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QUANTITATION OF CORTICOSTEROID BINDING GLOBULIN (CBG) BY STEADY STATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SS-PAGE)

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SUMMARY

A method suitable for quantitating corticosteroid binding globulin (CBG) in rat plasma has been developed using steady state polyacrylamide gel electrophoresis (SS-PAGE). The method is sensitive, specific and very precise. Using this technique CBG levels have been determined in immature and mature male and female plasma as well as in pregnant female plasma.

INTRODUCTION

A variety of methods have been designed in order to study specific steroid binding proteins in serum, including equilibrium dialysis[1], Sephadex gel equilibration[2], charcoal absorption[3, 4] and ammonium sulphate precipitation[5]. A major drawback of these methods is that they all quantitate the total specific binding of a particular steroid, and/or the binding assay is performed at non-equilibrium conditions. Recently we have developed a method using polyacrylamide gel electrophoresis to quantitate testicular androgen binding protein (ABP)[6]. By including radioactive hormone into the gels before polymerization, it is possible to quantitate the binding capacity of this protein at steady state conditions. Steady state polyacrylamide gel electrophoresis (SS-PAGE) can be used for any problem dealing with ligand–protein interactions. The only requirements are that the radioactive trace does not have a mobility of its own in the electrophoretic system used, and that it does not interact with the polyacrylamide gel matrix[6].

In the present paper an assay is described for quantitating the binding capacity of corticosteroid binding globulin (CBG) in rat plasma, utilising the SS-PAGE technique. Moreover additional information regarding the technique is presented to render it applicable to the measurement of any specific steroid binding protein.

We believe that SS-PAGE can compete with any other technique presently available on the basis of practicability and precision. In relation to specificity, the SS-PAGE method is far superior to any other technique available.

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MATERIALS AND METHODS

Radioactive cortisol (S.A. 44 Ci/mmol) was purchased from New England Nuclear Corporation. Acrylamide and N,N^1-methylene-bis-acrylamide was obtained from Eastman Kodak Company.

Preparation of samples. Blood from male and female rats was obtained after decapitation and put into centrifuge tubes containing 50 μl of heparin. Plasma was prepared by centrifugation at 800 g for 15 min at 4°. The plasma samples were analysed by polyacrylamide gel electrophoresis after dilution in 10 mM Tris–HCl buffer containing 1.0 mM EDTA and 10% glycerol (v/v) (TEG), pH 7.4. The samples were kept at −70° until the day of analysis.

Determination of optimal plasma dilution. Pooled rat plasma was diluted 1:10, 1:20, 1:40, and 1:80 (v/v) in TEG buffer. Duplicate samples from each dilution were run into polyacrylamide gels containing 4 nM [^{3}H]-cortisol alone or 4 nM [^{3}H]-cortisol and a 1000 fold excess of unlabeled cortisol. The addition of the cold cortisol completely suppresses binding of [^{3}H]-cortisol to CBG, but does not significantly effect cortisol binding by albumin.

Removal of endogenous steroids. Endogenous corticosterone in rat plasma can be removed by absorption with dry charcoal (1 mg/mg of protein) for 6–16 h at 0°. However, at high plasma dilutions (1/40 or 1/80), and when 15–25 nM of [^{3}H]-cortisol was added to the stacking gel, it was not necessary to remove endogenous steroids. Quantitation performed with and without charcoal absorption gave identical results.

Polyacrylamide gel electrophoresis. Polyacrylamide gels containing $6\frac{1}{2}$% (w/v) acrylamide and 0.2% bisacrylamide (w/v) were prepared as described by Davies[7], with some modifications[6]. Glycerol (10%) was added to the gel solution before polymerisation in order to increase the solubility of the ster-

oids. Radioactive and non-radioactive cortisol were also added to the polyacrylamide gel solution before polymerisation, which was initiated by the addition of ammonium persulphate (0.19 g/100 ml) and allowed to proceed overnight at 0–2°. Cortisol concentrations in the acrylamide gel ranged from 1.6 to 16 nM. In the routine assay for CBG 4–5 nM of [^{3}H]-cortisol was used in the separating gel.

