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STEROID DETERMINATIONS IN HUMAN OVARIAN FOLLICULAR FLUID USING REVERSED PHASE LIQUID CHROMATOGRAPHY

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Summary—A method is presented, based on high performance liquid chromatography (HPLC) with u.v. absorbance detection, to simultaneously analyse all major unconjugated steroids in ovarian follicular fluids. The total analysis time is only 30 min. The use of a 3 mm i.d. column allows us to obtain detection limits for 3-oxo-4-ene steroids of 2 ng/ml. Calibration curves are linear in the 10–20,000 ng range per injection. Excellent agreement is obtained with the results using a previously published gaschromatography method.

INTRODUCTION

Although high performance liquid chromatography (HPLC) is a well-established analytical methodology, it is seldom used for the routine determination of androgens, oestrogens or progestagens (except cortisol) in biological samples [1–4]. The main reason for this may be that the steroid levels in serum are only slightly above the detection limit obtainable with the mostly used liquid chromatographic detector, namely the u.v.-absorbance detector. The relatively high steroid concentration present in ovarian follicular fluids (FF) however can easily be determined using this technique. Recently we described [5] the determination of total steroid profiles of ovarian FF using capillary gas chromatography (GC) and on the relation of steroid levels with the morphological maturity and the fertilizability of the oocytes [6]. The derivatization, necessary for the GC of steroid hormones, however leads to a long analysis time. To speed up the procedure, a new HPLC analysis was developed.

EXPERIMENTAL

Follicular aspiration was done under general anaesthesia by an echographically guided transvaginal pick-up. To enhance oocyte recovery, follicular walls were flushed with a solution containing a fixed concentration of Earle's balanced salt. Ovarian stimulation was with either clomiphene-citrate, hMG and hCG or the GnRH agonist goserelin, hMG and hCG.

Following oocyte retrieval the fluids were centrifuged at 300 *g* and the supernatants stored at −20°C before analysis.

Sample preparation

Acetonitrile (100 μl) was mixed with 50 μl FF. After adding 50 μl saturated sodium bicarbonate solution and 1 μg promegesterone (100 μl of a 10 μg/ml solution in ethyl acetate), the fluid was extracted with 500 μl ethyl acetate. The upper layer was evaporated to dryness and redissolved in 50 μl HPLC eluent consisting of acetonitrile, water and methanol (35:56:9 by vol). To avoid column plugging, samples are centrifuged and care is taken not to inject any undissolved residues.

To evaluate the contamination of FF with flushing medium, their concentration of phenol red (the indicator contained in Earle's salt) was evaluated by measuring u.v. absorbance at 560 nm after mixing the FF with an equal volume of buffer at pH 10. The percentage flushing in a FF sample, was calculated using a calibration curve, constructed with "uncontaminated" FF.

The amount of fluid taken for steroid analysis, as well as the quantities of acetonitrile, sodium bicarbonate solution and ethyl acetate, were increased according to increasing dilution with flushing fluid.

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Chromatography

A Varian 9010 ternary gradient solvent delivery system and a Spectra–Physics SP 8440 varable u.v.-detector were used. The 20 × 0.3 cm glass column, filled with 5 μl octadecylsilica (ChromSpher from Chrompack, The Netherlands) was fitted in a ChromSep housing (Chrompack) together with a 1 × 0.3 cm guard column. Injection was performed with a 20 μl sample loop and a Rheodyne 7125 injection valve. Peak integration was performed using a DS 654 datasystem from Varian, U.S.A.

HPLC grade solvents were from Lab-Scan, Ireland. The internal standard (I.S.) promegesterone (17α,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione) was a gift from Roussel-UCLAF, France.

