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EFFECT OF SERUM DILUTION ON BINDING OF CORTISOL TO THERMOLABILE AND THERMOSTABLE SERUM PROTEINS

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SUMMARY

In this study, we assessed the suitability of diluted serum for the determination of free and protein-bound cortisol using an equilibrium dialysis method. This technique was applied to measure the unbound and protein-bound cortisol in the mother and her newborn.

INTRODUCTION

Pregnancy, several diseases and drugs, particularly estrogens, are known to alter the plasma corticosteroid binding globulin (CBG) and albumin concentrations [1–4]. In these conditions, the physiological meaning of the total cortisol concentration is rather unclear. Measurement of unbound cortisol concentration becomes therefore mandatory. For this purpose, several methods are currently available. The charcoal method [5] has the advantage of rapidity and simplicity but is lacking in accuracy. Indeed, the free cortisol index obtained with this method does not correspond to true equilibrium and therefore strongly depends upon methodological conditions. By contrast, methods based on ultrafiltration and equilibrium dialysis are widely recognized as yielding valid data [6–8]. The irreversibility and changes of volume inherent to the ultrafiltration method prevent its use for stringent thermodynamic evaluation. On the contrary, complete reversibility in the equilibrium dialysis method allows the determination of thermodynamically valid binding data [9]. These reasons led to our choice of the equilibrium dialysis method.

In order to measure unbound cortisol on small serum samples, it was found convenient to dilute the serum. Indeed, serum dilution increases the percentage of dialysable cortisol [5–10] permitting a more accurate measurement of the tracer inside and outside the dialysis chamber. Possible artefacts in unbound ligand determination on diluted serum have been reviewed by Tait and Burstein[11]. These authors concluded that it would be safer to accept values obtained with undiluted serum until the actual number of binding sites per molecule is determined after dilution. Similarly, Brunkhost and Hess[12] observed different affinity constants of albumin for cortisol when albumin was present at different concentrations. In this paper, we investigated the effect of serum dilution on the binding parameters of cortisol to physiological concentrations of CBG and albumin. To illustrate the interest of these measurements, the CBG content, the total, unbound and albumin— and CBG—bound cortisol were determined in the serum of pregnant women and of their newborn babies.

MATERIAL AND METHODS

Reagents. [1,2-^{3}H]-Cortisol (S.A. 40 Ci/mmol) was purchased from the New England Nuclear Corporation, stored at 4° in benzene–methanol (9:1, v/v) and chromatographed on Sephadex LH 20 (Pharmacia—Uppsala Sweden) in a benzene–dichloromethane–methanol (65 : 35 : 2, by vol.) system. All reagents were of analytical grade and purchased from Merck Co. Dichloromethane was redistilled before use. Visking dialysis tubing was supplied by Thomas Co. (Philadelphia) and extensively washed before use as described by Westphal[13].

Patients. Blood samples were drawn from the antecubital vein of 4 healthy male volunteers, of pregnant women at various stages of gestation and of 12 mothers just after the babies' expulsion and before placental delivery. The placentas were ice cooled and the two umbilical arteries separated from the vein. Selective blood sampling was performed with butterfly microneedles gauge 26. For convenience, the sera of the pregnant women were grouped into 8 pools, each corresponding to a five week period of gestation.

Total cortisol determination. Total cortisol concentration was measured by radioimmunoassay using an antiserum which cross reacted significantly with progesterone (6,5%) and 17 hydroxyprogesterone (41%).

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Since these steroids reach high levels during pregnancy (12 μg/100 ml and 1 μg/100 ml respectively [6, 14, 15]), a preliminary hexane extraction was performed. This step removed over 95% of progesterone and 80% of 17α-hydroxyprogesterone without causing significant loss of cortisol. To 0.5 ml serum were added 1 ml 9‰ NaCl and 3 ml hexane. After 1 min. vigourous vortex shaking, the organic phase was discarded. A 0.1 ml aliquot of the aqueous phase was mixed with 5 ml of distilled water and cortisol extracted with 5 ml dichloromethane. After solvent evaporation, cortisol was measured by immunoassay. All samples were processed in triplicate, with a 3% (S.E.M.) intra-assay variation in the results.

