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# Reliability of Progesterone Measurements in Urine

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**Summary:** A radioimmunoassay (RIA) of progesterone in urine is described. After the addition of labelled progesterone, morning urine was extracted with *n*-hexane and the residue was either directly subjected to RIA, or chromatographed on celite prior to RIA. The progesterone from celite chromatography was radiochemically pure. RIA after chromatography was therefore considered valid. The non-chromatographed procedure resulted in overestimations, the degree of which was inversely proportional to progesterone content. The results obtained by the two procedures were well correlated (r = 0.88 and 0.93, for 2 different groups of samples).

*Zuverlässigkeit von Progesteronbestimmungen im Harn*

**Zusammenfassung:** Ein Radioimmunassay zur Bestimmung von Progesteron im Harn wird beschrieben. Morgenharn wurde mit *n*-Hexan extrahiert und der Rückstand entweder direkt oder nach Chromatographie an Celit dem Radioimmunassay unterzogen. Ein im Wanderungsbereich von Progesteron durchgeführter Test auf radiochemische Reinheit zeigte, daß radiochemisch reines Progesteron gemessen wurde. Der Radioimmunassay nach Chromatographie wurde deshalb als gültig angesehen. Ohne vorangehende Chromatographie wurden zu hohe Werte erhalten, deren Abweichung vom Zielwert umgekehrt proportional zum Progesterongehalt war. Beide Verfahren korrelierten gut miteinander (r = 0,88 und 0,93 für zwei Gruppen von Proben).

## Introduction

Radioimmunoassay (RIA) of progesterone in blood plasma has been a major analytical tool for the assessment of the corpus luteum function in women (e. g. l. c. (1)). Blood letting is, however, an invasive method, frequently difficult to perform, especially when serial samples are needed. Therefore, alternative approaches are of importance.

Urine is a body fluid which is easy to obtain and which may be expected to yield information equivalent to that provided by assays in plasma. Recently techniques were developed for the radioimmunoassay or chemiluminescence assay of a progesterone metabolite – pregnanediol glucuronide (2, 3). These assays require, however, special reagents (a labelled glucuronide and an antiserum to the glucuronide) which are not generally available. In addition, the purification of the glucuronides, both labelled and non-labelled, is laborious and difficult. From this point of view, a RIA progesterone in urine would be much more covenient.

RIA of progesterone in urine has been described earlier (4–7). In none of the publications, however, was a valid procedure presented, i. e. none of the procedures was shown to produce accurate results. The aim of the present investigation was to develop, for progesterone in urine, a RIA procedure validated by the test of radiochemical purity (8).

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## Material and Methods

### Urine samples

Samples of early morning urine were collected from 11 apparently healthy women (aged 24–32 years) with histories of regular menstrual cycles. The sampling was daily, during the entire menstrual cycle. A total of 316 samples were obtained. The samples were brought daily to the Department of Obstetrics and Gynaecology, Karolinska Hospital, Stockholm. They were frozen and kept at −20 °C until analysed.

A pool of urine was prepared by mixing urine samples of 5 normally menstruating women. The samples were collected during the follicular phase of the menstrual cycle. This pool was kept frozen in appropriate aliquots and used for quality control and for the test of radiochemical purity.

### Reagents

[1,2,6,7-$^3$H]Progesterone (3.66 TBq/mmol) was purchased from the Radiochemical Centre (Amersham, UK) and non-radioactive progesterone from Steraloids (Wilton, NH 03086, USA). Progesterone antiserum (Batch 82K) was a gift from the World Health Organization, Matched Reagents Programme, Geneva, Switzerland. The cross-reactions exhibited by this antiserum were as follows: 17-hydroxyprogesterone 2.0%, 20α-dihydroprogesterone 2.6%, testosterone 0.2%, cortisol < 0.1%. All other reagents were of analytical purity. The radioactivity was measured in a scintillation fluid consisting of 5.5 g of "Permablend III" (91 g of 2.5-diphenyloxazole and 9 g of *p*-bis(O-methylstyryl)benzene; Packard Instrument Co., Downers Grove, IL 60515, USA) in 1 litre of toluene. The composition of the phosphate assay buffer was as described earlier (9), but the pH was 7.4.

### Extraction and chromatography

Urine (0.5 ml) was equilibrated (25 °C, 15 min) with a solution of labelled progesterone (approximately 40 Bq in 50 µl of assay buffer) and extracted with *n*-hexane (5 ml). The aqueous layer was frozen in an ethanol : dry ice mixture, the hexane phase was decanted and evaporated under nitrogen. The residue was dissolved either in 0.5 ml of assay buffer (60 °C for 10 min) for RIA ("rapid" procedure), or in 1 ml of isooctane for chromatography.

The chromatography was performed using celite/propylene glycol columns (1 + 1, weight/volume) as described earlier (9), except that a 3.5 ml progesterone zone (instead of 4 ml) was collected. The eluent (isooctane) was evaporated and the residue was dissolved in assay buffer (0.5 ml) for RIA ("chromatographic" procedure).

