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A FILTER ASSAY FOR THE CORTICOSTEROID BINDING GLOBULIN OF HUMAN SERUM*

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SUMMARY

A filter assay for corticosteroid-binding globulin (CBG or transcortin) in human serum was developed using DEAE-cellulose filter paper discs to bind the [^{3}H]-cortisol–CBG complex. The CBG binding capacity is determined by incubating diluted serum samples with a saturating concentration of [^{3}H]-cortisol in the presence and absence of 100-fold molar excess of radioinert cortisol and quantitating the amount of [^{3}H]-cortisol remaining specifically bound to the filter. After washing filters with buffer to remove unbound and non-specifically bound (albumin bound) steroid, 82% of this complex is retained on the filter. The specificity of this assay is demonstrated by the ability of various steroids, known to bind to CBG, to compete for [^{3}H]-cortisol. Endogenous steroids, which would otherwise dilute the specific activity of the [^{3}H]-cortisol, are removed from the undiluted serum by charcoal treatment (50 mg/ml serum). The assay is linear with serum dilutions of 1:20 or greater. The procedure is particularly useful at high serum dilutions where the sensitivity depends only upon the specific radioactivity of the steroid. The calculated concentrations of CBG in serum from men and pregnant and non-pregnant women are 37 mg/l, 70 mg/l and 37 mg/l, respectively; these values agree with those reported in the literature obtained by other methods. The equilibrium association constant of the cortisol–CBG complex as determined by the filter assay is $3{\cdot}5 \pm 0{\cdot}3 \times 10^9 M^{-1}$ at 4°C which is slightly higher than the reported values determined by equilibrium dialysis.

This new method is reproducible (CV 2·0–4·9%), rapid, accurate, and requires less than 0·5 ml serum. The procedure is not only useful for clinical investigation, but also for biochemical characterization of the protein.

INTRODUCTION

The existence of a corticosteroid-binding protein (CBG or transcortin) in the plasma of many species including man has been known for many years (see ref. 1 for review). The protein has been purified from human [2, 3], rat [4, 5], and rabbit [6] serum. A variety of methods for quantitating the CBG binding capacity of human serum has been published. These include equilibrium dialysis [7, 8], competitive adsorption [9, 10], gel filtration [11, 12], ultrafiltration [12–15], and immunochemical procedures [16]. In this paper we describe a simple and accurate method for quantitating CBG capacity in human serum based on a recently published filter assay procedure for measuring the sex steroid binding protein (SBP) of human serum [17].

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MATERIALS AND METHODS

Reagents and chemicals. [1,2-^{3}H]-cortisol (S.A. 53 Ci/mmol) was purchased from New England Nuclear. Radioinert steroids were obtained from Steraloids, Inc. or Sigma Chemical Co. Human serum albumin was purchased from Sigma Chemical Co. and defatted with charcoal-acid treatment. DEAE-cellulose filter paper discs (DE-81, 2·3 cm. dia.) were purchased from Reeve Angel & Co.

Serum samples. Venous blood was obtained from healthy volunteers and allowed to clot for 1 h at 25°C. The serum, obtained by centrifugation at 1500 rev./min for 5 min, was either refrigerated and used within 24 h or stored at −20°C.

Assay procedure. The principle of the method is based on the adsorption of the [^{3}H]-cortisol–CBG complex onto DEAE-cellulose filter paper discs at pH 7·4. Except where noted all serum samples are treated similarly. Endogenous steroids are removed by gently shaking 0·2–0·5 ml of undiluted serum with 50 mg/ml charcoal (Norit A) at 25°C for 30 min [9]. After centrifugation, the samples are diluted 50-fold with the 0·01 M Tris–Cl pH 7·4 buffer. Aliquots (0·5 ml are placed in tubes containing saturating amounts of [^{3}H]-cortisol (standard assay, $2{\cdot}8 \times 10^5$ d.p.m.; S.A. $5{\cdot}9 \times 10^4$ d.p.m./ng) in the presence and absence of 100-fold molar excess of a radioinert cortisol, allowed

to remain at 25°C for 15 min and cooled in ice for an additional 15 min. All subsequent operations are carried out in the cold room. DEAE-filter paper discs are equilibrated overnight with two changes of the buffer and stored at 4°C. The paper discs are placed on a plexiglass filtration apparatus composed of a series of 2·6 cm. dia. holes designed to retain stainless steel wire cloth discs. The apparatus is sealed with vacuum grease onto a collection chamber (bottom half of a vacuum desiccator) to provide proper vacuum. The filter discs are washed two times with 1 ml aliquots of buffer, and excess moisture is removed by applying a gentle vacuum. The vacuum is removed and 100 μl aliquots of the diluted serum are applied to each filter paper. After 2 min the vacuum is applied gently and the discs are washed with ten 1 ml aliquots of buffer. The filter paper discs are removed with the vacuum on and placed in vials with 10 ml of scintillant (5 g PPO,100 g naphthalene/l. dioxane) and the radioactivity counted (37% efficiency). The CBG binding capacity of a sample is calculated from the radioactivity bound to the filter, the "efficiency" of the filter to retain the [^{3}H]-cortisol–CBG complex, and the mol/mol stoichiometry of binding [2, 3]. The concentration of CBG is calculated from the binding capacity assuming a molecular weight of 51,700 Daltons for CBG [2].

