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NEW SIMPLIFIED PROCEDURES FOR THE DETERMINATION OF PROGESTERONE BY COMPETITIVE PROTEIN BINDING AND RADIOIMMUNOASSAY

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SUMMARY

In the present assay procedures a new and improved method for the separation of bound from unbound progesterone has been employed. A sample containing protein bound and unbound progesterone is simply filtered through a membrane filter which completely adsorbs the unbound fraction. By making a few other modifications in the existing competitive protein binding (CPB) and radioimmunoassay (RIA) of progesterone, the assay procedures were rendered simpler and more sensitive. These modifications included the rapid removal of endogenous progesterone from plasma, used as the source of corticosteroid binding globulin (CBG), and the preparation of stable [^{3}H]-CBG and [^{3}H]-antiserum solutions.

INTRODUCTION

At present, competitive protein binding (CPB) method is probably the most widely used technique for the assay of steroid hormones. More recently, however, radioimmunoassays (RIAs) of various steroids have been developed [e.g. 1–3]. The two methods are based essentially on the same principle, namely the specific binding of the steroid to certain proteins. The difference lies in the fact that in the CPB method, in contrast to the RIA method, non-antibody protein with specific binding sites is used. With either method, however, one must separate the protein-bound steroid from the unbound steroid at the end of the incubation period. Out of the numerous available techniques for the separation of bound from unbound steroid, the use of solid adsorbants such as Florisil or charcoal is, for the lack of a better technique, most extensively practised. The short-comings of these and other available separation methods were pointed out in an earlier report from this laboratory[4] in which a new, simpler and more reliable method for the separation of free from protein-bound progesterone was described. Use of this new technique for the separation of free from protein-bound progesterone (by adsorption of the free progesterone on a membrane filter) along with other modifications described in this report, resulted in procedures which are less cumbersome and, in the case of CPB, more sensitive than those available at present.

EXPERIMENTAL

Since the purpose of this article is to demonstrate the superiority of the new, or modified, steps incorporated in the RIA and CPB assay, procedures for constructing standard curves only for these assays will be described. There are several procedures available for the extraction of progesterone, preceding its determination by either RIA or CPB assay. The use of a particular extraction method depends essentially on the source of progesterone and the choice of the investigator. This will not be the subject of the present report.

Chemicals. Radio-labelled [1, 2, ^{3}H]-progesterone (44Ci/mmol) was purchased from New England Nuclear Corporation. Progesterone was given by Schering AG.

Standards. A series of standards containing 50–500 pg and another series containing 0·2–2 ng of progesterone were used for RIA and CPB assay respectively. The required amount of progesterone was pipetted in tubes from a stock solution in ethanol and dried subsequently under air at 37°C.

Buffers. Buffer I contained 150 mM NaCl and 50 mM Tris (pH 8·0) and was used for CPB assay. Buffer II contained 0·1% sodium azide and 0·1% gelatin in buffer I and it was used for RIA.

Antiserum. Lyophilized rabbit antiserum to progesterone (11-hemisuccinate-BSA) was given by Calbiochem. The powder was dissolved in 5 ml of buffer II and stored in aliquots of 0·5 ml at −20°C until needed.

Corticosteroid-binding globulin (CBG). The source of CBG used was blood obtained from healthy women taking oral contraceptives. After centrifugation of the blood, the plasma was aspirated and stored in aliquots (0·5 ml) frozen at −20°C until needed.

Stripping of plasma. It was previously shown that Millipore filters retained free progesterone completely and other steroids to varying degrees from aqueous

or plasma solutions [4]. This filtration procedure, which is used to separate the free from protein-bound progesterone in the assays described here (see below), was also used to strip plasma of its endogenous progesterone. This was done by diluting 0·2 ml of plasma to 2 ml with buffer I and after keeping at 40°C for 10 min, the solution was filtered through Millipore filters.

Filters. Standard Millipore filter discs (HAWP 02500), the corresponding glass base with neoprene stopper and the filtering flask were purchased from Millipore AB Gothenburg, Sweden. Occasionally, membrane filters manufactured and given by Sartorius-Membranfilter (W. Germany) were also used for comparison (see Discussion).

Labelled CBG solution. [^{3}H]-progesterone corresponding to approximately 16,000 c.p.m./ml of the final solution was dried in a 100 ml volumetric flask. Subsequently 750 μl of stripped plasma was added and the solution made up to the volume with bufferI. After shaking, gently, the solution was kept at 4°C until needed in the assay.

Labelled antiserum solution. [^{3}H]-progesterone corresponding to approximately 18,000 c.p.m./ml of the final solution was dried and after adding 37·75 ml of buffer II in it, the contents were mixed. Subsequently 250 μl of antiserum (originally stored at −20°C) was added. The combined solution was left in a water bath at 25°C for 10 min, then shaken on a Vortex mixer for 15 s and stored at 4°C until needed.

Separation of bound from unbound progesterone. This is a crucial step both in terms of sensitivity and precision in any protein binding assay[5]. Other important attributes of a good separation method are simplicity and rapidity of its operation. The present separation method fulfils the criterion for ideal conditions (see Discussion). The procedure used for the separation of free from bound progesterone both in RIA and CPB assay was as follows.

