a serum blank. Polyethylene glycol is employed to speed up the reaction rate and enable analyses to be completed in only a few minutes.[31]

Apparatus

1. Kinetic light-scattering measurements are made with a laser-modified Aminco Rotochem II centrifugal fast analyzer (American Instrument Co., Silver Spring, Md. 20910).
2. Data processing is accomplished by the PDP 8M computer of the Rotochem II.

Reagents

1. Antiserum. Antihuman IgG, IgA, and IgM are obtained from a commercial source (Technicon Instruments Corp., Tarrytown, N.Y. 10591). A 1:20 dilution of each antiserum in phosphate buffer saline containing polyethylene glycol is used.
2. Buffer solutions. Phosphate-buffered physiologic saline (PBS), (pH 7.4, 10 mM in phosphate, 0.15M in NaCl) is prepared by dissolving 1.18 g of Na_2HPO_4, 233 mg of NaH_2PO_4, and 9.0 g NaCl in 1 L distilled deionized water.
3. Polyethylene glycol solution (PEG-PBS). Solutions of polyethylene glycol (MW 6000-7500) are prepared by dissolving 40 g polymer in 1 L PBS.
4. Standards. Four different dilutions of commercially available reference serum (Technicon Instruments Corp., Tarrytown, N.Y. 10591) spanning the normal range are employed for standardization.

Procedure

IgG

1. Dilute samples and standards 1:100 with PBS-PEG.
2. Transfer 250 µl diluted antihuman IgG to reagent wells of transfer disk, and 50 µl diluted standard or

sample plus 200 μl PBS-PEG diluent to sample wells.

3. On command from the computer, the rotor containing transfer disk is accelerated and reactants are mixed. After initial mixing, solutions are again mixed for 0.5 s, 5 s prior to each reading (first reading at 15 s, second reading at 65 s).

IgA. The procedure for IgA differs from that described for IgG in the initial sample dilution (1:40) and the fixed-time interval chosen (15 s and 115 s).

IgM. Initial sample dilution (1:15) is made in physiologic saline. Diluted antiserum, diluent, and sample volumes are the same as those used for the measurement of IgG. The fixed-time interval chosen for analysis is 170 s (15 s and 185 s).

Calculations

1. The difference between the relative intensity of the first and second readings may be plotted as a standard curve versus concentration of standards. Unknowns may be determined based on the standard curve. As in most immunochemical determinations, this curve is nonlinear.
2. Alternatively, data reduction may be accomplished by a computer programmed to do linear interpolation based on standard curve.

Remarks

1. The major advantages of the kinetic method are the speed of measurement, elimination of blank measurements, and ease of data reduction.
2. Precision (95% limits) for IgG, IgA, and IgM is 5%, 4%, and 9%, respectively, for samples within the normal range.
3. Antigen excess samples must be detected using a 1:1 dilution of the sample (see manual methods), although screening of agarose electrophoresis gels serves as an adequate means of detecting antigen excess.

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