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A Homologous Radioimmunoassay for Guinea Pig Corticosteroid-Binding Globulin[1] (42540)

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Abstract. A rapid, specific, and sensitive (requiring only 20 fmole of antigen equivalent to 0.007 μl of serum) radioimmunoassay (RIA) was developed for the measurement of guinea pig corticosteroid-binding globulin (CBG). CBG was purified to homogeneity from guinea pig serum by affinity chromatography and used for immunization, as the standard and as the radiolabeled trace in the RIA. The antiserum to CBG was raised in rabbits. It was judged specific by immunoelectrophoresis and by comparison of RIA values with steroid-binding assay profiles obtained on serum separated on the basis of size and ion-exchange properties. The results of the radioimmunoassays agree with those of a steroid-binding assay run on identical samples. The sensitivity of the assay allows detection of CBG in serial serum samples, other biologic fluids such as milk, and cell culture supernatants.

Corticosteroid-binding globulin (CBG) is a plasma glycoprotein which binds corticosteroids and progestins with high affinity. Numerous techniques have been employed to quantitate the levels of CBG in plasma. These include equilibrium (1, 2) and multiple equilibrium dialysis (3), competitive protein binding (4), gel filtration (5, 6), and immunological techniques (7, 8). The development of radioimmunoassays for human (9, 10) and rat (11) CBG has been described.

The guinea pig represents an interesting system for the study of the role and regulation of corticosteroid-binding globulin. Like the human but unlike the rat, the cellular immune system of the guinea pig is relatively insensitive to moderate levels of glucocorticoid and the animal is considered to be corticoresistant. In addition, the circulating levels of CBG increase significantly during pregnancy in both the human and the guinea pig (corticoresistant species) while little or no change is observed in the rat (a corticosensitive species). The possible role of CBG in determining the degree of corticosensitivity remains to be determined. Because of their similar degrees of sensitivity, the guinea pig represents an excellent model system for studying the characteristics of the human system. To conduct such studies, a sensitive and specific assay system for the determination of CBG is required. All current RIAs for CBG lack species cross-reactivity and preclude the use of a heterologous assay system. We now report the development of a homologous radioimmunoassay for the accurate measurement of CBG in as little as 0.007 μl of serum.

Methods and Materials. All chemicals were reagent grade and obtained from J. T. Baker (Phillipsburg, NJ), except Tris which was from Sigma Chemical Co. (St. Louis, MO) and all electrophoretic quality chemicals which were from Bio-Rad (Richmond, CA). Sephacryl S-200 was from Pharmacia Fine Chemicals (Piscataway, NJ). Tritiated cortisol (97 Ci/mmole) and $Na^{125}I$ (100 mCi/ml) were obtained from New England Nuclear Corp. (Boston, MA) while all nonradioactive steroids were from Steraloids (Wilton, NH).

Third trimester pregnant albino guinea pigs were obtained from a local supplier and were housed in the university animal care facilities throughout the study.

Isolation of guinea pig CBG. Guinea pig CBG was isolated from serum obtained during the last third of pregnancy essentially as previously described for the isolation of rat CBG (11). The purity of the isolated CBG was demonstrated by the presence of a single band after electrophoresis under both native (12) and denaturing conditions (13).

Iodination of CBG. Guinea pig CBG was iodinated by a variation of the methods of

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Franker and Speck (14) and Markwell and Fox (15). In a 10 × 75-mm test tube were mixed guinea pig CBG (5×10^{-10} mole), KI (1.8 μl of a 1 mg/100 ml 0.125 *M* sodium borate buffer, pH 8.2, containing 0.15 *M* NaCl), 400 μCi $Na^{125}I$ (100 mCi/ml), and sufficient borate buffer to bring the final volume to 100 μl. The contents were cooled to 4°C and transferred to a second cold tube containing 200 μl of 2 mg/100 ml Iodogen in dichloromethane. The dichloromethane was removed subsequently by a stream of nitrogen. The iodination reaction was allowed to proceed at 4°C for 10 min with frequent agitation. Covalently bound ^{125}I was separated from free iodine on a column (1 × 45 cm) of Sephadex G-100 previously equilibrated with PBS (0.01 *M* sodium phosphate containing 0.15 *M* NaCl, pH 7.4). Incorporation of iodine ranged from 86 to 95%, yielding sp act 1.2–1.6 × 10^6 Ci/mole.