After incubation of the assay tubes, the contents of each were simply filtered through a Millipore filter placed on its glass base. The filtrate was collected in a test tube placed inside the filtering flask.* Filtration was done under slight vacuum applied to the filtering flask and took about 2 s for each sample. Unbound progesterone was adsorbed to the membrane (cellulose nitrate) filter and thereby resulting in an instantaneous and a complete separation of bound and unbound steroid. The radioactivity in the filtrate or on the filter could be counted for bound and unbound progesterone respectively. We found it more convenient to count an aliquot of the filtrate for the bound progesterone.

CPB assay. One ml of the labelled CBG solution was added to each tube containing a progesterone standard. The tubes were kept in a water bath at 25°C for 10 min and after shaking on a Vortex mixer for 15 s were left at 4°C for 1 h. The contents were then filtered through Millipore filters and 500 μl was counted for [^{3}H]-progesterone bound to CBG. A standard curve was constructed as shown in Fig. 1.

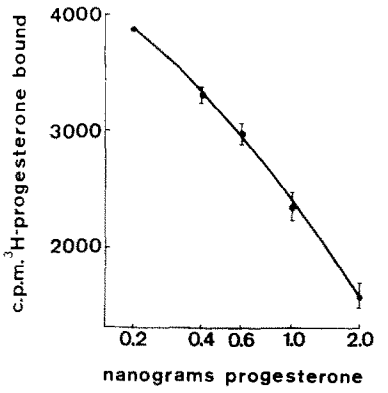

Fig. 1. Standard curve for CPB assay of progesterone. Each point is the mean of 3 determinations. Vertical bars represent S.E.M., and in the absence of a bar S.E.M. is included within the point.

RIA. Eight hundred μl of the labelled antiserum solution were added to each progesterone standard. After equilibrating for 30 min at room temperature, the contents were mixed and the tubes were left at 4°C over night. Next morning the contents were filtered through Millipore filters and 500 μl of the filtrate was counted for [^{3}H]-progesterone bound to the antibody.

Radioactivity measurements. After adding 10 ml of Aquasol (New England Nuclear Corp.) in the vials containing samples, they were counted in a Packard Tri-Carb (Model 3320) liquid scintillation counter. The counting efficiency was about 40%.

RESULTS AND COMMENTS

CPB assay. It has been reported previously that there is a decline in the binding of [^{3}H]-progesterone after storage of [^{3}H]-CBG solution[6]. A significant decrease in [^{3}H]-progesterone bound to CBG occurred after 5 days of storage at 5°C, which resulted in the reduction of sensitivity and useful reading range of the standard curve[6]. We have also observed similar instability of the [^{3}H]-CBG solution to storage at pH 7. However, when the solution was prepared in a buffer of pH 8 or higher, it was stable at least for 15 days, stored at 4°C, since the standard curves from this and the fresh solution were superimposable. This finding may also explain why the instability of the [^{3}H]-CBG solution to ageing was not observed consistently by Johansson[6] as in his method the solution was prepared in water and not in a buffer of a known pH (see also Ref. 7).

A standard curve using the present assay method is shown in Fig. 1. Plasma which was used as a source of CBG was stripped of endogenous progesterone by a simple and quick procedure (see Methods).

* Multiple samples (up to 30) can be filtered simultaneously by using a sampling manifold (Millipore).

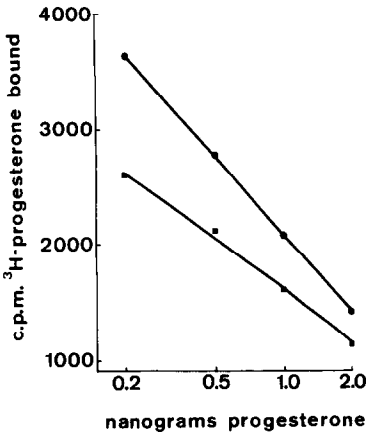

Fig. 2. Standard curves using stripped (●) and unstripped (■) plasma. Each point is the mean of a duplicate determination.

Although we did not check the extent to which endogenous progesterone was removed, it is not likely that any significant amount remained (see Discussion) since the membrane filters are capable of binding over 96% of free progesterone in solution[4]. The filters are also capable of binding a significant amount of corticosteroids and estrogen present in an aqueous solution[4]. As shown in Fig. 2, the stripped CBG bound significantly higher [^{3}H]-progesterone. Recently Baranezuk *et al.*[8] reported a procedure for the removal of endogenous steroids from CBG prior to its use for progesterone assays. They used gel filtration for stripping and their data showed that the binding of progesterone increased after stripping of CBG.

RIA. Preliminary experiments were performed to test the possibility of using a combined antiserum and [^{3}H]-progesterone solution. A combined solution (antiserum + [^{3}H]-progesterone) was prepared (see Methods) and stored in a refrigerator (4°C). It was checked at various times up to 30 days for bound [^{3}H]-progesterone. The data shown in Fig. 3 clearly indicated that there was no significant change in the binding of [^{3}H]-progesterone during storage.

