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A HIGHLY EFFICIENT PROCEDURE FOR THE EXTRACTION OF PROGESTERONE FROM UTERUS AND ITS COMPATIBILITY WITH SUBSEQUENT RADIOIMMUNOASSAY

SATISH BATRA and LARS PHILIP BENGTSSON
Department of Obstetrics and Gynecology, University of Lund, Lund, Sweden

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SUMMARY

A method for the determination of progesterone concentration in human or rabbit myometrium is described. The tissue is first chemically digested in a mixture of NaOH and sodium dodecyl sulphate. After digestion which is relatively rapid in this mixture, progesterone was recovered completely by extraction with ethyl acetate. After a minor purification step (Sephadex LH-20 chromatography), progesterone was determined by radioimmunoassay. The method is capable of determining progesterone concentration in pregnant human myometrial tissues as small as 50 mg wet weight. The details of the method along with the data on its reliability are presented.

INTRODUCTION

In recent years there has been a vast amount of quantitative data available on the level of progesterone in plasma of various species, including man, under physiological, pathological and experimental conditions. There is, however, relatively little information on the concentration of progesterone in uterine or in other target organs. This lack of information on the progesterone content of the myometrium or endometrium, particularly those of human, is probably due to the fact that the progesterone content of the tissue is very low and until recently the methods were not sensitive enough to determine precisely the amount of extracted progesterone from rather small samples of the material available. With the recent advent of radioimmunological techniques, which have a very high sensitivity and inherent specificity, the determination of progesterone (or estrogen) in the small samples of tissues such as the human endometrium and myometrium should be feasible. However, crucial to the determination of the progesterone concentration in a tissue by these highly sensitive techniques is the availability of a reliable method for the extraction of progesterone, and the subsequent preparation of a suitable sample for the desired assay.

In the past generally three variants of a basic extraction procedure have been used (for example see 1–4). They comprise (a) extraction of the tissue directly with organic solvents, (b) homogenization of tissue in water or strong alkali followed by extraction with organic solvents and (c) quick freezing or freeze-drying of tissue before extraction with organic solvents. However, with none of these methods is there a complete recovery of the endogenous progesterone. In several cases numerous additional steps were incorporated which not only were time consuming but increased variability in the amount of progesterone extracted from the tissue[1]. Our preliminary experiments showed that with the use of any of the above mentioned procedures, the recovery of progesterone was not only low (40–80%) but there was a considerable variation from sample to sample. The procedure in which tissue was first homogenized in sodium hydroxide gave the best results, however.

In the present method, the tissue was first chemically digested in a mixture of alkali (NaOH) and a cationic detergent (sodium dodecyl sulphate) which was followed by an extraction with ethyl acetate. With this simple method, the extraction of tissue progesterone was rapid and virtually complete. The progesterone in the extract, after a minor purification step, could be determined by radioimmunoassay. The details of the method along with the data on its reliability and precision are presented.

EXPERIMENTAL

Tissues. Pieces from rabbit uterus or human myometrium were used. These tissues, due to the presence of high collagen in them, are generally difficult to homogenize or digest chemically. Occasionally endometria from pseudopregnant rabbits or non-pregnant women were also used. Human tissues were obtained at hysterectomy operations or Cesaerean section deliveries.

Incubation medium. In order to allow the tissues to take up [^{3}H]-progesterone from the medium, they were incubated for 1 h at room temperature in Krebs–Ringer bicarbonate medium[5] which was bubbled with 95% CO_2 and 5% O_2.