Both procedures were run in duplicate for every urine sample. In both procedures a 0.2-ml aliquot of the 0.5 ml assay buffer solution was taken for the RIA proper and another 0.2-ml aliquot for a recovery measurement.

### Radioimmunoassay (RIA)

The RIA was performed as described in detail earlier (9). Briefly, the incubation of the standard or unknown solutions with the antiserum and the tracer (total volume 0.3 ml) was carried out at 60 °C for 10 min, followed by 30 °C for 30 min. The bound and free fractions were separated by charcoal at 0 °C. For the calculation of results, a logit-log transformation was used. The lowest detectable concentration (sensitivity) was 75 pmol/l.

### Test of radiochemical purity (8)

An aliquot (5.0 ml) of the urine pool was equilibrated with radioactive progesterone (approximately 500 Bq in 0.5 ml assay buffer). Ten portions (0.5 ml) of this solution were separately extracted with hexane and chromatographed as described above, except that 0.5 ml fractions were collected in the region of the 3.5 ml progesterone zone. The corresponding fractions from the 10 columns were combined (in order to accumulate sufficient amounts of radioactivity and mass), the solution was evaporated and the residue dissolved in 0.5 ml of assay buffer. Aliquots (0.2 ml) were used for the RIA on the one hand, and for the radioactivity measurements on the other. In the calculation of the RIA results, a correction for the mass of the tracer added was made (10). For each fraction, specific activity (Bq/pg) was calculated.

## Results

In order to test the validity of the progesterone assay in urine, radiochemical purity was tested by measuring the specific activities (Bq/pg) of progesterone in the first 5 consecutive half-milliliter fractions of the progesterone chromatographic zone. These fractions contained 94–95% of the recovered radioactivity. (The last 2 fractions were not used for further calculations due to a low content of radioactivity and mass). The test was repeated three times in order to make possible a statistical evaluation. Since there was no indication of an isotopic effect (11), the difference between the means of specific activities in individual fractions was tested by a one-way analysis of variance. It was found that – at the 95% confidence level – the difference was not significant; the F-value found was 2.53 (df: 4,10). Consequently, the chromatographic procedure was considered valid and the results yielded by this procedure were taken as reference values for those obtained by the rapid procedure.

The validity of the latter procedure was tested by a comparison of the results with those obtained by the chromatographic method. For this comparison, 316 samples were assayed by both methods. The measurements were divided into 2 groups according to the values indicated by the chromatographic procedure. In figure 1a, values (n = 145) below 1 nmol/l are shown, and in figure 1b, measurements (n = 143) higher than 1 nmol/l are depicted. The first group represents the majority of measurements during the follicular phase of the menstrual cycle, the second group is typical for luteal phase values.

Regression analysis indicated that the correlation coefficients (r) were high in both groups; they were 0.88 and 0.93, respectively. The slopes and their standard errors for the calculated best-fit straight lines (1.36 ± 0.06 and 1.15 ± 0.04, respectively) indicated a highly significant difference ($P < 0.001$ in both cases) from the theoretical slope = 1. Comparison of the calculated straight lines with the theoretical

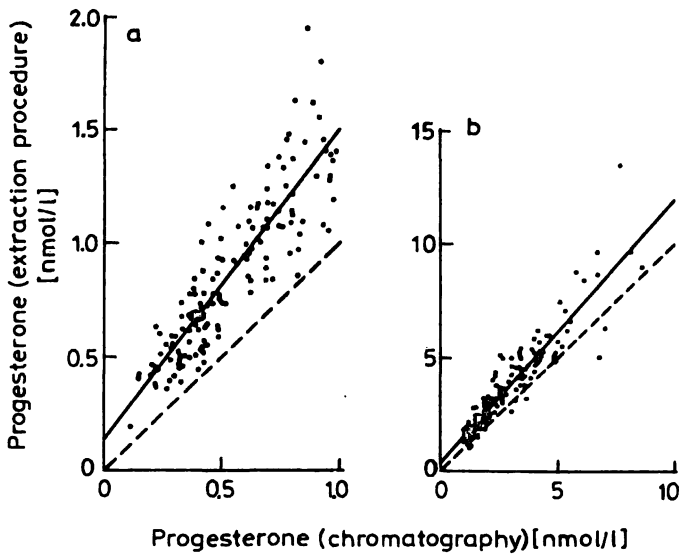


Fig. 1. Scatter diagrams of values obtained by the chromatographic (abscissa) and rapid (extraction only; ordinate) procedures. The theoretical straight lines ($y = 1x$; $r = 1$) are represented by the broken lines, the best-fit straight lines by the fully drawn lines. Scatter diagrams a and b are drawn for regions of chromatographic values 0–1 nmol/l and 1–10 nmol/l, respectively. The equations of the best-fit straight lines and the correlation coefficients are as follows:
$y_a = 0.137\ (\pm 0.036) + 1.360\ (\pm 0.061)\ x_a$; $r_a = 0.882$,
$y_b = 0.452\ (\pm 0.133) + 1.152\ (\pm 0.040)\ x_b$; $r_b = 0.925$,
where ± values in parentheses are standard errors.

