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EFFICIENT METHOD FOR PURIFYING PROGESTERONE FOR COMPETITIVE PROTEIN-BINDING TECHNIQUE

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SUMMARY

A simple and efficient method of purification of progesterone from plasma extracts on Sephadex LH-20 column is described. The chromatographic system can be applied to any ligand assay for the determination of progesterone. Progesterone emerges as a compact peak well separated from related compounds. A visual dye which has the same mobility as progesterone was used to monitor the steroid fraction. This dye does not interfere with the binding assay. If redistillated solvents are used in all steps, the nonspecific blank can be eliminated. The complete absence of the procedural blank is a prerequisite for accurate determinations of plasma progesterone in man, postmenopausal woman or the follicular phase of the normal menstrual cycle.

Several methods have been published for estimating human plasma progesterone by the use of competitive protein-binding. Some of these [1–3] use naturally occurring plasma proteins, others [4–7] the more specific antisera.

An attempt to eliminate the purification step by increasing the specificity of the antibodies has recently been made [8, 9]. This approach is satisfactory for routine determinations, but in studies which require determinations of low plasma concentrations, such as in men or menopausal women, the purification step remains essential. This paper describes a rapid and practical method for purifying progesterone on Sephadex LH-20 columns using a marker dye to locate the fraction.

MATERIALS AND METHODS

[1,2-^{3}H]-Progesterone-(53 Ci/mmol), [1,2-^{3}H]-17α-hydroxyprogesterone-[1,2-^{3}H]-deoxycorticosterone [1,2-^{3}H]-corticosterone and [1,2-^{3}H]-11-deoxycortisol (all 40–50 Ci/mmol) were obtained from Amersham–Searle Co. Before use, the radiochemical purity on the compounds was checked by paper chromatography and the compounds were purified if needed. Unlabelled progesterone (Sigma) was recrystallized from methanol and dissolved in methanol at the proper concentration.

Sephadex LH-20 (Pharmacia Co.) was used as supplied.

All solvents were of reagent grade and were further purified before use as described previously [10, 11]. 0·5 ml samples of aqueous solutions were counted in 10 ml of Bray's scintillation fluid [12, 13] in a Packard Tricarb Liquid Scintillation Spectrophotometer (model 3375).

Sudan III (Chroma–Gesellschaft Schmid & Co., Stuttgart-Untertürkheim) was purified on a column (10 × 500 mm) of Sephadex by the method about to be described for the purification of progesterone. The dye was resolved into two bands. The red band migrating with the liquid front was discarded. The eluate containing the slower moving orange band was evaporated to dryness and the residue was used as the reference marker.

Column chromatography: Sephadex LH-20 (7 g) was allowed to swell overnight in a mixture (200 ml) of isooctane, benzene–methanol (85:10:5 by vol). All columns (5·5 × 180 mm) were prepared just before use. The purified dye fraction was dissolved in the same solvent mixture and 0·1 ml of the dye solution was used to redissolve all samples. The sample was stirred on a Vortex Mixer and applied to the column with a Pasteur pipette. The tube was rinsed with the same volume of solvent.

The column was eluted with the solvent mixture of 1 part of that used for the swelling of the Sephadex and 1 part of freshly prepared mixture of the same composition. After the emergence of about 7 ml, progesterone and the dye were eluted together as a very compact peak (2 ml). They were collected in assay tubes and dried under reduced pressure at 40°C. The elution time was about 20 min.

The binding assay was carried out as previously described [14].