Sephacryl S-200 chromatography. Samples (0.5 ml) of whey, plasma, or trace were applied to a column (1.6 × 90 cm) of Sephacryl S-200 equilibrated with 0.01 *M* Tris buffer, pH 7.4, containing 0.3 *M* KCl. The column was developed with the same buffer at a constant flow rate of 6 ml/hr (maintained with a Technicon autoanalyzer pump, Technicon Instrument Co., San Francisco, CA). Dextran blue and [^{14}C]valine were added to each sample before chromatography in order to determine the void volume and internal volume respectively. Fractions (1 ml) were collected and analyzed for protein and radioactivity.

Anion-exchange chromatography. Samples (0.5 ml) of plasma were dialyzed overnight against 0.01 *M* Tris, pH 7.4, and were applied to a column (1 × 5 cm) of DEAE–cellulose equilibrated with the same buffer. After washing with 6–9 column vol of the starting buffer, the column was developed with a linear salt gradient to 0.3 *M* KCl. Fractions (2 ml) were collected and analyzed for conductivity, protein concentration, and radioactivity.

Nondenaturing polyacrylamide electrophoresis. Electrophoresis was carried out in 7.5% acrylamide gels at pH 8.3 as described by Ornstein (16) and Davis (12). Gels were electrophoresed at 4 mA/tube until the bromphenol blue marker was within 0.5 cm of the bottom of the gel tube. The gels were removed and stained with Coomassie brilliant blue.

Sodium dodecyl sulfate (SDS) gel electrophoresis. The procedure of Weber and Osborne (13) was used with the following modifications. Prior to electrophoresis, the protein samples were boiled for 5 min and then incubated for 2 hr at 37°C in 0.01 *M* sodium phosphate (pH 7.0) containing 1% SDS and 1% β-mercaptoethanol. Instead of the original gel buffer, a buffer composed of 1.45 g of $NaH_2PO_4 \cdot H_2O$, 9.66 g of $Na_2HPO_4 \cdot 7H_2O$, and 2 g of SDS diluted to 1 liter with distilled water was used. Following electrophoresis at 6 mA/tube, the gels were removed, severed at the ion front, and stained with Coomassie brilliant blue.

Steroid-binding assays. Steroid-binding assays were performed as previously described by Raymoure and Kuhn (19).

CBG standard concentration. A pool of four preparations of guinea pig CBG, stored in 250-μl aliquots at −70°C until use, served as the standard in the RIA. A known amount of norleucine was added to a sample of this pool to serve as a standard for volume determination. Aliquots were hydrolyzed *in vacuo* at 110°C for 24 hr and amino acid analysis was performed on a Beckman Model 120C. The amount of norleucine present in each analysis was used to determine the volume of the standard CBG solution which was analyzed. The concentration of CBG in the pool was determined in two ways. First, the total weight of protein present in each determination was calculated from the amino acid analysis, and the molarity of the standard solution was then derived by using the known molecular weight of the protein (44,000) (20). Second, from the previously determined amino acid composition of pure guinea pig CBG (20) and the amount of alanine present in each analysis, it was possible to calculate the concentration of CBG in the standard pool. The two methods gave essentially identical results and revealed the concentration of the CBG in the standard pool to be 0.44 mg/ml.

Antiserum production. Intact, adult male New Zealand White rabbits were used to produce antiserum (21). Purified guinea pig CBG (300 μg in 1 ml 0.15 *M* NaCl) was emulsified with 1 ml Freund's complete adjuvant (GIBCO, Grand Island, NY) and injected into multiple intradermal sites on the backs of two rabbits. Half of the above amount of CBG was diluted in normal saline and emulsified in Freund's incomplete adjuvant (GIBCO) and used for booster injections given in both in-

tradermal and intramuscular sites. Blood was collected from the marginal ear veins at intervals of 1 week after each boost, allowed to clot, and centrifuged. The serum was collected and stored frozen at −70°C until use.