After polymerisation of the separating gel, a stacking gel[7] containing 15 nM of [^{3}H]-cortisol was prepared on top of the separating gel. The vol. of the stacking gel was at least twice the vol. of the sample. The gels (8 × 70 mm) were polymerised in round glass tubes and samples of 100–300 μl were layered. The electrophoresis was run at 0° for 4 h at $2\frac{1}{2}$ mAmp/tube until the bromphenol blue marker had come to the end of the gel. Following electrophoresis, the gels were sliced into 2.3 mm segments and placed directly into counting vials containing 5 ml of scintillation fluid (2,5-diphenyl-oxazole, 0.5%, and 1,4-bis [2-(4-methyl-5-phenyloxazolyl)] benzene, 0.005% in toluene). For practical reasons only ten slices (5 on each side of the albumin band) were counted. After standing overnight at room temperature, more than 98% of radioactive steroid in the gel was extracted into the toluene counting fluid. Radioactivity was measured in a Nuclear Chicago Mark II liquid scintillation counter with a counting efficiency of 55%.

Theoretical. The theoretical basis for steady state gel electrophoresis has been published previously[6]. However, some important points should still be mentioned. Steady state between binding sites and radioactive hormone is attained when the rate of association is equal to the rate of dissociation. Such a condition is usually obtained in the classical methods like equilibrium dialysis[1] and batchwise Sephadex gel equilibrium technique[2]. In SS-PAGE, steady state between association and dissociation has been achieved when the level of radioactive hormone in front of the peak equals that behind the peak. A lower level of radioactivity behind the peak indicates that the binding protein has not yet reached the steady state with the free ligand in the gel, and that the association still exceeds dissociation. This may be overcome by running the electrophoresis for a longer time, or preferably by adding a suitable amount (15 nM) of radioactive hormone to the stacking gel so that the binding protein reaches a steady state more rapidly during the electrophoresis.

Certain properties are required of the ligand before it can be used in a SS-PAGE system. It must not have any electrophoretic mobility of its own, and it must not bind significantly to the polyacrylamide gel. If there is binding of the radioactive ligand to the polyacrylamide gel matrix, the partition coefficient[6] between gel and buffer must be measured. [^{3}H]-Cortisol did not bind to the polyacrylamide matrix, and the partition coefficient was 0.998 ± 0.01 (S.D.). Finally it is important that the ligand must not be destroyed or metabolized during the polymerization process. These requirements are all fullfilled in the case of [^{3}H]-cortisol.

At steady state, when the law of mass action is applied:

$$[BP_{tot}] = [S_b]\left(\frac{K_d}{[S_u]} + 1\right) \quad (1)$$

where $[BP_{tot}]$ = concentration of total binding protein, $[S_b]$ = concentration of bound steroid, K_d = equilibrium constant of dissociation, $[S_u]$ = concentration of unbound steroid. From this equation it is obvious that the concentration of binding protein in the sample is proportional to the concentration of the bound steroid. However, the concentration of bound steroid in the gel can never be stated since the vol. of buffer containing the binding protein is unknown due to the unknown width of the protein band. If a given amount of binding protein is distributed within a narrow band, the concentration will be high, compared to the same number of binding sites in a wide band. However, since the bound steroid (S_b) and the total amount of binding protein (BP_{tot}) are identically distributed within the same vol., total amounts in moles may be substituted for molar concentrations in the equation above:

$$BP_{tot} = S_b\left(\frac{K_d}{[S_u]} + 1\right). \quad (2)$$

If K_d is known, the total number of binding sites in the sample can be calculated from equation 2.

In order to determine the K_d, one first has to calculate the total number of binding sites in the sample from Scatchard plot analysis (see later Fig. 3) where identical samples are run into gels containing increasing amounts of radioactive steroid. The ratio of bound to free steroid in the Scatchard analysis in SS-PAGE is only relative, due to the difficulties in determining the actual concentration of binding protein in the gel. Therefore, the slope of the regression line drawn through the various points does not indicate K_d. However, this error in the determination of slope does not influence the intercept at the abscissa which provides a value for the theoretical maximum number of binding sites (BP_{tot}). Knowing the BP_{tot} and measuring the concentration of free steroid in the gel S_u and the mass of bound steroid (S_b) in the CBG peak, K_d can indirectly be arrived at from equation 2. Thus, when K_d is known, the number of binding sites for a specific protein, in this case CBG, may be determined from one SS-PAGE gel.