RESULTS

The elution pattern of the column (Fig. 1) shows that progesterone is well separated from compounds

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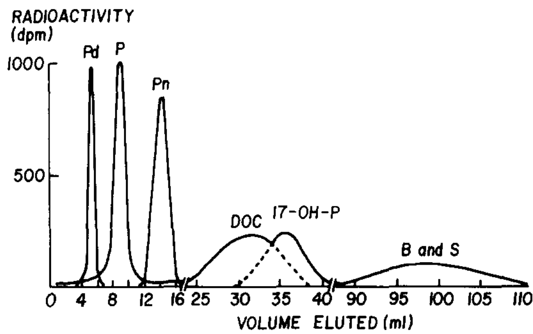

Fig. 1. Elution pattern of progesterone and related compounds. Pd: Pregnane-3,20-dione, US Pharmacopeia, USP. Steroid reference substance No. 402 (non-radioactive); P: progesterone; Pn: pregnenolone; DOC: 11-deoxycorticosterone; 17-OH-P: 17-hydroxyprogesterone; B: corticosterone; S: 11-deoxycortisol.

which may interfere with the assay. The recovery of the 2-ml progesterone fraction was 90·2% (±0·8% S.E.M., $n = 17$). The blank was consistently negligible and not detectable on the standard curve (<0·1 ng).

That the dye in the samples does not interfere with the binding assay is illustrated by the fact that the percentages of progesterone bound as determined in 2 ml of double-distilled water, 2 ml of solvent mixture, and 2 ml of the dye solution were 65·0 ± 0·46, 65·2 ± 0·30 and 65·5 ± 0·62, respectively (S.E.M.; $n = 4$ in each case).

Figure 2 shows the logit plot of percentage progesterone bound against the log of amount of unlabelled progesterone. There is no significant difference between the curves with and without the dye.

The amount of dye used is so minute that no quenching effect could be detected in the counting step.

DISCUSSION

The chromatography of progesterone on Sephadex LH-20 with isooctane–benzene–methanol 85:10:5 (by vol.) is very efficient. Progesterone appears in a compact peak and interfering compounds such as pregnanedione, pregnenolone, DOC and 17-hydroxyprogesterone only emerge before or much later.

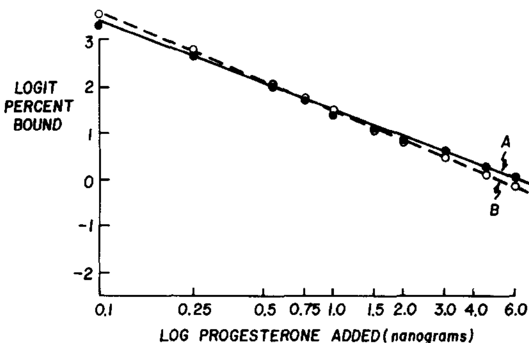

Fig. 2. Effect of the marker dye on assay of bound progesterone, plotted by the method of Rodbard *et al.*[15]. A—without dye ○; B—with dye ●.

The use of a component of Sudan III as a marker makes the monitoring of the elution very simple. Ten columns can be run simultaneously in less than 1 h, including the time required for sample application. Each column received about 1 g of Sephadex and the cost is therefore so small that the columns were not reused with further saving of time, since elution can stop as soon as the coloured band has emerged. Collins, Sommerville and their co-workers have used dyes isopolar with other steroids [16] before in chromatographic techniques.

With protein-binding techniques, the present system has the advantage of eliminating the non-specific blank which is usually present if paper or t.l.c. is used. Even after careful washing, paper blanks often remain a problem [17], and there are variations from one paper batch to another. Sephadex LH-20 is an inert product and if redistilled solvents are used in all steps of our procedure, the blank is consistently undetectable. A blank-free Sephadex LH-20–water column was previously developed for the radioimmunoassay of aldosterone [13].

In studies of human plasma requiring the determination of low progesterone concentrations, the purification step is essential. We have used the described method successfully in studying physiological variations of progesterone in men [18], menopausal women, and women during the follicular phase of the menstrual cycle [18, 19]. This method of chromatography has been used for the isolation of progesterone from plasma extracts in ligand assays utilizing both steroid binding globulin [14] or a more specific antiserum. The antiserum obtained from New England Nuclear Corp. was the lyophilized progesterone antiserum prepared in rabbits against progesterone-11β-succynilbovine serum albumin. The results on the same plasma samples were identical by both approaches confirming the specificity of the purification step.

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