Immunoelectrophoresis. Grabar–Williams type immunoelectrophoresis (22) was performed in a Bio-Rad 1415 electrophoresis cell in gels of 1% agarose in Tris-buffered tricine (0.025 *M* tricine, 0.08 *M* Tris, 0.001 *M* calcium lactate, pH 8.6). Gel size was 1.5 × 100 × 100 mm. Anode and cathode tanks also contained Tris-buffered tricine. Samples were diluted 1:2 in 0.01% bromphenol blue in the same buffer, placed in 2.5-mm wells cut with a Bio-Rad template, and electrophoresed at 10 V/cm. After overnight incubation with antiserum in the troughs, gels were washed in distilled water, pressed, and stained (0.5% Coomassie brilliant blue R-250, 45% ethanol, and 10% glacial acetic acid in distilled water). The gels were destained with a solution of 45% ethanol, 10% glacial acetic acid, and 45% distilled water.

RIA procedures. Serum samples were diluted 1:1000 in PBS containing 1% BSA. Usually, 20 or 80 μl of diluted sample, 80 or 20 μl PBS/BSA, 100 μl ^{125}I-labeled guinea pig CBG (50,000–75,000 cpm in PBS/BSA) containing 51 μg rabbit IgG, and 200 μl diluted (1:10,000) rabbit anti-guinea pig CBG were mixed together and allowed to incubate 18 hr at 4°C. Goat anti-rabbit IgG (150 μl of a 1:10 dilution in PBS/BSA) was added and the mixture incubated for 1 hr at room temperature. Polyethylene glycol (150 μl of a 6% solution of PEG 6000 in PBS) was added and the solution was incubated at room temperature for an additional 30 min. The tubes were then centrifuged at 3000*g* for 30 min in a Beckman J-6B centrifuge. The supernatants were decanted and the pellets were counted for ^{125}I in a Searle (Chicago, IL) gamma-counter at 87% efficiency. The assay results were analyzed on a variation of the RIA analysis program developed by David Rodbard at the NIH, written by M. R. Radisich for the HP 9830A (Hewlett–Packard, Palo Alto, CA), and edited by M. C. Aubert.

Collection of milk and plasma. Milk was collected from lactating guinea pigs using the milking device described by Gupton *et al.* (17). Milk samples were centrifuged for 1 hr at 145,000*g*, and the whey was carefully removed with a Pasteur pipet. Blood from the footpads of guinea pigs was collected in heparinized capillary tubes according to the procedure of Lopez and Navia (18). The blood was centrifuged in 6 × 50-mm Pyrex tubes for 5 min at 12,000*g* in a Brinkman Model 5412 microfuge (Brinkman Instruments, Westbury, NY). The plasma was removed and stored frozen at −20°C until use. In some instances where larger volumes of serum were required, blood was collected from decapitated animals that had been anesthetized with ether. The blood was allowed to clot for 1 hr at room temperature and incubated for 4 hr at 4°C, and the serum was separated by centrifugation for 20 min at 15,000*g*.

Results. *Evaluation of the antiserum.* The antiserum raised against pure guinea pig CBG was examined by immunoelectrophoresis and by comparison of the elution patterns of CBG as detected by RIA or steroid-binding assays following chromatographic separation of serum on Sephacryl S-200 or DEAE–cellulose. These chromatographic procedures were chosen because neither of these was involved in the purification of the CBG used as standard and iodinated tracer in the RIA.

Graber–Williams type immunoelectrophoresis (22) (Fig. 1) demonstrated a single precipitin arc between the purified CBG and antiguinea pig serum (Lane 2 vs trough A). In addition, a single arc was observed between the anti-CBG serum and either purified CBG (Lane 6 vs troughs E and F) or whole guinea pig serum (Lane 4 vs trough D). These results confirm the purity of the isolated CBG. Significantly, no precipitin line was observed between the antiserum and purified guinea pig albumin (Lane 5 vs troughs D and E). The results suggest that the antiserum is directed against only one component of the guinea pig serum, CBG. To examine the size of the antigen recognized by the antibody, pregnant guinea pig serum was passed through a Sephacryl S-200 column. Fractions (1 ml) were collected and analyzed for protein and CBG content by both RIA and steroid-binding methods. Figure 2 shows that the CBG content profiles obtained by steroid-binding and RIA assays follow identical patterns. Thus the antigen detected by the antibody is the same size (60,000 Da) as the molecule responsible for the binding of ^{3}H-cortisol. Identical results were obtained when either male or nonpreg-