RESULTS

[^{3}H]-Cortisol binding to CBG

As illustrated in Fig. 1, [^{3}H]-cortisol binds to a protein with a mobility (relative to bromophenol blue; *Rx*). of 0.55. The dotted area within the peak Fig. 1, right) represents specific binding, since an excess of cold steroid added to the gel completely abolishes the binding (Fig. 1; right). The electro-

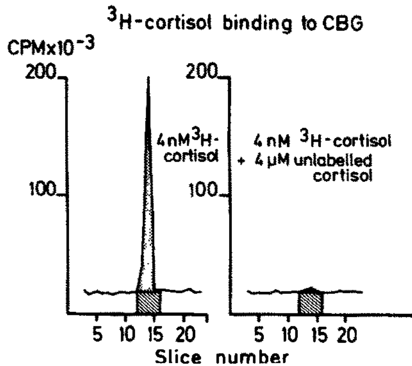

Fig. 1. Specific binding of [^{3}H]-cortisol by CBG analysed by SS-PAGE. Male serum diluted 1:20 (v/v) was run into gels containing 15 nM [^{3}H]-cortisol in the stacking gels and 4 nM [^{3}H]-cortisol in the separating gel. Left: [^{3}H]-cortisol alone (4 nM). Right: [^{3}H]-cortisol + 1000 times excess of unlabelled cortisol. Note; For the Scatchard plot analyses the steroid bound in the peak (▨) is used as S_b, and that under the peak (■) as free (S_u).

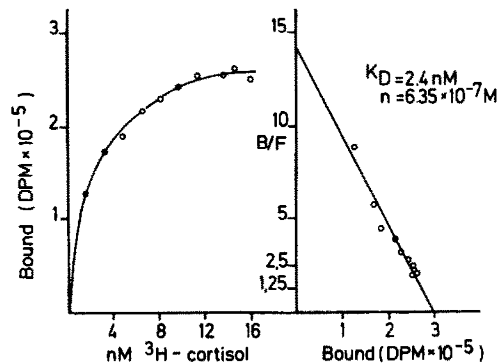

Fig. 3. Saturable binding to CBG. Immature male serum diluted 1:40 (v/v) was run in SS-PAGE with gels containing varying concentrations of [^{3}H]-cortisol (1.6–16.1 nM). The electrophoresis was run as described in Materials and Methods. When radioactivity in the peak was considered as bound, and radioactivity under the peak as free, Scatchard plots could be constructed. Left: Saturation curve. Right: Scatchard plot.

phoresis has in this case been run to equilibrium, since the level of radioactivity in front of the peak equals that behind the peak. The hatched area under the peak represents the free steroid.

Determination of optimal serum dilution

Aliquots of rat plasma were diluted 1:10, 1:20, 1:40 and 1:80 and run into gels containing 4 nM [^{3}H]-cortisol with or without a 1000 fold excess of non-labelled cortisol. As illustrated in the lower curve of Fig. 2, there is negligible binding to non-saturable binding sites in the CBG region of the gel at all the plasma dilutions studied. However, at the lowest plasma dilution (1/10), a lower CBG level was measured. At plasma dilutions of 1/20 and higher, the effect of endogenous steroids was less important, and similar CBG binding capacities were measured regardless of the plasma dilution used (Fig. 1). Thus, in all subsequent studies plasma dilutions of 1:40 or 1:80 (v/v) were used.