Digestion of tissues. A mixture of 5% sodium dodecyl sulphate and 0.5 N sodium hydroxide (NaOH)

was used for the digestion (or dissolution) of the tissues. Preliminary experiments showed that this combination of detergent and alkali gave most satisfactory results. This mixture will be referred to as SDS. Tissue samples, weighing 200–300 mg were dropped in tubes containing 0.5 ml (or 1 ml) of SDS, cut into small bits with scissors and allowed to stand in a water bath at 40°C. The contents were mixed intermittently, about every 20 min for a few seconds, on a Vortex mixer until the tissues were digested. When no particulate material was visible in the tubes, the digestion of the tissue was considered complete, which took 2–3 h. For larger pieces of tissues proportionately greater vol. of SDS was used for digestion. The tissue/SDS (w/v) ratio can be varied depending on the predicted amount of steroid in the tissue sample but adequate time should be allowed for a complete digestion. Non-pregnant human myometrium, among the tissues analysed, took the largest time for digestion.

Extraction of progesterone from the digested tissues. Depending on the predicted progesterone content of the tissue, an appropriate aliquot, generally 0.5 ml, of the digested tissue was taken for extraction. Extraction was performed by shaking with 3 vol. of ethyl acetate in glass-stoppered tubes. Two extractions were done and the supernatant was transferred to disposable glass tubes. The supernatant was evaporated to dryness under air at 40°C. The dried extract, however, contained a significant amount of sodium dodecyl sulphate (a whitish powder at the bottom of the tube) as this detergent is to some extent soluble in ethyl acetate. Sodium dodecyl sulphate present in the extracted samples strongly interfered in the radioimmunoassay of progesterone, but it was easily removed by Sephadex gel chromatography.

Sephadex gel chromatography. Sephadex LH-20 was soaked over night in ethyl acetate. Microcolumns (Pasteur pipettes) i.d. 5 mm were packed, using about 1 g of Sephadex per column, to a total length of 7.5 cm. Columns were rinsed with ethyl acetate for a period of 10 min before applying the sample. The evaporated extract from the previous step was redissolved in 0.5 ml of ethyl acetate and 0.3 ml of this was applied to the top of the column which was subsequently eluted with ethyl acetate. On the basis of the preliminary experiments (see results section) the first 3.5 ml of the eluate was collected. A new column was used for each sample. After evaporation of the collected eluate it was dissolved in 1 ml of ethyl acetate and a suitable amount, depending on the predicted progesterone content, was taken for radioimmunoassay.

Radioimmunoassay. The method for progesterone determination was essentially similar to that described by Youssefnejadian *et al.*[6]. The antiserum (FO 22.5.73) which, was a gift from Dr. Kjell Martinsson (Royal College of Veterinary Medicine, Stockholm) and found to be highly specific for progesterone, was used in a dilution of 1:1500 v/v. The antiserum was raised in sheep against BSA conjugated with 11α-hydroxyprogesterone. After addition of antiserum and of labelled progesterone to samples, or standards, they were mixed and equilibrated at 4°C overnight. The unbound steroids were removed by addition of Dextran-coated charcoal. Occasionally, the method described recently by Batra [7] was used. The radioactivity in these and all other samples was counted with liquid scintillation spectrophotometer (Packard-Tri-Carb, Model 3320) using Aquasol (NEN) as the scintillation solution. When necessary, Channels ratio was used to correct for quenching.

Chemicals. Sodium lauryl sulphate (sodium dodecyl sulphate) and progesterone were purchased from Sigma Chemical Co. Radioactive [1,2,6,7,^{3}H]-progesterone (105 Ci/mmol) was purchased from New England Nuclear Corporation and Sephadex LH-20 from Pharmacia Fine Chemicals. Human chorionic gonadotropin (HCG) was given by Leo, Sweden.

RESULTS

Recovery of ^{3}H-progesterone. The recovery in the extraction procedure was calculated by determining the fraction of the total radioactivity found in the extract and was expressed as per cent. As a double-check, radioactivity remaining in the aqueous phase was also counted. Data are presented as mean values ± standard deviation (S.D.).

Table 1 shows a complete recovery of progesterone which was not influenced by the length of digestion in SDS. With NaOH, on the other hand, recovery was not only lower but decreased with increasing digestion period. Only 80% of progesterone from NaOH solution could be recovered after 2–3 h, a period of time essential to digest even small pieces (about 200 mg) of tissues. Since, recovery remains unchanged even after 24 h in SDS, it has the additional advantage that samples, particularly those difficult to digest, could be left overnight before extraction.