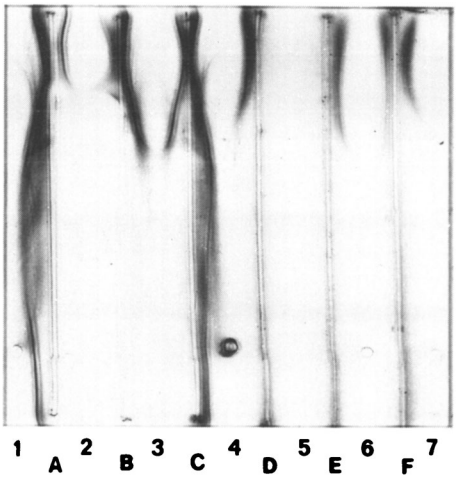

FIG. 1. Characterization of antiserum by Grabar–Williams type immunoelectrophoresis. Immunoelectrophoresis was performed as described under Materials and Methods. The samples examined were as follows: whole guinea pig serum, Lanes 1, 4, and 7; purified guinea pig CBG, Lanes 2 and 6; purified guinea pig albumin, Lanes 3 and 5. The antiserum troughs contained either antiwhole guinea pig serum (troughs A–C) or anti-guinea pig CBG (troughs D–F).

nant female guinea pig serum was analyzed by this method.

The results of the chromatography of pregnant guinea pig serum over the anion-exchange resin, DEAE–cellulose, are shown in Fig. 3. After elution with a linear salt gradient, a single peak of cortisol-binding activity was detected. When the fractions were analyzed by RIA, a single peak corresponding to that detected by steroid binding was observed. This suggests that the antigen detected by our antibody had charge characteristics similar or identical to those of the molecule which was responsible for cortisol-binding activity. Identical results were obtained when either normal female or male sera were analyzed in a similar manner.

The results of these three procedures strongly suggest that the antibody is directed against a single component of guinea pig serum which is identical in properties to CBG as determined by steroid-binding techniques.

Evaluation of RIA. A typical standard curve is depicted in Fig. 4. Data were transformed to log–logit plots and subjected to weighted least-squares analysis by computer (see Materials and Methods). In a typical experiment, the amount of CBG required to reduce B/B_0 to 0.9 was 20 fmole of CBG per tube corresponding to the amount of CBG present in 6.7 nl of normal guinea pig serum or 2.1 nl of pregnant guinea pig serum. The bound/free ratio ($B/B_{0\,max}$) was kept between 30 and 50% in all assays and the mean nonspecific binding was 0.9% of the total binding.

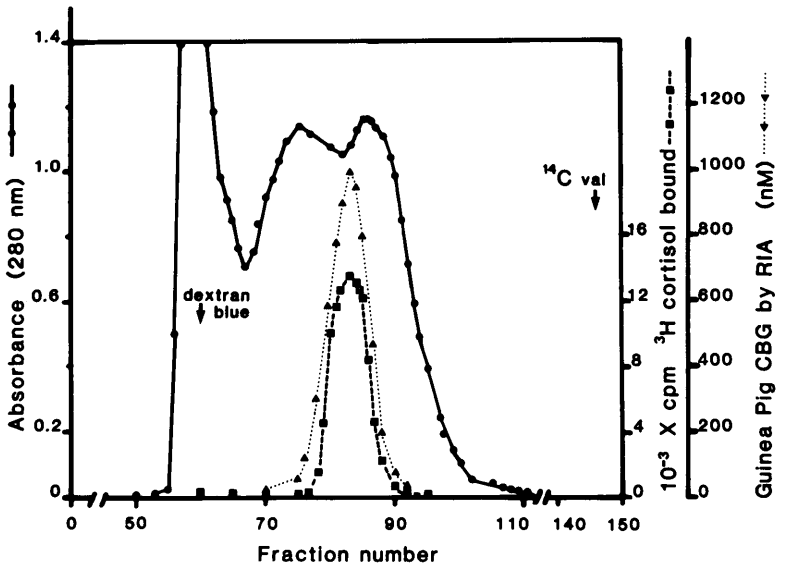

FIG. 2. Sephacryl S-200 chromatography of pregnant guinea pig serum. Serum was separated as described under Materials and Methods and analyzed for CBG content by both RIA and steroid-binding assays.