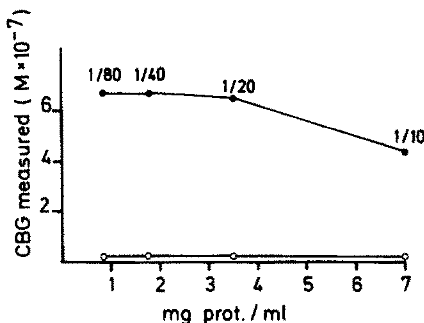

Fig. 2. Effect of serum dilution on specific and non specific binding of [^{3}H]-cortisol by SS-PAGE. Immature male serum was diluted 1/10, 1/20, 1/40 and 1/80 (v/v) and run in duplicate in SS-PAGE with 15 nM [^{3}H]-cortisol in the stacking gels and 4 nM in the separating gel (upper curve). In parallel gels a 1000 fold excess of cold cortisol was added both to the stacking gel and the separating gel to assess non-specific binding (lower curve).

Determination of equilibrium constant of dissociation (K_d)

In order to determine the K_d, aliquots of pooled male rat plasma diluted 1:40 were run into prelabelled gels containing from 1.6 to 16 nM of [^{3}H]-cortisol. As illustrated in Fig. 3, the binding in the CBG peak was saturable and minimal increase in the CBG binding occurred at gel concentrations higher than 8 nM. When the data were plotted according to Scatchard (bound/free versus bound) a straight line was obtained, indicating a maximum binding capacity of approximately 500,000 d.p.m. From a knowledge of the S.A., molecular weight, sample vol. and dilution, this is equivalent to a binding capacity in whole plasma of 6.35×10^{-7} M. From the total binding capacity of the sample, equation 2 can be resolved so that

$$K_d = [S_u]\left(\frac{BP_{tot}}{S_b} - 1\right). \qquad (3)$$

Thus, when BP_{tot} is known, S_b is obtained from the peak size and the $[S_u]$ is determined by taking aliquots of the gel solutions for counting, K_d can be calculated. For rat CBG, the K_d was 2.4×10^{-9} M $\pm$ 0.34 (S.D.). In all subsequent measurements a K_d of 2.4 nM was used for calculations.

As illustrated in Fig. 4, similar binding capacities for this male rat plasma were obtained in the range from 1.6 to 16 nM in the gel. For routine assays 4 nM [^{3}H]-cortisol was used in the separating gel.

Precision

Samples containing varying concentrations of binding sites (3–23×10^{-7} M) were analysed in duplicate by SS-PAGE in 4 nM gels. The within assay coefficient of variation was 8.5%. The interassay variation is dependent on the hormone concentration used in the gel (the lower the hormone concentration the

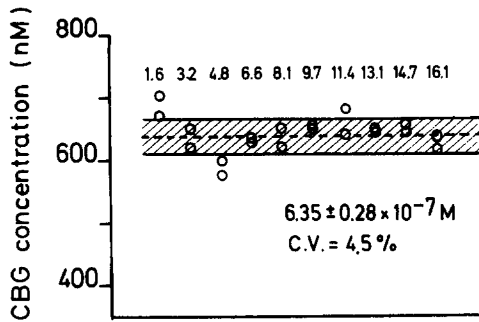

Fig. 4. Estimated CBG levels in one plasma sample at various concentrations of [^{3}H]-cortisol in the gels (same experiment as in Fig. 2). Round circles show the individual values. The numbers above the line indicate the concentrations of [^{3}H]-cortisol in the separating gel (in nM). The dotted line gives the mean binding capacity (6.35×10^{-7} M) and the hatched area indicates one standard deviation. The coefficient of variation in this assay was 4.5%.

Table 1. CBG levels in male and female rats measured by SS-PAGE before and after charcoal extraction (mean ± S.D.)

	Before charcoal ($M \times 10^{-7}$)	After charcoal ($M \times 10^{-7}$)	No. of animals
22 days ♂	6.9 ± 1.2	7.6 ± 1.4	10
45 days ♂	7.6 ± 4.3	7.6 ± 4.3	8
80 days ♂	9.2 ± 1.7	8.6 ± 1.3	7
360 days ♂ (castrated)	21.3 ± 2.8	19.2 ± 3.6	7
45 days ♀	10.2 ± 5.5	10.9 ± 5.5	12
80 days ♀	25.7 ± 4.0	23.2 ± 3.7	6
360 days ♀	22.5 ± 5.4	20.1 ± 4.5	7
Pregn ♀ (16–19d)	19.2 ± 1.8	21.3 ± 1.8	7

The samples were all diluted 1:40 (v/v) and measured in two different assays. Details are described in the Materials and Method.

larger the interassay variation); but when using 4–5 nM[^{3}H]-cortisol in the gel, the interassay variation is less than 15%.

