

“Rocket” Immunoelectrophoresis Assay of Vitamin D-Binding Protein (Gc Globulin) in Human Serum

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The anodic properties of the binding protein for vitamin D and its metabolites in human serum prompted us to develop a “rocket” immunoelectrophoresis assay for this alpha globulin. Only 0.2 mL of antiserum is required for assay of 72 samples in 5-μL wells on two plates. The assay is sensitive (0.1 μg) and precise (intra-assay CV 1.4%; inter-assay CV 7.9%), and permanently recorded assay plates can be produced in 24 h. These features are attractive for the convenient measurement of this protein, which is important in anti-rachitic steroid transport.

The binding protein for vitamin D and its metabolites (DBP) in plasma is now recognized to be identical to the plasma group-specific component (Gc) (1–4). A 58 000-dalton alpha globulin, binds anti-rachitic sterols with mole per mole stoichiometry (2–4), but circulates predominantly in the apo form (5). Absence of this protein is believed to be a lethal mutation, and genetic variations, inducing co-dominant alleles, are recognized (6). Quantitation of plasma DBP has received attention recently, and radial immunodiffusion (7–9) and radioimmunossay (5) methods have been reported. The concentration of DBP in plasma is normally approximately 6 μmol/L, so immunoprecipitation techniques are suitable for small plasma volumes. Although we have achieved good results with a radial immunodiffusion assay (10), the anodic property of DBP suggested we could develop a more precise, rapid, and reproducible assay by using the technique of “rocket” immunoelectrophoresis (11).

Materials and Methods

Materials

Pure DBP was prepared from Cohn Fraction IV of human plasma as previously described (2), and monospecific antisera to it were raised in rabbits as reported (5). Alternatively, anti-Gc antisera is available from Calbiochem-Behring Co., La Jolla, CA 92112, or from Dako Corp., Santa Barbara, CA 93103.

Agarose, type III (high electrodosmosis), and Tris (Trizma) base were from Sigma Chemical Co., St. Louis, MO 63178. Diethyl barbituric acid (barbital) was from Fisher Scientific Co., Fair Lawn, NJ 07410, and Coomassie Blue R250 was from Miles Laboratories, Inc., Elkhart, IN 46514.

For electrophoresis we used a Model 1415 electrophoresis cell with electrode/buffer chambers (Bio-Rad Laboratories, Richmond, CA 94804) and a dc power supply (Buchler Instruments, Inc., Fort Lee, NJ 07024). Glass plates (200 × 100 × 3 mm) were cut locally, and solvent saturation pads (Gelman Sciences, Inc., Ann Arbor, MI 48106) were used as electrode wicks. A plate-leveling apparatus was purchased from Hoeffer Scientific Instruments, San Francisco, CA 94107, and wells were cut into the agarose with a 2-mm (o.d.) gel punch (LKB Instruments, Inc., Rockville, MD 20852). We used Plexiglas template with 40 perforations, 5 mm apart, made for us by Graham Morris, MRC Dunn Nutritional Laboratory, Cambridge, CB4 1XJ England.

Barbital, 25 mmol/L in Tris, 75 mmol/L, pH 8.6, was the buffer used in preparing gels, in the electrophoresis, and for dilutions of reference DBP and serum. Gels were stained in a solution of Coomassie Blue R250, 250 mg/L, in glass-distilled water/isopropanol/acetic acid (65/25/10 by vol).

Methods

A 10 g/L solution of agarose was prepared in the Tris–barbital buffer by heating until clear, and then cooled to 55 °C. An appropriate volume of antiserum (0.1–0.3 mL) was stirred into 30 mL of the agarose solution, and the solution was poured onto an ethanol-washed, pre-warmed (55 °C) glass plate that had been leveled. After the gel formed, the template was moved over the plate and wells were cut with the vacuum-connected gel punch positioned 20 mm from the long edge of the plate. Allowing for 10-mm margins, 36 wells were made.

The plate was then placed into the tank, with the wells nearest the cathode. Pre-moistened wicks were gently pressed onto the 10-mm margins at the long edge of the plate, and the wick edges were saturated with buffer from a Pasteur pipette.

