J. steroid Biochem. Vol. 35, No. 1, pp. 83–89, 1990
Printed in Great Britain.

0022-4731/90 $3.00 + 0.00

STEROID DETERMINATIONS IN HUMAN OVARIAN FOLLICULAR FLUID USING CAPILLARY GAS CHROMATOGRAPHY

E. VANLUCHENE,* A. HINTING, M. DHONT, R. SERREYN and D. VANDEKERCKHOVE
University Hospital, Department of Gynecology, State University of Ghent, De Pintelaan 185, B-9000 Ghent, Belgium

(*Received* 26 *December* 1988; *received for publication* 7 *August* 1989)

Summary—A method is presented based on capillary GLC using both a thermionic and a flame ionization detector to simultaneously analyse all major unconjugated steroids in ovarian follicular fluids (FF). Although specificity can not always be guaranteed for the smaller concentrations of androstenedione and cortisol, accuracy and reproducibility are excellent for the major progestagens and estrogens (progesterone, 17- and 16α-hydroxyprogesterone, pregnenolone, 20α-dihydroprogesterone, estradiol and estrone). Above all the analysis is performed with relatively cheap instrumentation and products. Apart from the "profiles" of unconjugated steroids, a semi-quantitative analysis of steroid conjugates is possible if a preliminary group separation with disposable anion exchanger columns is included.

INTRODUCTION

The steroid content of follicular fluid (FF) has frequently been used in *in vitro* fertilization studies to discriminate oocytes which can be fertilized from those which cannot. In the majority of these studies, the analytical methods are based on radioimmunoassay using methods which were proven to afford reliable results for serum. Possible cross reactions of the antibodies used, occurring even after adequate chromatographic isolations influence the results. Dehennin and coworkers [1, 2] used highly specific GC–MS methods based on stable isotope dilution. Even concentrations in the low ng/ml level were accurately determined. However, the cost of instrumentation and labels and the complexity of the method do not allow general application of their methods. We chose to apply a capillary GLC analysis method whereby, for all steroids convertible to *O*-methoximes, the specific nitrogen thermionic detector was used. This method should provide accurate and reproducible results for steroids occurring in concentrations above 100 ng/ml and a semi-quantitative estimation for steroids present at lower levels. Relatively cheap instrumentation may be used, thus enabling any small laboratory to apply the method. The relatively large quantities of unconjugated steroids in FF can be analyzed directly after a simple and fast octadecylsilica adsorption step. For the much lower concentrations of sulphate and especially glucuronide conjugates, a recently described [3] group separation using disposable anion-exchange columns has been included.

*To whom correspondence should be addressed.

EXPERIMENTAL

Follicular fluids were obtained by laparoscopy from 75 patients (aged 24–39 yr) whose ovaries had been stimulated by a clomiphene-citrate, hMG and hCG combination. Following oocyte retrieval the fluids were centrifuged at 300 *g* and the supernatants stored at −20°C before analysis, which proceeded as follows.

(A) Routine analysis of unconjugated steroids in FF

To 500 μl FF were added: 2.1 ml water, 0.4 ml 1.5 M potassium acetate buffer, 1 ml 0.5 M triethylammonium sulphate solution (both pH 5.0) and finally 1250 ng (in 100 μl ethanolic solution) tetrahydrocortisol (THF) as internal standard. The diluted sample was then sucked through a 500 mg (1.5 × 0.9 cm i.d.) Bond Elut C18 (Analytichem Int., Harbor City, Calif.) disposable column, primed with 2 ml residue-free methanol (E. Merck, Darmstadt, F.R.G.) and 4 ml water. A 10-place Vac-Elut manifold (also from Analytichem Int.) was used. Flow rate was 2 drops/s. Impurities were washed out by three 2.5 ml volumes 0.15 M potassium acetate buffer. Thereafter the steroids were desorbed with 4 ml methanol. The methanol and some water residues were evaporated at 50°C using a nitrogen stream, and the residue was redissolved in 2 drops of water. 20 ml redistilled ethyl acetate were then added and after homogenization, water was removed with 5 g anhydrous sodium sulphate. Following transfer to conical test tubes and evaporation of the ethyl acetate, *O*-methyloximes were formed by reaction with 100 μl of a 100 mg per ml warm (62°C) pyridine solution of *O*-methylhydroxylamine hydrochloride at 62°C over 30 min.

The pyridine was blown off and trimethylsilylation was carried out with 100 μl *N*-trimethylsilylimidazole at 100°C over 2 h. The samples were finally purified using small Lipidex 5000 (Packard Instr., The Netherlands) columns and could be stored for several months, if necessary, at −10°C.