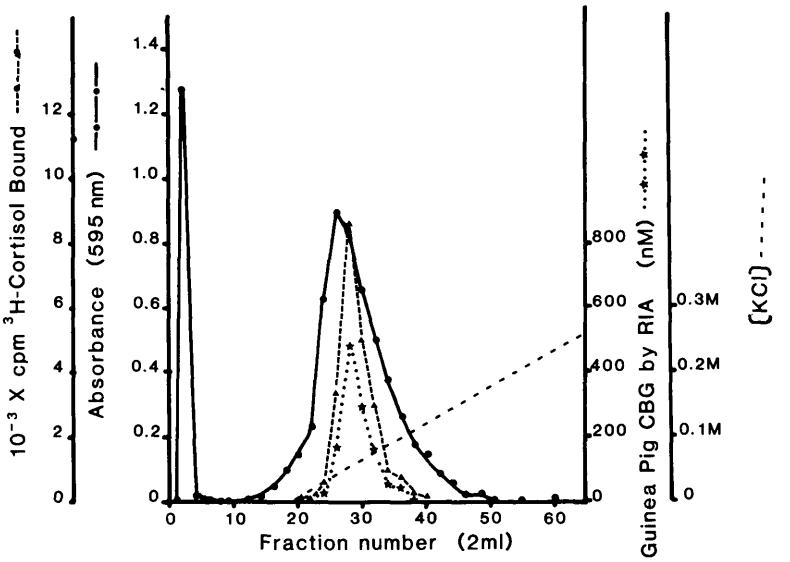

FIG. 3. Analysis of pregnant guinea pig serum chromatographed over DEAE-cellulose. Serum (0.5 ml) as separated by chromatography on a 1 × 5-cm column of DEAE-cellulose. Fractions (2 ml) were collected and the content of CBG was determined by both RIA and steroid-binding assays.

The reproducibility of the assay was determined by measuring the quantity of CBG in a pregnant and normal serum pool sample six times in each of 10 separate assays. The within-assay variation was 8% while the interassay variability was 12.4%.

The ability to measure a known amount of pure guinea pig CBG added to an unknown serum sample (cold recovery) was determined following the addition of 166 fmole of CBG standard to each of five sets of duplicate tubes with varying amounts of diluted sample (to achieve a B/B_0 range of 35–70%). The mean recovery was 177 fmole; SD = 33.3 fmole.

Since previous methods of guinea pig CBG measurement have relied primarily on the quantitation of steroid binding, a comparison of the values obtained by RIA with those from a steroid-binding assay on identical samples covering a wide range of CBG values was undertaken. The values obtained were in excellent agreement with a correlation coefficient of 0.935.

To ensure that there was no difference in immunological reactivity between the CBG–corticosterone complex and steroid-free CBG, samples were treated with charcoal to remove any steroid and their RIA values were compared to those obtained prior to steroid removal. There was no measurable difference between the two suggesting that the antibody does not differentiate between the CBG–steroid complex and free CBG.

In the interest of both the degree of immunologic homology and the possibility of using the assay to measure CBG in other species, the cross-reactivity of sera from other species in the RIA was examined. As can be seen in Fig. 4, the competition curves for guinea pig serum and the standard were parallel. Competition curves for other competing sera showed either no competition (sheep, turtle, and chicken) or only a small amount of nonparallel competition (human, rat, dog, and rhesus monkey). The degree of any competition was very slight and less than 0.005% as a maximum.

The RIA was used to examine CBG levels in normal male, female, and pregnant sera and in whey collected from lactating guinea pigs at varying times after parturition. The mean value for 10 male serum samples was 1037 ± 101 n*M* while that for normal female sera was 1265 ± 285 n*M* (n = 10). The value obtained for 12 samples from the last third of pregnancy was 5958 ± 373 n*M* confirming the considerable increase in CBG levels which accompanies pregnancy in this species. The values measured in the whey samples varied considerably with the time after parturition. The