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to ensure a visibly continuous contact for the entire wick. Power was initiated and adjusted to provide a current of 20 mA/plate; then 4-μL samples were applied from a Hamilton syringe. The tank was closed, and the electrophoresis was allowed to proceed overnight (~15 h). We routinely ran two plates in the chamber. Thus, allowing for duplicate sets of standards per plate, 56 test samples could be accommodated.

After the run, the plate was covered with a wet (distilled water) filter paper cut to the same size as the plate, after filling the wells with distilled water to prevent air trapping and gel rupture. Twenty additional dry filter sheets were placed over the wet sheet, and uniform pressure (20 g/cm² or 10 lb/plate) was applied for 15 min. The gel plate was then dried in an oven at 55 °C, and transferred to an immersion tank containing the Coomassie Blue stain solution for 5–10 min. The stained gel was washed in hot soapy water and rinsed in tap water. (Destaining can be made more rapid by adding ethanol to the wash.) Finally, the plate was dried in an oven at 55 °C.

Sera from normal, cirrhotic, and pregnant subjects were analyzed and compared to known amounts of pure DBP with respect to rocket height × mid-height width. Intra-assay variation was determined. Double determinations of separate samples in two different assay were analyzed for variation (Sa) according to the formula (12):

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Sa = \sqrt{\frac{\sum d^2}{2n}}
\]

where \( d \) signifies the difference between two single determinations on the same sample, and \( n \) signifies the number of double determinations.

**Results**

Single rocket precipitates were observed with either the high-titer (0.1 mL per plate) or lower-titer (0.3 mL per plate) antiserum. With 0.1 to 0.4 μg of DBP, 10- to 40-mm rockets were produced (Figure 1). At normal concentrations of DBP, the serum can be diluted five- to 20-fold to provide 0.2 to 0.8 μL in the 4 μL assay volume used. This range usually encompasses most serum values and provides a convenient schedule.

By minimizing diffusion during sample application, we observed little difference in rocket height (\( r = 0.96 \) to 0.99) or rocket height × width (\( r = 0.99 \) or greater) in correlating rocket precipitates to antigen amounts.

Intra-assay variation was studied for low and high rocket heights. As indicated in Table 1, the CV for sera containing approximately 300 μg of DBP per liter (A and B) was 1.4%. At lower concentrations (C), a CV of 4.1% was observed.

Inter-assay variation was studied with 10 different sera in two separate assays, including the dilution step. The analytical CV was 7.9%.

Table 2 shows the concentration of DBP in sera from normal male and female subjects. Normal values were lower than those observed in sera from pregnant women and higher than those observed in sera from patients with liver disease.

**Discussion**

This method provides a convenient and reproducible quantitation of serum DBP. In 24 h, 56 samples can be measured and a permanent record of the assay obtained. In practice, duplicates are not necessary because they are almost always identical. Because rocket peak height correlated almost as well with the amount of DBP as did height × mid-height width, rocket height is now routinely used. In unknown samples, use of replicates at various dilutions ensures that the immunoprecipitation measurement will fall within the standard curve.

Compared with our radial immunodiffusion assay (10), we can reliably measure smaller amounts of DBP with less antiserum. In practice, this permits a more efficient utilization of antiserum. Precision and reproducibility appear to be comparable with others’ experience (12), and suitable for measurement of this serum protein.

The values we observed with this technique compare well with those obtained with radial immunodiffusion techniques (7–9, 13), but are slightly lower than those found by radioimmunoassay (5). The latter method, although sensitive to 1–2 ng amounts of DBP, requires a great dilution of sera, and this may account for the differences. All methods, however, have provided data consistent with the relative values shown in Table 2.

Recently, the vitamin D sterols have received increasing attention with the availability of assays to measure these compounds in human sera (14). The interpretation of these assays may be enhanced by the concurrent measurement of serum D-sterol binding capacity or serum DBP (15, 16).
seems reasonable to expect that increasing attention will be paid to methods for quantitation of serum DBP.

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References