For the separation of 3-oxo-4-ene steroids the eluent composition (flow rate 0.4 ml/min) is changed from a 25:66:9 acetonitrile–water–

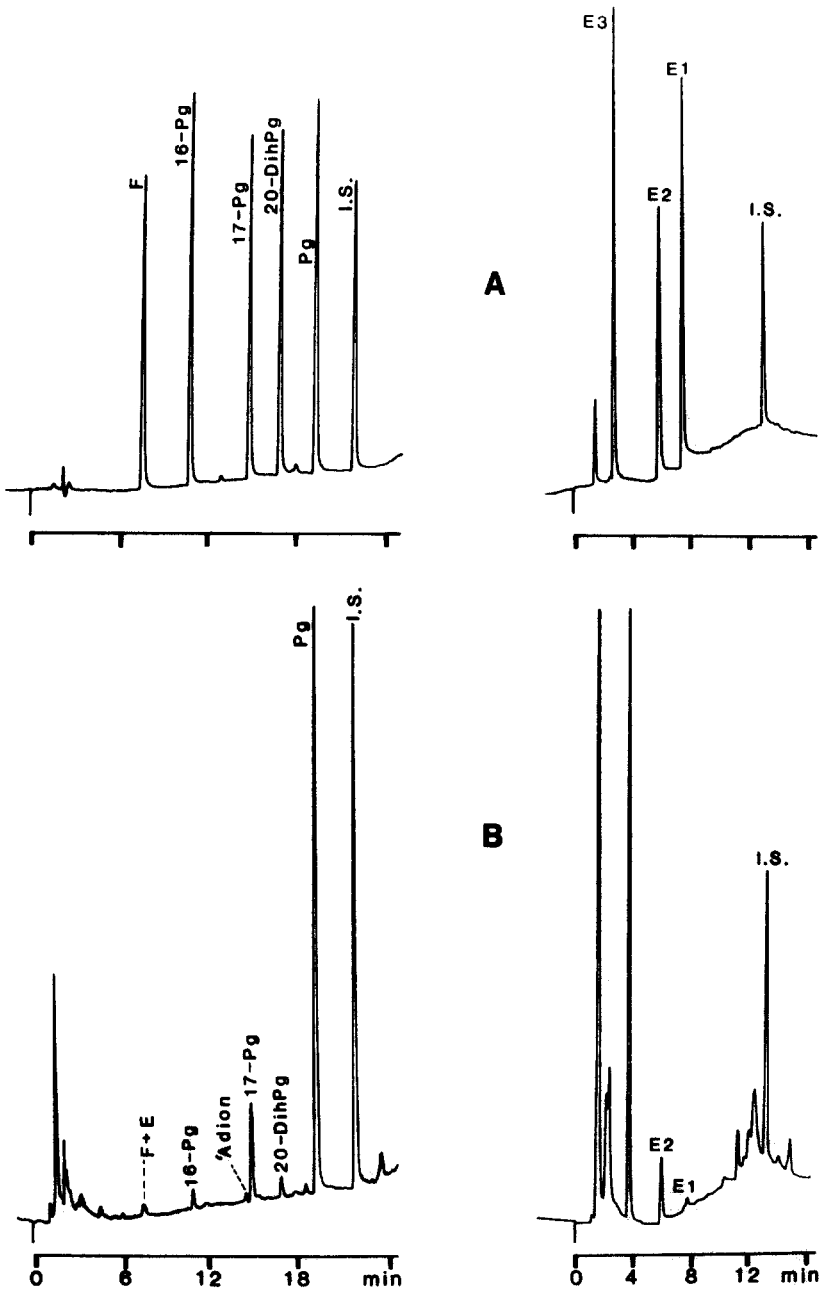

Fig. 1. Chromatograms of reference steroids (A) and steroids in a FF (B). 3-Oxo steroids were detected by absorbance measurement at 242 nm, and oestrogens at 206 nm. F, cortisol; E, cortisone; 16-Pg, 16α-hydroxyprogesterone; 17-Pg, 17-hydroxyprogesterone; 20-DihPg, 20α-dihydroprogesterone; Pg, progesterone; I.S., internal standard promegesterone; E3, oestriol; E2, oestradiol; E1, oestrone.

methanol ratio to a 70:21:9 ratio within 19 min. Detection is determined by absorbance measurement at 242 nm. For the measurement of oestradiol and oestrone, a different gradient is used without methanol: starting from a 45:55 acetonitrile–water ratio, to a 76:24 ratio within 8 min. Detection is determined at 206 nm.

Calibration

The I.S. (1 μg or 100 μl methanolic solution) was mixed with 5–1000 ng steroid, evaporated to dryness, redissolved in 50 μl water and extracted as described earlier.