(B) Analysis of conjugated steroids

Ion exchange. 5–10 ml FF or serum are processed. To the biological fluid were added water, 1.5 M potassium acetate buffer and 0.5 M triethylammonium sulphate solution (pH 5.0) so that final concentrations of 0.15 and 0.125 M respectively were obtained. This mixture was applied to a BE C18 column (primed as above) and sucked through at 2 drops/s. After rinsing with 7.5 ml 0.15 M potassium acetate buffer (pH 5.0), the column was sucked dry and removed from the Vac Elut. A 500 mg Bond Elut SAX column (1.5 × 0.9 cm i.d., from Analytichem Int.) was washed successively with 4 ml methanol, 5 ml water, 25 ml 0.5 M acetic acid in water and again with 25 ml water in order to replace the original chloride counter-ion by the acetate ion. Then 4 ml methanol were applied and sucked through, except for 1 ml which was left in the anion-exchange column. The BE C18 column upon which the steroids had been adsorbed before was attached air tight to the BE SAX column. A 4 ml volume of methanol was applied to desorb the free and conjugated steroids from the C18 column and simultaneously load them on the SAX column (rate = 2 drops/s). Unconjugated steroids are not retained and can be collected here. After removing the C18 column an additional 1 ml methanol was sucked through the SAX column to complete elution of the unconjugated steroids.

The glucuronic acid conjugates were eluted with 8.4 ml 0.02 M formic acid in methanol:water (1:1). The fraction assumed to contain steroid sulphates was desorbed with 8.4 ml of a 0.25 M TEAS solution (pH 5) in methanol:water (1:1).

Enzymatic hydrolysis and solvolysis. After adding 5 μg testosterone as internal standard, the steroid glucuronide fraction was taken almost to dryness using a rotary evaporator. The residue was dissolved in 4.5 ml water, brought to pH 4.8 with 0.5 M sodium hydroxide and incubated with 40 μl *Helix pomatia* extract (from Industrie Biologique Française, Clichy, France) at 37°C for 24 h. The incubate was saturated with solid sodium bicarbonate and extracted with 20 ml ethyl acetate. Finally, the extract was washed with 5 ml water.

To the steroid sulphate fraction were added 5 μg testosterone as internal standard. It was then taken to dryness and redissolved in water (4.5 ml). One g of sodium chloride was added and the pH adjusted to 0.9 using conc. sulphuric acid. A 30 min extraction with 20 ml ethyl acetate (automatic shaking apparatus) was then performed. After centrifugation at 1500 *g* the water layer was removed and the upper layer was incubated for 16 h at 37°C. Finally, the ethyl acetate was washed with saturated bicarbonate and water. Derivatization was done as described above.

Recovery of added radioactive dehydroepiandrosterone sulphate was always above 90% in accordance with our earlier investigations on serum [3].

(C) Gas chromatography

The gas chromatograph was a Varian 3500 (Varian Ass. Inc., Calif.) equipped with a flame ionization (FID) and a nitrogen-sensitive thermionic detector. The column (a 25 m polydimethylsiloxane CP-Sil 5 CB, i.d. 0.32 mm, film thickness 0.12 μm from Chrompack, The Netherlands) was connected to the falling needle injector (also from Chrompack) using polyimide glue. Carrier gas (hydrogen) flow was 2 ml per min. The oven temperature was initially 160°C and was programmed: first at 20°C/min to 200°C and then at 4°C/min to 290°C. Injector and detectors were heated at 300°C. To enable the analysis of the low quantities of e.g. androstenedione and cortisol, nearly the total sample (8 μl out of a 15 μl sample) had to be injected. All steroids were quantitated using the nitrogen specific detector except for estradiol for which FID was used. A Varian 654 datasystem provided accurate peak area determinations and offered the possibility of storing and recalculating chromatograms using interactive graphics. Appropriate response factors in relation to tetrahydrocortisol or testosterone were determined and taken into account for calculation of concentrations using the internal standard method.

RESULTS

(A) Unconjugated steroids

The chromatograms in Figs 1 and 2 show that, apart from the major unconjugated steroids (progesterone, estradiol, 16α- and 17-hydroxyprogesterone, pregnenolone) which could be quantitated with both FID and nitrogen specific detector, the smaller quantities of androstenedione, testosterone, estrone, cortisol and deoxycorticosterone can only or at least far more accurately be detected with the nitrogen detector and on condition that nearly half of the total sample is injected. Blank samples (Fig. 3) do not contain any peaks at the retention times of the steroids to be determined. Specificity can be checked by comparison of both detector responses relative to the internal standard peak: steroid derivatives possessing 2 methoxime-functions (androstenedione, cortisol) exhibit 2-fold higher relative response than those with only one methoxime-function.