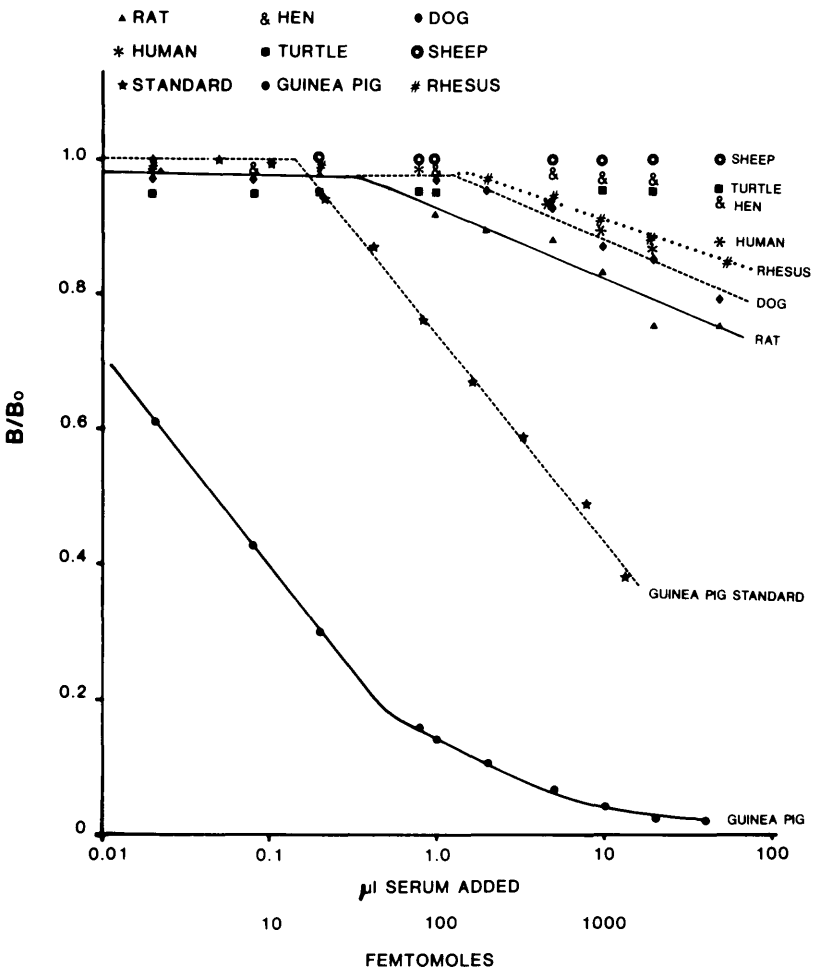

FIG. 4. A typical RIA dose–response curve and analysis of species cross-reactivity. Samples of standard, guinea pig serum, or various amounts of sera from other species were analyzed in the RIA. Data were transformed by a log–logit plot and subjected to weighted least-squares regression analysis by computer as described under Materials and Methods.

highest levels were found in those samples collected shortly after parturition with decreasing values being observed as time increased after parturition. In each case, the whey samples were about 10% of the corresponding serum samples. These results confirm our previous observations using a steroid-binding assay (19).

Discussion. The assay described is rapid, sensitive, reproducible, and highly specific for guinea pig CBG. The antiserum used was found to be reactive against only CBG in serum and to be highly species specific. When compared with results obtained by a steroid-binding assay, there was consistently good agreement. The RIA, however, is free from the potential interference caused by the binding of steroids by other plasma proteins (such as albumin) and the presence of competing endogenous steroids in the sample. Both of these would be expected to interfere with steroid-binding assays. While charcoal treatment can be used to remove endogenous steroids, loss of binding activity can occur particularly with samples which have low protein concentrations. Utilizing the RIA procedure, identical results are obtained in the absence or presence of steroid. In addition, antibody–antigen binding is not as severely pH sensitive as is steroid binding (3, 23, 24) nor is it as heat sensitive. Furthermore, the RIA may be useful in detecting aggregates, nonbinding populations, or varied populations of CBG present

during different physiologic states when used in conjunction with the steroid-binding assay.

The assay described requires much less than a microliter of serum to determine CBG values although in practice at least 1 μl to be diluted 1:1000 is required. This is considerably less serum than is required for the steroid-binding assay (5–10 μl) (19, 25) and even less than required in the radioimmunodiffusion method described by Goodman *et al.* (8). The small volumes required mean that this assay will be extremely useful for studies requiring sequential sampling or for fetal blood samples where only a limited amount of blood can be taken. It will also prove to be extremely useful for assaying the low concentrations of CBG found in the media of liver cultures and some other biological fluids.

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