Recovery of progesterone added to SDS and NaOH treated tissues (non-pregnant human myometrium) was 98.45% ± 4.4 (n = 14) and 86.63% ± 1.41

Table 1. Recovery of progesterone dissolved in NaOH or SDS solution and extracted at various times with ethyl acetate

Time (h)	Recovery % from SDS	NaOH
0.5	104.1	90.6
1	103.2	87.9
2	99.7	81.4
3	99.2	80.6
24	100.0	67.6

Five ng progesterone labelled with [^{3}H]-progesterone (10.000 c.p.m.)/ml of the respective solution was kept in a water bath at 40°C. Aliquots 0.5 ml were taken at the times shown and extracted with ethyl acetate. Values are means of duplicate determinations.

Table 2. Recovery of [^{3}H]-progesterone from tissues priorly incubated in Krebs–Ringer medium containing [^{3}H]-progesterone

Tissue	Number of determinations	Recovery % from SDS	Recovery % from NaOH
Rabbit uterus	10	97.10 ± 0.21	72.42 ± 6.90
Human myometrium (non-pregnant)	13	93.12 ± 0.97	71.82 ± 5.36

Tissue pieces (about 200 mg) were incubated for 1 h, rinsed in Krebs–Ringer for 15 s and dropped in 0.5 ml of the respective solution. Tissues were cut into small bits (c.f. Table 4) and left to be digested. Progesterone was extracted after digestion of the tissue and radioactivity in the extract was counted.

(n = 12) respectively. The recovery of progesterone, or other steroids, added to tissue homogenates, however, is not comparable to the recovery of endogenous steroid, particularly when the tissues are homogenized in a medium, such as NaOH, where the binding of progesterone to tissue is drastically changed and probably is completely eliminated. Further recovery experiments were therefore done on tissues which were allowed to accumulate [^{3}H]-progesterone under physiological conditions *in vitro* (see Methods). Table 2 shows recovery of progesterone from such tissues. About 95% progesterone could be recovered from tissues digested in SDS whereas only about 72% could be recovered from tissues digested in NaOH. Not only was there a poor recovery from the NaOH digested tissues, there was also a greater variation in the amount recovered.

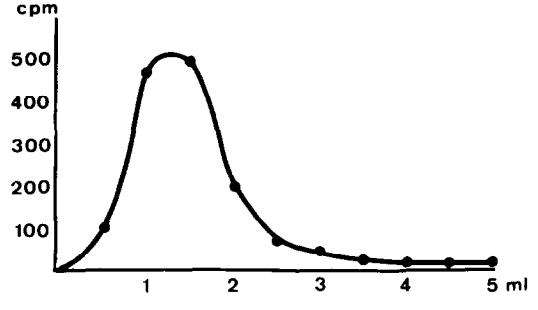

Fig. 1. Elution pattern of [^{3}H]-progesterone from Sephadex LH-20 microcolumn (Pasteur pipette), i.d. 5 mm. After the introduction of 0.3 ml of the sample which was contaminated with SDS, it was eluted with ethyl acetate and eluate was collected in 0.5 ml aliquots.

The elution profile of progesterone from Sephadex is shown in Fig. 1. On the basis of this information first 3.5 ml of the effluent was collected and the recovery of progesterone determined. Table 3 shows that the recovery of [^{3}H]-progesterone added to tissues was unaffected by passage through Sephadex column.

To further reinforce the above evidence for the completeness and high precision in the extraction of endogenous progesterone, radioactive progesterone was determined in pieces of myometrium obtained from rabbits injected with [^{3}H]-progesterone. The results in Table 4 show that 95% of the tissue radioactivity, since only 5% was found in the aq. phase, could be extracted when uterine pieces of rabbits given an intravenous injection of [^{3}H]-progesterone were analysed. The precision was also high. The recovery of radioactivity in ethyl acetate extract from tissues after a subcutaneous injection was lower, presumably due to higher concentration of water soluble metabolites as the remaining radioactivity was found in the aq. phase (see Discussion). However, here also the precision (see S.D.) was high.