The standard curve for RIA is shown in Fig. 4. The precision of the CPB assay and RIA is shown in Table 1.

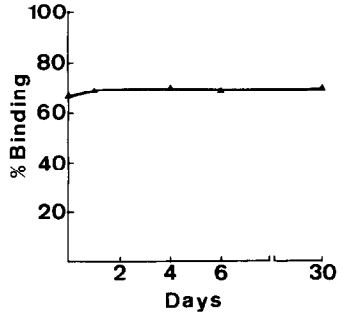

Fig. 3. Binding of [^{3}H]-progesterone to antiserum after storage of the combined [^{3}H]-progesterone and antiserum solution at 4°C.

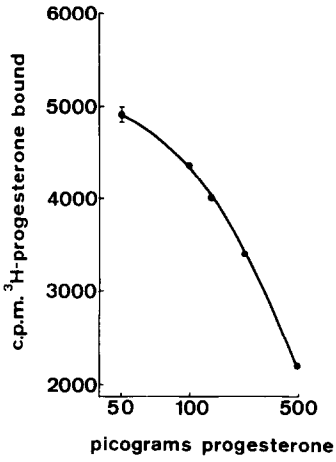

Fig. 4. Standard curve for RIA of progesterone. Each point is the mean of 3 determinations. Vertical bars represent S.E.M., and in the absence of a bar S.E.M. is included within the point.

DISCUSSION

The primary and most important improvement in the present procedure was made in the method of separation of bound from unbound progesterone, a step critical to the precision, sensitivity and simplicity of all protein (including antibody protein) binding assays. This separation was achieved by a novel approach in which membrane (cellulose nitrate) filters were used to adsorb the unbound progesterone. These filters, as discovered recently[4], are capable of adsorbing virtually all the free progesterone from aqueous solutions containing up to 1 μg of progesterone. The protein-bound progesterone on the other hand is neither retained nor its binding influenced when passed through the membrane filter[4].

Advantage of this steroid adsorbing property of the filters was taken also to strip the plasma completely of endogenous progesterone and at least partly of corticosteroids[4]. The stripped plasma was subsequently used as the source of CBG in the CPB assay. Due to an increase in the number of progesterone binding sites, the sensitivity of the assay using stripped CBG is increased (see Ref. 8). It is important to point out, however, that although stripping of

Table 1. Replicate analysis of a known amount of progesterone by CPB assay and RIA

CPB ASSAY

Amount taken	Number of determinations	Amount calculated	Coefficient of variation (%)
0.4 ng/ml	10	0.41 ng/ml	4.08
1.0 ng/ml	11	1.01 ng/ml	4.37

RIA

100 pg/ml	9	98 pg/ml	6.63
250 pg/ml	10	258 pg/ml	3.14

plasma by the present procedure resulted in a significant increase in the [^{3}H]-progesterone binding, this could not be due only to the removal of endogenous progesterone since there is relatively little progesterone in plasma of non-pregnant women. The increase in binding could also have resulted from the removal of corticosteroids[4], as well as progestins in the case of women taking oral contraceptives. However, the extent to which the binding sites in CBG were freed from these steroids by the present stripping method cannot be known with certainty. In order to exploit the full capability of the present stripping procedure, plasma obtained from women in late pregnancy would seem to be an ideal source of CBG.

An important practical advantage in using membrane filters was that, the need for a centrifuge, which is called for when using charcoal for RIA, was eliminated. Dextran-coated charcoal has been used in almost all the previously reported procedures for RIA and a great number of those reported for CPB assay of progesterone. The only undesirable feature of the membrane filters is their relatively high cost. However, the presently available membrane filters are manufactured to meet certain requirements such as uniform pore size, high porosity, strength and durability etc., resulting in extraordinarily high cost. Since the retention of the free steroid is most probably due to adsorption, it is unlikely that these requirements need to be stringent for filters to be used to separate bound from unbound steroid. It is therefore foreseeable that modified and cheaper membrane filters could be made commercially available in the future for their application in analytical methods similar to the one described here. Recently, we have used cellulose nitrate filters manufactured by Sartorius-Membranfilter (Göttingen, W. Germany), which are considerably less expensive, and found that they performed as well as the Millipore filters.

Another important modification made in the present procedures was in the preparation of a stable CBG solution with bound [^{3}H]-progesterone. This resulted from our observation that these solutions, when prepared in a buffer of pH 8 or higher, were stable to ageing. This finding may also explain the discrepancy reported in the literature[6,7]. Similarly, a combined solution of [^{3}H]-progesterone and antiserum could be prepared and stored at 4 C without a change in the bound progesterone. This combined solution obviated the need for pipetting of antiserum and [^{3}H]-progesterone separately in assay tubes, a procedure used in previously reported RIA of progesterone.

The above modifications along with the new approach of separating free from bound progesterone overall reduced the time taken for the assays and resulted in greater simplicity and sensitivity of the assay procedures.

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