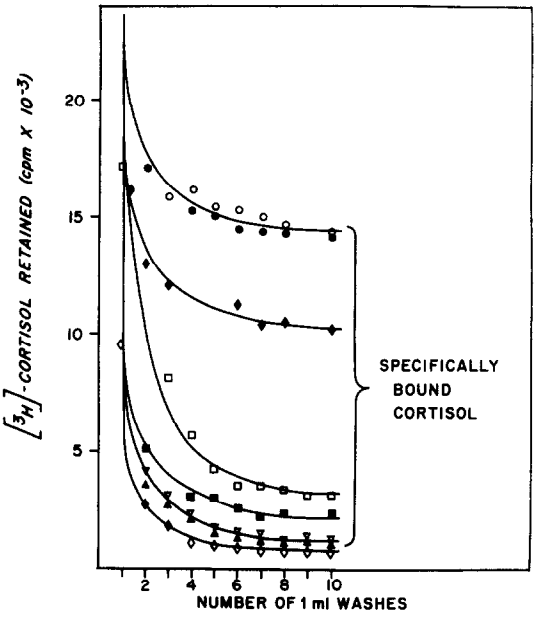

Fig. 1. Binding of the [^{3}H]-cortisol–CBG complex to DEAE-cellulose filter papers at 4°C. 1·5 ml of diluted serum is incubated with 8·5 × 10^6 d.p.m. [^{3}H]-cortisol (S.A. 5·9 × 10^4 d.p.m./ng) in the absence of radioinert steroids (○—○), or in the presence of 100-fold molar excess of radioinert cortisol (▽—▽), corticosterone (▲—▲), progesterone (■—■), testosterone (□—□), 5α-dihydrotestosterone (◆—◆), or estradiol-17β (●—●). [^{3}H]-cortisol incubated with 0·8 mg/ml human serum albumin (◇—◇).

RESULTS

Figure 1 demonstrates the retention of [^{3}H]-cortisol when 100 μl of diluted serum previously incubated with saturating amounts of [^{3}H]-cortisol are applied to filter paper discs. Approximately ten 1 ml washes are required to remove excess radioactivity, comprised of unbound, albumin-bound, and other non-specifically bound steroid. The presence of 100-fold molar excess of radioinert cortisol or corticosterone dilutes the specifically bound [^{3}H]-cortisol and results in a 92% loss of radioactivity bound to the filters after 10 washes. The remaining [^{3}H]-cortisol bound to the filter must be subtracted from the total amount bound to the filter in the absence of radioinert cortisol to obtain the correct [^{3}H]-cortisol specifically bound to CBG. The washing procedure also eliminates [^{3}H]-cortisol bound to albumin (Fig. 1).

Addition of 100-fold molar excess of radioinert progesterone and testosterone also significantly displaces the specifically bound [^{3}H]-cortisol, whereas 5α-dihydrotestosterone is not as efficient and estradiol-17β has no effect. Based on the binding specificity of CBG (see ref. 1 for review), these data strongly indicate that the radioactivity retained by the filters corresponds to [^{3}H]-cortisol bound to CBG.

In order to determine if the cortisol–CBG complex is completely retained by the filter paper during the washing procedure, filter assays were carried out with increasing concentrations of [^{3}H]-cortisol but under non-saturating steroid concentrations with respect to CBG. Under these conditions all the radioactivity added to each filter should be completely retained after the washing procedure if 100% of the complexes were bound to the filter. The results are shown in Fig. 2. The expected linear relationship is obtained; 82% of the cortisol–CBG complexes is retained by the filters as determined from the slope of the line. This value represents the "efficiency" of the assay after 10 washes and should be used as a correction factor to obtain the total binding-capacity of a particular sample. It should be pointed out that the "efficiency"

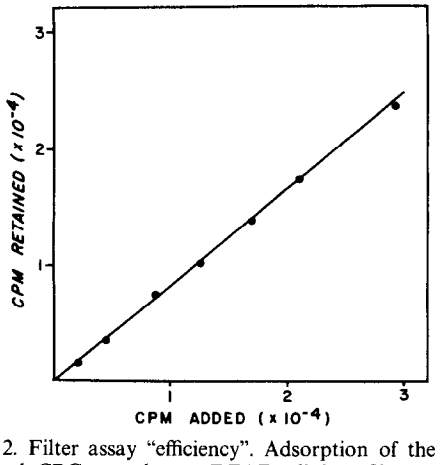

Fig. 2. Filter assay "efficiency". Adsorption of the [^{3}H]-cortisol–CBG complex to DEAE-cellulose filters at non-saturating steroid concentrations with respect to CBG. [^{3}H]-cortisol (S.A. 1·16 × 10^5 d.p.m./ng).