Recovery as determined by RIA. It was essential to determine the actual progesterone concentration in recovery experiments to ascertain that the radioactivity determined in the previous set of experiments did in fact represent progesterone and not its metabolites or chemically hydrolized products. The recovery of a known amount (10 ng) of progesterone added

Table 3. Effect of the passage of tissue extract through Sephadex LH-20 (to remove SDS contamination) on the recovery of progesterone

Tissue	Number of determinations	Recovery % Before chromatography	Recovery % After chromatography
Human myometrium (non pregnant)	4	100.43 ± 2.53	97.55 ± 2.85
Human myometrium (pregnant)	4	108.10 ± 4.78	103.60 ± 2.90

Tissues were incubated in Krebs–Ringer medium containing [^{3}H]-progesterone, and subsequently digested and extracted. An aliquot of the extract was chromatographed. The radioactivity in the extracts before and after chromatography was counted.

Table 4. Recovery of radioactivity from uterine tissues of rabbits injected with [^{3}H]-progesterone

Mode of administration	Number of determinations	Recovery % of total in tissue*
i.v.	19	94.94 ± 0.18
s.c.	10	81.93 ± 0.47

Two hundred μCi of progesterone was injected either intravenously or subcutaneously to estrogen primed rabbits. The animals were killed after 10 min of i.v. injection or after 1 h of s.c. injection. The uterine horns were removed and several pieces of the uterine tissue weighing about 300–400 mg were cut. The pieces were rinsed in cold Krebs-Ringer solution for 1 min, weighed and radioactivity extracted with ethyl acetate after digestion in SDS for 3 h.

* Total radioactivity is the sum of that found in the extract and the aqueous phase.

Table 6. Progesterone concentration in pregnant myometrium as determined by the present procedure

Tissue sample	Wet weight (g)	Progesterone (ng g wet weight)
1	0.2057	89.9
2	0.2382	88.1
3	0.2050	92.7
4	0.2047	90.4
5	0.2022	98.9
6	0.2124	82.4
		90.40 ± 4.95

Progesterone concentration was determined by RIA in six different pieces of a myometrial strip obtained at a Cesaerean section delivery.

in SDS solution as determined by RIA, following the complete procedure of SDS digestion (2 h), extraction and chromatography, was 107% ± 7.3 (n = 10).

Table 5 shows the recovery of progesterone added to tissue digests (pregnant myometrium at term). The results show a complete recovery of added progesterone determined by the present extraction and RIA procedures.

In Table 6 progesterone concentration of tissue samples, weighing between 204–230 mg, cut from the same myometrium is shown. In spite of the possibility of some variation between each individual piece[1], the precision was relatively high. The localization, with respect to placenta, of this myometrium obtained at a Caesarean section was not determined.

The progesterone concentration of rabbit uteri obtained from two groups of pseudopregnant rabbits is shown in Table 7. As would be expected, the progesterone concentration in early (6-day) pseudopregnancy was considerably higher than that towards the end (18-day), of pseudopregnancy.

DISCUSSION

In view of the fact that detergents have recently been found to be very effective in dissolving cellular proteins, which are otherwise difficult to dissolve, for example cell membrane proteins[8, 9], it was not surprising to have obtained excellent results with SDS for digestion of the tissue. Among a number of agents tested as solubilizers for cellular membranes[8], sodium dodecyl sulphate was found to be one of the two most effective in solubilizing erythrocyte membranes. The organic nature of dodecyl sulphate as well as its high capacity for binding to proteins[10] may further contribute to dislodge steroids bound to tissue proteins. Recently, by using sodium dodecyl sulphate, Higgs and Vane[11] observed a considerable improvement in the extraction of prostaglandins from synovial fluid.