Relative response factors were determined by injecting 20 μl of a mixture containing 15 μg I.S. and 5 μg of each steroid per ml eluent of 35:56:9% composition.

Recovery was determined using 1 μg quantities of steroids and the I.S. dissolved in 50 μl water followed by the normal extraction procedure.

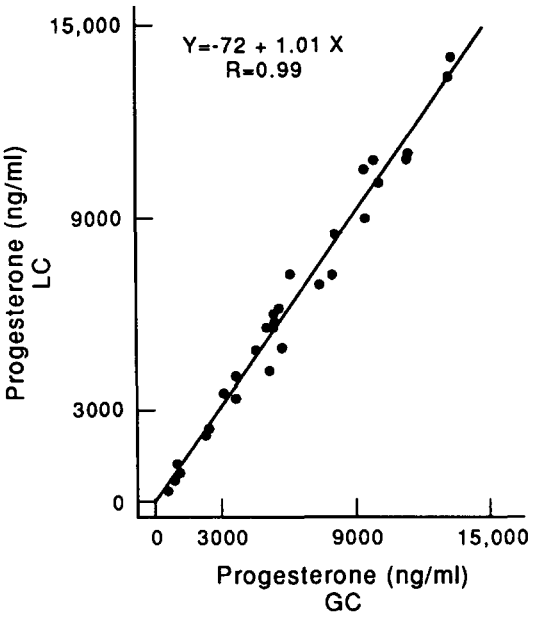

Fig. 2. Comparison of results for progesterone determinations in FF using the described HPLC method with those obtained with capillary GC.

RESULTS

Chromatograms of reference and FF steroids are represented in Fig. 1. The steroids present in FF are well separated and allow accurate peak area measurements by using interactive graphics.

The calibration curve for progesterone (Pg) is linear in the 50–20,000 ng/ml range, regression line equations being:

$$\frac{\log \text{Area Pg}}{\log \text{Area I.S.}} = -3.5 + 0.95 \log (\text{conc. Pg}) \qquad (R = 0.998)$$

or

$$\frac{\text{Area Pg}}{\text{Area I.S.}} = -0.0055 + 0.00019 (\text{conc. Pg}) \qquad (R = 0.99).$$

The detection limit (defined as a signal-to-noise ratio of 3) for the reference compound progesterone with our (older) detector is 200 pg per injection. If 50 μl FF are analysed the detection limit is 10 ng/ml. If 250 μl are analysed the detection limit for progesterone decreases to 2 ng/ml.

Table 1. Reproducibility of steroid determinations in FF

Steroid	Mean conc. (ng/ml)	% CV
Progesterone	11,595	0.93
17-Hydroxyprogesterone	1544	2.97
20α-Dihydroprogesterone	406	0.91
16α-Hydroxyprogesterone	310	1.3
Androstenedione	14.5	4.5
E2	665	0.78
E1	52.3	2.9

E2, oestradiol; E1, oestrone.

Relative response factors are 3.05–3.70 for the 3-oxo-4-ene steroids and 4.02 for oestradiol. As the calibration graphs are linear and pass through the origin, the daily determination of the response factors is a valuable substitute for the calibration curves. The recovery of a range of steroids from water was >96%. Reproducibility was tested by repeating the analysis of FF 6 times (Table 1).

Determinations using HPLC agree well with those obtained using our previously published [5] GC procedure. In Fig. 2 results of 29 follicles, determined with both methods, are presented.

DISCUSSION

HPLC offers easy and rapid analysis of all major steroids in FF. The low detection limits obtained are to a great extent due to the use of a 3 mm instead of the usual 4.6 mm i.d. column. Oestrogens are frequently detected at 280 nm. Their measurement at 206 nm is, however, at least 15 times more sensitive, especially when the eluent does not contain methanol.

Addition of saturated sodium bicarbonate solution proved adequate for the removal of interfering peaks due to phenol red and other acidic impurities without impairing the recovery of phenolic steroids. Acetonitrile

protein denaturation improves the extractability of some steroids, especially when the FF is contaminated with blood.

As other blood and FF pigments also absorb at 560 nm, the measurement of phenol red as an indicator of FF contamination with flushing fluid provides only an approximative parameter. As a consequence it is not possible to use this parameter to calculate the original steroid concentration in the FF.

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