Tab. 1. Degree of overestimation of results by the rapid procedure, as calculated from the regression lines (cf. fig. 1).

| Results (nmol/l) | | Percentage of overestimation |
|---|---|---|
| Chromatographic procedure | Rapid procedure | |
| 0.2 | 0.41 | 105 |
| 0.5 | 0.82 | 64 |
| 1.00 | 1.50 | 50 |
| 5.00 | 6.21 | 24 |

Tab. 2. Precision and recovery. Within-assay variation was calculated as an average from 316 true duplicates. The excessive error was expressed as the percentage of duplicates in which coefficients of variation exceeded 25% and which were excluded from the calculations. Between-assay variation was calculated from quality control samples in 7 assays. Recovery is expressed as means ± standard errors of percentages (n = 626).

| Procedure | Within-assay variation (%) | Excessive error in duplicates (%) | Between-assay variaton Mean (nmol/l) | (%) | Recovery (%) |
|---|---|---|---|---|---|
| Rapid | 8.5 | 5.1 | 0.54 | 12.9 | 69.5 ± 0.4 |
| Chromatography | 9.3 | 5.2 | 0.32 | 17.0 | 51.7 ± 0.5 |

one shows that the measurements yielded by the rapid method were, on average, higher than those obtained by the chromatographic method, and that the degree of overestimation was inversely proportional to the progesterone content of the sample (tab. 1). These observations make the rapid procedure invalid in the sense that it consistently overestimates the true content and therefore does not yield accurate results.

The data for within-assay and between-assay variations, and for recovery are summarized in table 2. It has to be pointed out that the assessment of the within-assay variation was obtained by averaging individual, within-duplicate coefficients of variation (CV), after excluding all CV-values exceeding 25%. This percentage was considered to be a limit necessitating the repetition of measurements. The between-assay variation was assessed by assaying a low-progesterone quality control pool in 7 assays. It follows from the data of table 2 that the between-assay variation was lower and recoveries higher in the rapid procedure in comparison with the chromatographic method. Relatively low recoveries for both procedures (70% and 50%, respectively) are ascribed to the use of hexane as extractant.

## Discussion

It is well established that extraction as the only purification step does not always provide valid estimates in a RIA (12–13). This happens in those cases when the extraction does not completely separate the compound assayed from cross-reacting compounds present in plasma. Not even the inclusion of a chromatographic step, however, can guarantee that the method will invariably yield valid estimates. Such a case was observed with the RIA of low levels of progesterone in the plasma of women in the follicular phase of the menstrual cycle (8).

In the present case, however, chromatography did achieve radiochemical purity and thus validity of the progesterone assay in urine. Because the chromatography used in the present study was in principle the same as that for plasma, and the specificity of the antisera employed here and in the previous study (8) was apparently similar, the radiochemical purity achieved by a single chromatography of urinary hexane extracts seems to be due to a lower content of interfering cross-reacting compounds in urine than in plasma, and/or to the use of hexane instead of ether as a solvent.

The rapid procedure was found invalid, because it produced a significant overestimation of the results. This finding is not surprising in view of the data published by *Johnson* et al. (5). Their data showed a marked overestimation of progesterone in urine when measured by RIA after hexane extraction, in comparison with the results obtained by gas chromatography-mass spectrometry.

The changing degree of overestimation by the rapid method in the present study is in accordance with our earlier observations that the specificity of a rapid assay decreases with decreasing plasma levels (13, 14). The common factor causing overestimations in the assays of plasma and urine samples seems, therefore, to be a relatively large concentration of cross-reacting factors when the concentration of progesterone is low.

The present investigation shows that valid measurements of progesterone in urine can be achieved by radioimmunoassay, provided the progesterone is subjected to preliminary purification by celite chromatography. This procedure is of a great value whenever accurate measurements are needed.

In the clinical practice, however, prompt information on significant – albeit relative – changes is sometimes more important than accurate values. It is conceivable in these cases that the inherently invalid, but much more practicable procedure without chromatography (rapid method) is used. This comment is made in view of the fact that there exists a good correlation of the results yielded by both procedures, and that the average overestimates resulting from the rapid procedure are moderate in absolute terms (e.g., 0.4 nmol/l vs. 0.2 nmol/l, 6.2 nmol/l vs. 5 nmol/l).

A clinical study comparing measurements in urine of progesterone by the chromatographic and rapid methods on the one hand, and of pregnanediol glucuronide on the other hand is in progress. This study is expected to demonstrate whether or not direct measurements of pregnanediol glucuronide can be replaced by progesterone measurements using a slightly more laborious technique but achieving assays of known degree of validity with the use of commonly available reagents.

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