Calibration curves are straight lines (intercept = 0) in the 1–500 ng (amount injected) interval.

Recoveries of radioactive steroids from octadecylsilica disposable columns were always over 90% as has been described earlier [3].

Repeated analysis of a FF pool (12 different follicles obtained from 5 patients) generated the

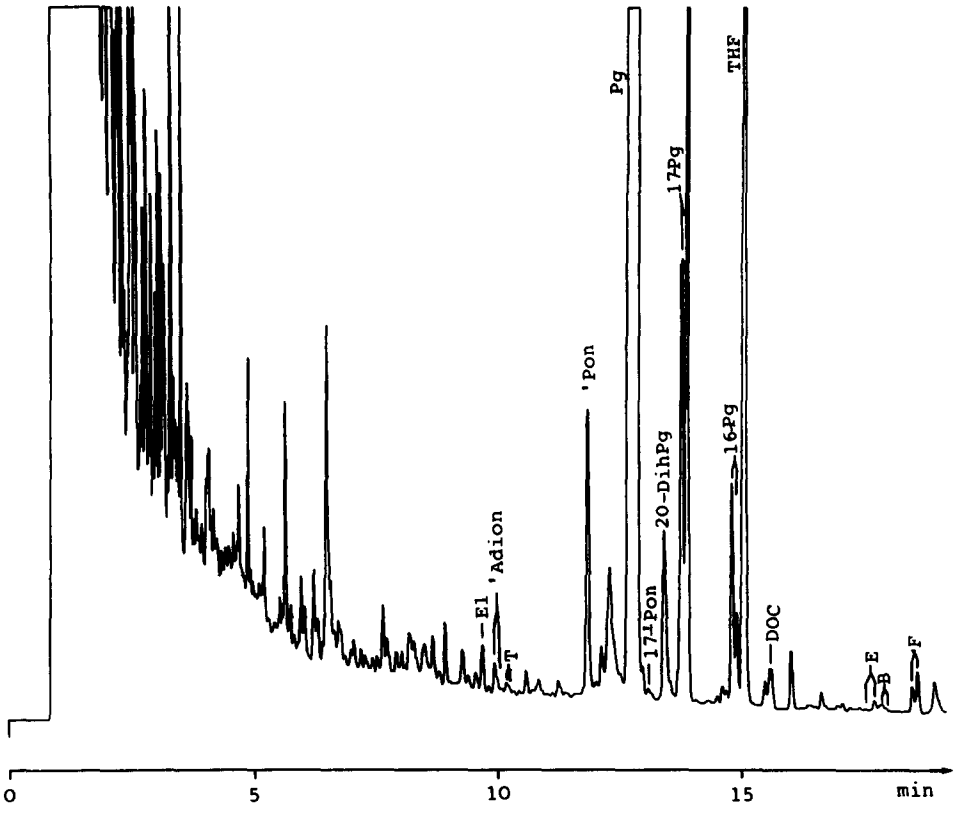

Fig. 1. Chromatogram of (*O*-methoxime) trimethylsilylethers of unconjugated steroids in follicular fluid detected by the nitrogen sensitive thermionic detector. Only methoxime containing steroid derivatives are detected and the response is proportional to the number of methoxime groups in the molecules. List of abbreviations used in the figures: El: estrone, 'Adion: androstenedione, T: testosterone, Pon: pregnenolone, Pg: progesterone, 17-Pon: 17-hydroxypregnenolone, 20-DihPg: 20α-dihydroprogesterone, 17-Pg: 17-hydroxyprogesterone, 16-Pg: 16α-hydroxyprogesterone, THF: tetrahydrocortisol, DOC: 11-deoxycorticosterone, E: cortisone, B: corticosterone, F: cortisol, E2: estradiol, CH: cholesterol, Andr: androsterone, Etio: etiocholanolone, eAndr: epiandrosterone, 16-DNA: 16α-hydroxydehydroepiandrosterone, KA: 11-oxoandrosterone, HA: 11β-hydroxyandrosterone, 17-Pon: 3α,17α-dihydroxy-5β-pregnan-20-one, THE: tetrahydrocortisone, THA: tetrahydro-11-dehydrocorticosterone, THB: tetrahydrocorticosterone, aTHB: 3α,11,17α,21-tetrahydroxy-5α-pregnan-20-one.

precision data of Table 1. Each sample was analyzed with a new Bond Elut C18 column. When we re-used these columns the same repeatability could be obtained: for 2 FF pools of different origin, analyzed 5 times on a same column, the variation coefficients were between 3.0 and 4.2% for estradiol, progesterone, 17-hydroxyprogesterone, pregnenolone and estrone.