Equilibrium dialysis. We essentially followed the method of Westphal[13]. One ml of diluted or undiluted serum was dialysed 48 h at 37° against 1.7 ml 0.05 M phosphate buffer pH 7.4 containing labelled cortisol and antibiotics. Prior to dialysis, another aliquot of serum was treated at 60° for 20 min to destroy CBG, a thermolabile α1-globulin. A sample of the CBG free serum was dialysed as described above to determine the albumin-bound cortisol fraction. Under our experimental conditions, equilibrium was reached after 16 h of dialysis and identical results were obtained when dialysis was prolonged up to 72 h [5].

Equilibrium constant for cortisol binding to CBG at 37°. Serum was depleted of endogenous steroid by a 2 h incubation at room temperature with activated charcoal (50 mg/ml). After centrifugation, the supernatant was divided into 2 parts, one of which was heated at 60° for 20 min to destroy the CBG. Undiluted or tenfold diluted serum samples were then dialysed in the presence of six concentrations (10–200 pmol) of [1,2-^{3}H]-cortisol. After correction for non specific binding [16], the results were expressed according to Scatchard. The calculations were performed using a multi 20 intertechnique programmable calculator.

C.B.G. Determination. C.B.G. Was measured using the immunodiffusion method of Van Baelen and De Moor [17].

Calculation of albumin bound cortisol

$$B_A/F = K_A(P_A - B_A) \quad \text{(i)}$$

(Scatchard equation [18])

B_A = albumin bound cortisol (ML^{-1})
F = unbound cortisol (ML^{-1})
K_A = equilibrium constant of cortisol binding to albumin (LM^{-1})
P_A = total number of cortisol binding sites on albumin (ML^{-1})

Since albumin has a high capacity but a low affinity for cortisol, B_A is much smaller than P_A and equation (i) may be simplified into

$$B_A/F = K_A P_A \quad \text{(ii)}$$

$K_A P_A$ was determined on CBG free serum.

Calculation of unbound and CBG-bound cortisol

$$B/F = K_T(P_T - B_T) + K_A P_A \quad \text{(iii)}$$

B = total bound cortisol (ML^{-1})
K_T = equilibrium constant of cortisol binding to CBG (LM^{-1})
P_T = total number of CBG binding sites available for cortisol (ML^{-1})
B_T = CBG bound cortisol (ML^{-1})

CBG bound cortisol may also be expressed as total cortisol (Σc) minus free cortisol (F) and albumin bound cortisol (B_A or $F K_A P_A$).

$$B_T = \Sigma c - F - K_A P_A F \quad \text{(iv)}$$

Equations (iii) and (iv) may be combined into equation (v):

$$\Sigma c = K_T F^2(1 + K_A P_A) + (K_T P_T - K_T \Sigma c + K_A P_A + 1)F \quad \text{(v)}$$

Knowing $K_A P_A$, Σc and $K_T P_T$, one can calculate F. B_T can then be derived from equation (iv).

RESULTS

Determination of equilibrium constant of cortisol binding to CBG at 37°

Figure 1 illustrates the Scatchard plots of CBG-cortisol interactions using the sera of healthy males and pregnant women at various periods of gestation. Several parallel lines were obtained, indicating the homogeneity of the cortisol binding sites and the absence of cooperativity. The calculated values of K_T are listed in Table 1. The differences of intercept with the abscissa indicate a progressive increase in the total number of binding sites (P_T) with advancing pregnancy. It is noteworthy that the Scatchard plots obtained with the serum of males and of the first

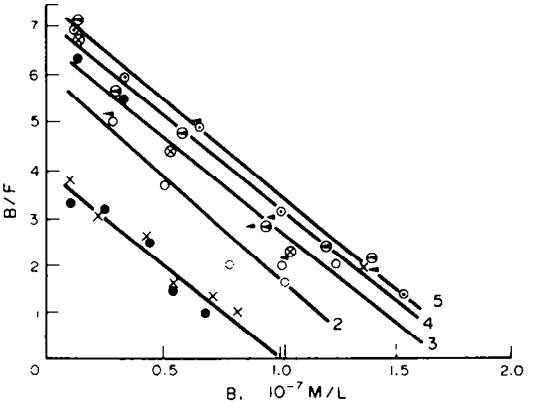

Fig. 1. Scatchard plots of cortisol binding (at 37°) to CBG of 7 different pools of serum. 1. Normal males serum—pregnant women serum (5–10 weeks). 2. Pregnant women serum (10–20 weeks). 3. Pregnant women serum (20–25 weeks). 4. Pregnant women serum (25–30 weeks). 5. Pregnant women serum (30–35 weeks and 35–40 weeks).