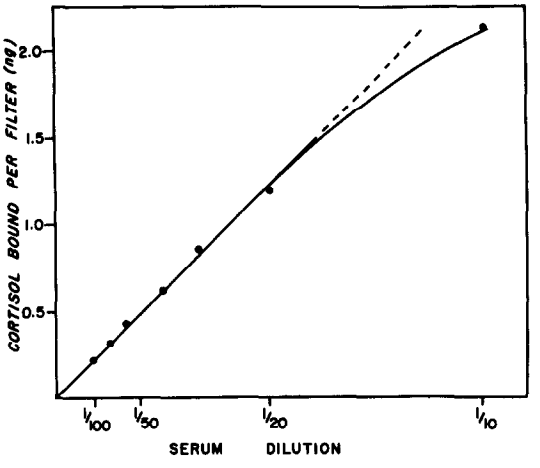

Fig. 3. Adsorption of the [^{3}H]-cortisol–CBG complex to DEAE-cellulose filters as a function of serum dilution. 0·5 ml samples of diluted serum from a pregnant woman was incubated with $2{\cdot}83 \times 10^6$ d.p.m. [^{3}H]-cortisol (S.A. $4{\cdot}8 \times 10^4$ d.p.m./ng) in the presence and absence or 100-fold molar excess radioinert cortisol. Data were corrected for 18% loss of complex as shown in Fig. 2.

can only be determined for the cortisol–CBG complex on serum purified of endogenous and exogenous steroids which bind to CBG. When one serum was not charcoal-treated, the "efficiency" was artificially lowered to 69%. Various factors such as pH, temperature, ionic strength, buffer ion and the nature of the steroid ligand will affect the "efficiency" of the assay, and therefore an experiment as shown in Fig. 2 should be performed in each laboratory to establish the correction factor. Values of 75% were obtained for the determination of the sex steroid binding capacity of human serum (86% is now obtained by improvements in methodology), and 85% for solutions of pure human sex steroid binding protein [17].

The influence of serum dilution on the total amount of [^{3}H]-cortisol bound to CBG under saturating conditions with respect to CBG is shown in Fig. 3. The assay is linear at serum dilutions of 1:20 or higher. At low protein concentration the sensitivity and accuracy of the assay depends only upon the specific radioactivity of the steroid. Deviation from linearity at high protein concentrations probably results either from "overloading" of the filter paper with protein, or from lack of saturation of CBG with [^{3}H]-cortisol in the initial incubation.

Table 1. Reproducibility of the filter assay*

	Mean (mg CBG/liter serum)	SEM	SD	CV	Range
male	24.8	0.38	1.21	4.9	23.2–26.6
female	30.6	0.20	0.62	2.0	29.8–31.8
pregnant (term)	64.2	0.72	2.27	3.5	60.6–68.6

* The values were calculated from 10 determinations on the same serum.

The determination of the equilibrium constant of association of the [^{3}H]-cortisol–CBG complex at 4°C by the filter assay is shown in Fig. 4. The calculated constant, Ka, obtained from the Scatchard analysis is $3{\cdot}5 \pm 0{\cdot}3 \times 10^9\ M^{-1}$. Extrapolation of the line to the abscissa of the Scatchard plot (20·3 nM) yields a CBG binding capacity of 37 μg cortisol bound/100 ml serum (1·02 μM) after correcting for the 50-fold dilution. The calculated concentration of CBG is 53 mg/l serum assuming a molecular weight of 51,700 Daltons [2]. The concentration of CBG in this particular sample carried out under the standard assay procedure (saturating condition) is 59 mg/l.

Table 1 shows the reproducibility of the filter assay as determined on serum from a man, and a pregnant and a non-pregnant woman. Table 2 compares our normal values with those obtained in other laboratories using different assay methods.