Accuracy

Since CBG in these experiments is determined exclusively by binding, it is impossible to measure the precise number of protein molecules. Thus, the values obtained by us have been obtained by calculating one binding site per molecule of CBG, assuming that the affinity between cortisol and CBG is the same in various sera. Fig. 5 illustrates a linear relationship between the sample size and the amount of CBG measured.

CBG levels in male and female rats

This method for quantitating the binding capacity of rat CBG has been practically applied by measuring CBG levels in rats. Table 1 gives CBG levels in the male and female rats at varying ages and during pregnancy. CBG levels in adult male rats are significantly lower than in females ($P < 0.01$). Castration of male rats for 6 months caused an increase in CBG almost to that of the adult female. There was no significant difference in CBG levels between adult females (80 days) and very old female rats (>360 days). The levels of CBG in the 16–19 days pregnant rats were not different from that of the non-pregnant adult females.

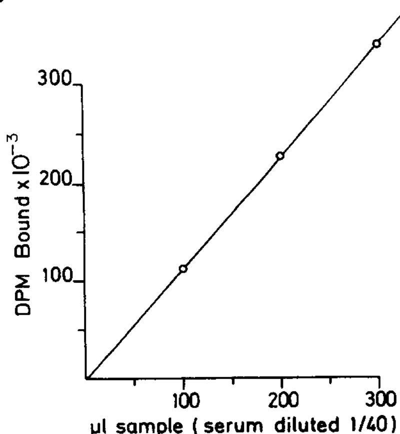

Fig. 5. Linear relationship between sample size and binding of [^{3}H]-cortisol to CBG. The electrophoresis was run as described in Materials and Methods.

Calculation results

The counts per minute provided by the scintillation counter were automatically punched on paper tape. This was directly processed by an IBM 370 computer. The program was designed to plot the radioactivity (c.p.m.) versus slice number. Furthermore, the area under each peak of radioactivity was calculated and free radioactivity ("background") subtracted. Binding capacity of rat CBG was expressed as molar concentration.

DISCUSSION

Steady state polyacrylamide gel electrophoresis (SS-PAGE) is a specific and sensitive method for the quantitation of specific steroid binding proteins. Quantitation of CBG by a high resolution technique like polyacrylamide gel electrophoresis has several advantages compared to other methods such as equilibrium dialysis[1], Sephadex gel equilibration[2], charcoal or florisil adsorption[3, 4] and gel filtration[8]. This electrophoretic technique combines the advantages of high resolution with a steady state condition which makes it possible to measure binding to CBG over a wide range of binding capacities. By its simplicity, this electrophoresis technique lends itself to multiple assays suitable for routine work.

It should be pointed out that SS-PAGE is useful only for proteins with a fairly rapid rate of association and dissociation. This type of technique is less suited for studies on intracellular steroid receptors, since the

exhange rate of steroid receptor complexes is very slow. When the rate of dissociation is slow, ($T_{\frac{1}{2}}^{0^\circ} > 1\text{–}2$ h), binding capacities of steroid binding proteins can be better measured at saturation[6]. The samples are then first saturated with radioactive steroid and then analysed by gel electrophoresis without any hormone in the gel. Due to the slow rate of dissociation and the high degree of reassociation within the peak, this gives a quantitative expression of the available number of binding sites.

This technique of SS-PAGE is practical since 60 gels can be run simultaneously under identical conditions. When the liquid scintillation counter is connected to a punch tape printer which can be processed by a computer, peak integration and calculation is done very quickly and data plotting take place within minutes.

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The computer program for SS-PAGE adapted to an IBM 370 and IBM 360 computer can be obtained from the authors.