Table 1. Affinity constant of CBG for cortisol measured at equilibrium and at 37° in different pools of serum

	Normal males	Pregnant women					
		5–10 weeks	10–20 weeks	20–25 weeks	25–30 weeks	30–35 weeks	35–40 weeks
$K_T \times 10^7$ (L/M)	4.06	4.02	4.48	3.87	3.78	3.89	3.89
	0.967	0.981	0.985	0.975	0.988	0.961	0.978

$K_T = 3.989 \pm 0.1 \times 10^7$ L/M (average ± S.E.M.).

group of pregnant women (5–10 weeks) were superimposable, indicating the similarity in P_T values.

Determination of $K_A P_A, P_T, B_T$ *and* F

Table 2 summarizes the measurements of $K_A P_A$ on 10 preheated (CBG free) serum dialysed either undiluted or 10 fold diluted. Statistical analysis did not reveal any difference between the results obtained with undiluted or diluted sera ($t = 0.99$; $n = 10$; $P < 0.4$).

The calculated values of P_T, B_T and F of the same serum are illustrated in Table 3. Again, statistical analysis did not reveal any difference between the results obtained with undiluted or diluted serum: P_T ($t = 1.2$; $P < 0.3$), B_T ($t = 0.31$; $P < 0.45$) and F ($t = 0.70$; $P < 0.45$).

The correlation between the P_T, B_T and F values obtained with undiluted and diluted serum was also assessed by a linear regression test (Fig. 2).

(P_T: $r = 0.86$; B_T: $r = 0.99$; F: $r = 0.92$).

Application of the method in some physiological conditions

The above described procedure has been used to measure the CBG total cortisol (Σc), unbound cortisol (F) albumin- and CBG-bound cortisol (B_A, B_T) concentrations in the serum of 12 healthy pregnant women at delivery and of the venous cord blood of their newborn babies. The mean values and standard deviations are listed in Table 4.

DISCUSSION

The equations applicable to undiluted serum may be applied to diluted sera, provided that dilution does not affect the equilibrium affinity constant of CBG and albumin for cortisol (K_T, K_A) nor the number of binding sites per molecule.

From Fig. 1 and Table 1, it may be concluded that the K_T values and the number of CBG-binding sites remain constant in several physiological conditions and are independent of the serum dilution. The number of CBG binding sites available for cortisol (P_T) is constant whether measured on undiluted or on diluted sera (Table 3).

The ratio of *albumin-bound to unbound cortisol* ($B_A/F = K_A P_A$) has been investigated by several authors [19–22]. Our results (Table 2) were not statistically

Table 2. Comparison between the $K_A P_A$ values obtained with undiluted and diluted transcortin-free sera

		1	2	3	4	5	6	7	8	9	10
$K_A P_A$	Undiluted sera	3.13	3.25	2.41	2.61	2.74	2.04	2.54	2.43	2.54	2.44
$K_A P_A$	10 Times diluted sera	2.7	2.75	2.25	2.08	2.37	2.68	2.72	2.1	2.34	2.86

Table 3. Comparison between the number of CBG binding sites available for cortisol (P_T), CBG bound cortisol (B_T) and unbound cortisol (F) measured in undiluted and diluted sera

	P_T ($\times 10^{-7}$ M/L)		B_T ($\times 10^{-7}$ M/L)		F ($\times 10^{-7}$ M/L)	
	Undiluted serum	Diluted serum	Undiluted serum	Diluted serum	Undiluted serum	Diluted serum
1	10.11	10.35	3.27	3.36	0.063	0.063
2	7.48	11.1	1.48	1.52	0.108	0.071
3	13.41	15.85	6.65	6.55	0.298	0.226
4	17.59	19.62	3.36	3.48	0.282	0.240
5	11.74	15.17	7.43	7.25	0.099	0.075
6	14.58	14.82	9.34	9.63	0.209	0.198
7	21.07	19.86	11.38	11.22	0.294	0.324
8	13.49	14.17	2.27	2.46	0.181	0.170
9	15.16	12.86	7.53	7.54	0.240	0.310
10	15.08	14.72	8.11	7.98	0.290	0.294