The organic character of SDS, however, also led to its carry over in the ethyl acetate extract, which

Table 7. Progesterone concentration in uteri of pseudopregnant rabbits

Number of animals	Days pseudopregnant	Progesterone (ng/g)
5	6	22.80 ± 7.59
5	18	4.88 ± 0.41

Pseudopregnancy was induced by an i.v. injection of 70 IU of HCG (Gonadex, Leo, Sweden).

Table 5. Recovery of progesterone added to tissue digest as determined by RIA

Progesterone in tissue sample (ng)	Progesterone added (ng)	Progesterone recovered (ng)	Recovery % of added progesterone
8.4	9.5	9.0	94.7
7.6	8.0	8.4	105.0
8.0	8.0	7.4	92.5
7.0	8.5	8.0	94.1
			96.58 ± 4.93

All four pieces weighing about 200 mg, were cut from a myometrial strip obtained at a Cesaerean section and processed for the determination of progesterone by RIA as described in the methods.

strongly interfered in RIA of progesterone. This was easily remedied by simply passing the extracts through Sephadex columns.

The mechanism by which Sephadex LH-20 retained SDS cannot be determined. It has been shown by Gaylor and Delwiche[12] that this Sephadex (LH-20) was very effective in retaining certain non-ionic detergents. It appears that the SDS is also retained by adsorption to the non-polar Sephadex rather than due to a molecular sieving process[12].

With the present procedure of extraction, about 100% of the tissue progesterone could be recovered. In none of the previously reported methods, such high recoveries were possible [1, 3, 4, 13, 14]. It is important while measuring recovery of added progesterone, as usually done by the addition of radioactive steroid, that sufficient time and appropriate conditions are provided for the radioactive progesterone to be able to bind to tissue proteins or be exhanged with the endogenous progesterone. For example, addition of progesterone to tissue homogenate in an unphysiological medium such as NaOH (Table 2), or when the addition of progesterone is followed rapidly by an extraction, would not be comparable to the recovery of endogenous progesterone, a point often overlooked. The method of incubating tissues with radioactive progesterone, rather than the addition of radioactivity in the homogenate, would appear to be the simplest and most acceptable method for determining the recovery of endogenous steroid. Recently Guerrero *et al.* [4] obtained only 60.2% ± 11.1 recovery of progesterone which was added to homogenate of freeze-dried tissue.

Another, but less convenient, method for checking the recovery would be to administer radioactive steroid to the animal, sacrifice the animal and remove the appropriate tissue for the recovery measurement, as was attempted in the present investigation. With this approach, however, there may also be additional complications, particularly when extensive water soluble radio metabolites are formed rapidly, since one cannot be certain that the radioactivity in aqueous phase represents inextractable progesterone or the water soluble metabolites. This may be the explanation for the apparent lower recovery in the experiment where tissues were removed after 1 h of a subcutaneous injection than that when they were removed after only 10 min of i.v. injection (Table 4).

With the present extraction procedure, when combined with radioimmunoassay as described, one is able to determine progesterone concentration in the myometrial and endometrial tissues weighing as little as 50 mg. Since the endogenous progesterone can completely be recovered in the extract, the sensitivity of the assay depends almost entirely on the method used for the determination of the steroid in the extract.

The limited data presented on the progesterone content of human myometrium at term is in agreement with those published previously where the authors have had to use several grams of tissue[1, 3, 15, 16]. The progesterone content of the uteri of 6-day pseudopregnant rabbit was much higher than that of 18-day pseudopregnant rabbit which corresponds to the level of progesterone in plasma[17, 18]. These data are also comparable to those reported recently by Challis *et al.*[14] on pregnant rabbit myometrium. These authors determined tissue progesterone by radioimmunoassay and the reported recovery from the extraction procedure, as measured by adding [^{3}H]-progesterone to the tissue homogenates, was only 70%.

The present method for the determination of progesterone in tissue is relatively simple and inexpensive. It can therefore, be easily undertaken by any laboratory using radioimmunoassay of steroids in plasma or other body fluids.

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