DISCUSSION

The quantitation of the binding of various steroids to specific proteins is of great importance in an effort to understand how steroid hormones are transported in blood and how they bind to their specific "receptors" at the cellular level. Many of the methods reported in the literature are tedious and time consuming. Recently a simplified procedure using DEAE-cellulose filter paper discs has been developed for measuring the sex steroid-binding protein of human

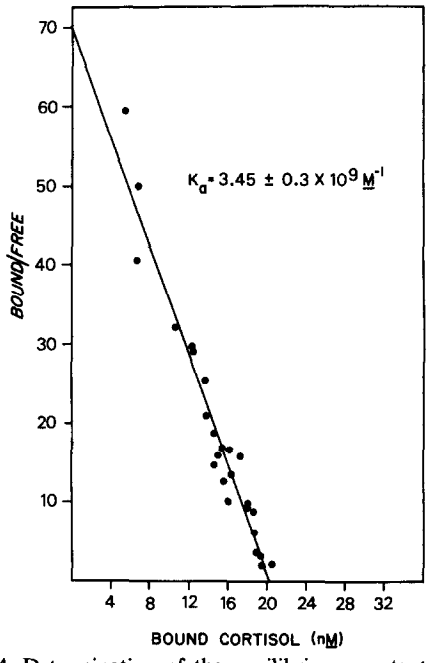

Fig. 4. Determination of the equilibrium constant of association of [^{3}H]-cortisol binding to CBG at 4°C by the filter assay. 0·5 ml samples of 50-fold diluted serum from a pregnant woman incubated with [^{3}H]-cortisol (S.A. $1{\cdot}18 \times 10^8$ d.p.m./nmol). Data corrected for 18% loss of complex as shown in Fig. 2. The "free" steroid is calculated by subtracting [^{3}H]-cortisol bound to the filter from that added to the filter.

Table 2. Concentration of CBG in human serum (mg/liter)

	Method[a]	Male	Female	Pregnant
Doe (12)	GF	35	33	70
De Moor (18)	GF	35[b]	35	62
Leybold (19)	EA	37	--	--
Westphal (20)	ED	37[b]	37[b]	--
De Moor (10)	CA	35	36	--
Van Baelen (16)	RID	42	42	89
This work	FA	37 ± 3[c]	37 ± 3[c]	70 ± 4[d]

a. GF, gel filtration; EA, enzymatic assay; ED, equilibrium dialysis; CA, competitive adsorption; RID, radial immunodiffusion; FA, filter assay.
b. serum from men and women.
c. ±SEM (10 subjects).
d. ±SEM (7 subjects).

serum [17] as well as glucocorticoid "receptors" in hepatoma cells [21]. A brief report on the use of the filter assay for measuring CBG has appeared in a recent publication [22]; however no data varifying the validity of the assay were presented. The data reported here establish the use of the filter assay for measuring the concentration of corticosteroid binding globulin in human serum. The simplicity, accuracy, reproducibility and low cost of the assay offer significant advantages over other methods previously described in the literature. Approximately 50 samples can be assayed in one working day. The procedure is particularly useful at low protein concentrations where the sensitivity depends only upon the specific radioactivity of the steroid. This advantage makes the assay useful not only for clinical investigations directed at measuring the binding capacity in various sera, but also for biochemical studies involved in the characterization of the pure protein. In fact, the development of the filter assay for measuring the sex steroid-binding protein of human serum was most helpful for establishing a purification procedure resulting in homogeneous protein [23].

Experience with the present assay indicates that endogenous cortisol must be removed with charcoal prior to the determination of CBG. If this precaution is not taken errors will be introduced resulting in low "efficiency," low calculated values of CBG, and low values for the equilibrium constant of association. Under the conditions of the assay, using 50-fold diluted serum, CBG is approximately 95% saturated with cortisol. This percent saturation is calculated from the principle of mass action using the concentration of both cortisol and CBG in the assay and the value of the K_a. Even in the case of sera from pregnant women or individuals receiving estrogen medication, a 50-fold dilution of these sera results in approximately 90% saturation of CBG.

Since the filter assay is not carried out under equilibrium conditions, it could be argued on theoretical grounds that the method may not yield a thermodynamically meaningful binding constant. However, determination of the binding constant by Scatchard analysis (Fig. 4) indicated a higher value than obtained by equilibrium dialysis at 4°C: $6 \times 10^8\ M^{-1}$ [18], $7 \times 10^8\ M^{-1}$ [24] and $1 \times 10^9\ M^{-1}$ [25]. The rate of dissociation of steroid-protein complex under the assay conditions is apparently too slow to account for significant changes in the actual concentration of the complex remaining on the filter. Therefore the method may also be used to study the physical chemistry of cortisol binding to CBG.

Note added to proof

It has recently been pointed out (*Analyt. Biochem.* **67** (1975) 422–427) that errors may be introduced in the evaluation of the "efficiency" of the filter assay unless non-saturating conditions with respect to the ligand are used. Under the conditions described here as well as for those of the sex steroid-binding protein of human serum [17], 99% of the steroid is protein bound. Therefore, correction factors need not be applied for our procedures.

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