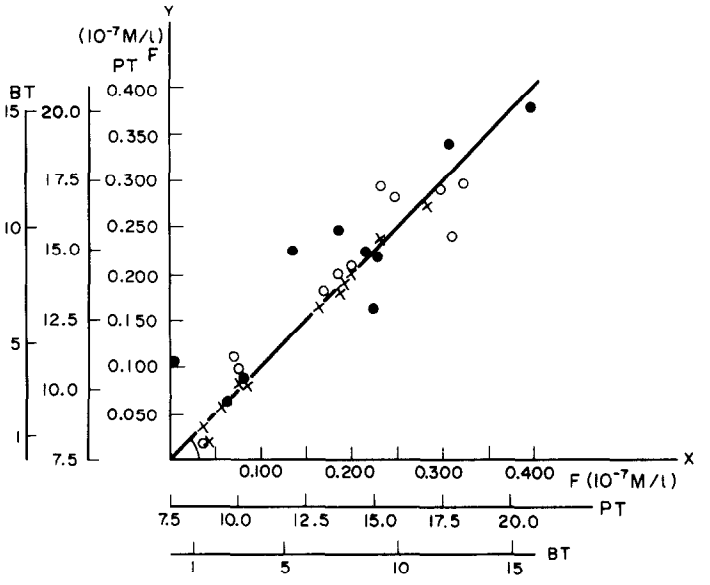

Fig. 2. Correlation between B_T (×), F (○) and P_T (●) determined in undiluted (abcissa) and diluted (ordinate) serum.

different when diluted or native sera were used. This finding is in agreement with that of Goldie[19] and stresses the need to assess the $K_A P_A$ value for each serum and not to extrapolate this value from theoretical considerations as proposed by Tait[11].

This study demonstrates the suitability of the theoretical equations employed to determine the unbound and protein-bound cortisol from diluted sera. The calculated P_T values do not correspond to the serum CBG concentration since they were obtained in the presence of other steroids also competing with cortisol for the CBG-binding sites: the calculated P_T values only represent the number of CBG binding sites available for cortisol.

Table 5 illustrates the correlations between the calculated P_T values and true CBG concentration, the CBG-bound cortisol and CBG concentrations, the unbound and total cortisol concentrations in the sera of pregnant women and their newborn babies.

Mothers' and newborn babies' sera contain different amounts of total cortisol and CBG. In maternal sera, characterized by high cortisol and CBG levels, a good correlation exists between calculated P_T and measured total CBG and between CBG-bound cortisol and CBG levels whereas total and unbound cortisol are but weakly correlated.

On the other hand, in newborn babies' sera, characterized by low cortisol and CBG levels, there are no correlations between P_T and CBG nor between CBG-bound cortisol and CBG levels whereas a statistically significant correlation exists between total and unbound cortisol.

These examples illustrate the need for measuring the distribution of total cortisol between bound and

Table 4. Concentrations (10^{-7} M/L) of total (Σc), unbound (F) and CBG-bound (B_T) cortisol, CBG (molecular weight assumed to be 40.000) and calculated CBG available for cortisol (P_T) in mothers and newborn babies sera

	Σc	F	B_T	CBG	P_T
Mothers' sera	18.34 ± 1.57	1.044 ± 0.141	13.28 ± 1.02	24.68 ± 1.09	16.7 ± 0.11
Newborn babies' sera	2.47 ± 0.28	0.360 ± 0.056	1.176 ± 0.112	4.66 ± 0.53	2.04 ± 0.012

Table 5. Correlations P_T: calculated CBG binding sites available for cortisol; CBG: Total CBG measured by immuno-diffusion; B_T: CBG-bound cortisol

	P_T versus CBG	B_T versus CBG	Total versus unbound cortisol
Mothers' sera (n = 12)	r = 0.920	r = 0.911	r = 0.794
Newborn babies' sera (n = 12)	r = 0.338	r = 0.494	r = 0.946

unbound forms in each serum and clearly demonstrate the unsuitability of extrapolating these values from total cortisol and CBG concentrations.

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