Analysis of 373 different follicular fluids provided the mean concentrations of Table 2 and Fig. 4. The androstenedione concentration is especially highly variable. Whereas a mature follicle contains less than 10 ng/ml androstenedione associated with high progesterone levels, an immature follicle can contain up to 200 ng/ml androstenedione while its progesterone levels are low.

(B) Conjugated steroids

A 10 ml FF pool (15 follicles in total from 10 patients), and a 10 ml serum pool (taken peripherally on the day of oocyte retrieval), when analysed with the same above described methods provided the concentrations listed in Table 3. Chromatograms are given in Figs 5 and 6. Testosterone is not a good choice as an internal standard because testosterone sulphate is present in FF [1]. The steroid sulphate levels of FF in Table 4 may be underestimated by some 10%.

DISCUSSION

The described methods provide concentrations of unconjugated steroids similar to those found in other studies using RIA [6 and references therein], except for androstenedione for which our results are markedly lower. Similar low androgen levels have been obtained by Dehennin *et al.* [1, 2] using isotope dilution GC–MS. The specificity of the present method is based merely on the use of a nitrogen specific detector. Control of interference is possible by comparing the results obtained with FID detection. Furthermore, we frequently reinjected samples on a slightly more polar capillary column

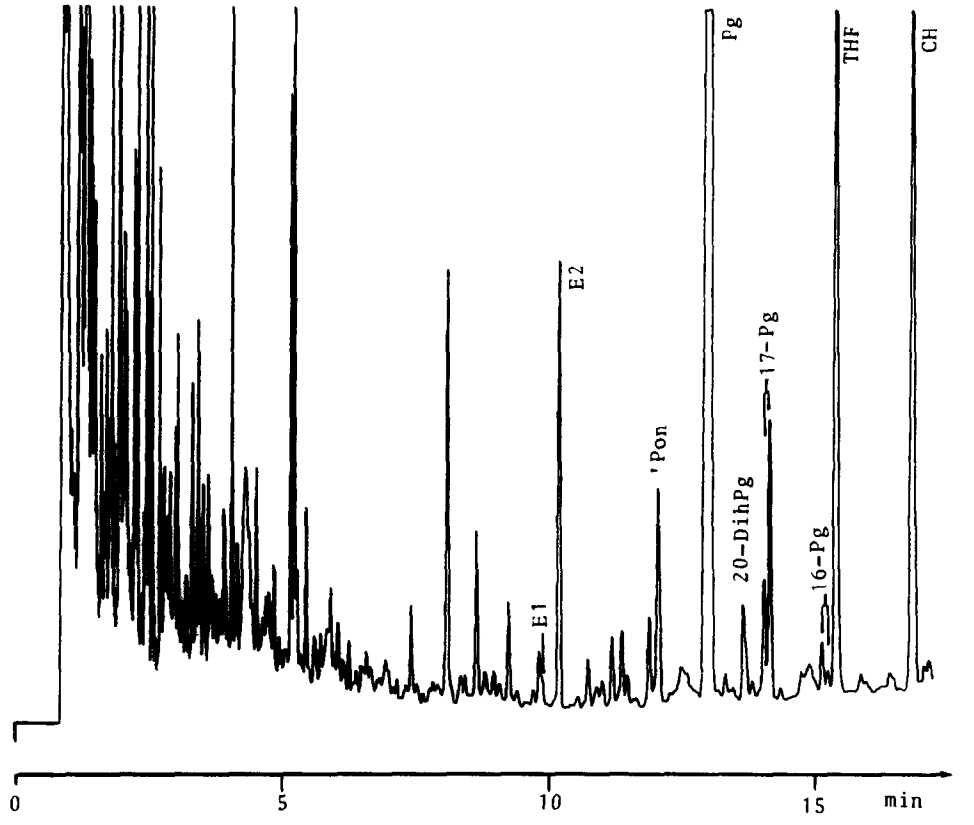

Fig. 2. Chromatogram of unconjugated steroids in follicular fluid. Detection by flame ionization: non-methoxime containing steroid derivatives are also detected.

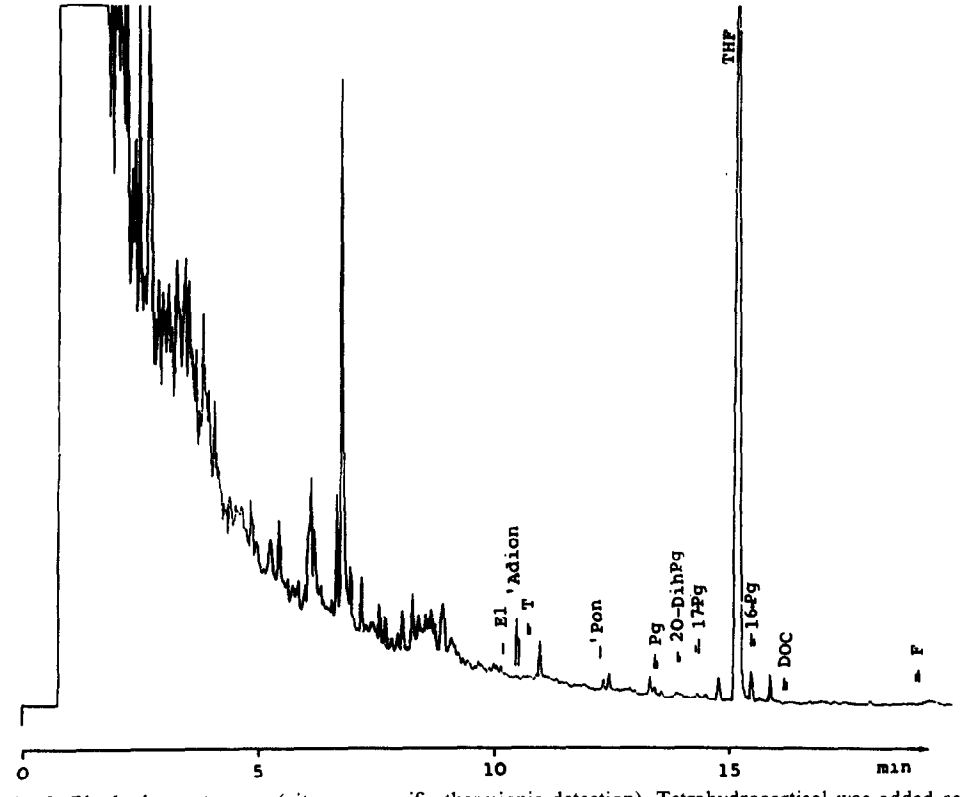

Fig. 3. Blank chromatogram (nitrogen specific thermionic detection). Tetrahydrocortisol was added as internal standard.

Table 1. Reproducibility of steroid quantition in a pool of follicular fluid. The analyses were repeated 5 times

Steroid	Mean (ng/ml)	CV %
Progesterone	10,993	4.5
17-Hydroxyprogesterone	1,679	4.5
Estradiol	785	4.2
16α-Hydroxyprogesterone	479	3.4
Pregnenolone	460	4.4
20α-Dihydroprogesterone	344	4.0
Estrone	89	3.0
Cortisol	64	12.2

Table 2. Steroid concentrations determined in 373 individual follicular fluid samples obtained after clomiphene-hMG-hCG stimulation in an *in vitro* fertilization program

Steroid	Mean (ng/ml)	SD
Progesterone	8206	4496
17-Hydroxyprogesterone	1289	740
Estradiol	712	359
Pregnenolone	332	266
16α-Hydroxyprogesterone	309	188
20α-Dihydroprogesterone	262	186
Estrone	85	90
Cortisol	46	19
Androstenedione	46	73

(RSL 200 from Alltech, Eke, Belgium) in order to make sure that no foreign substances were affecting the analytical methods.

The octadecylsilica adsorption used was adapted from the work of Heikkinnen *et al.* [5] and was

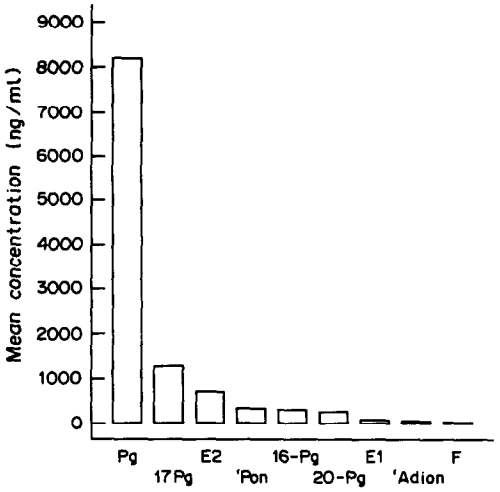

Fig. 4. Bar graph of relative steroid quantities in 373 samples of follicular fluid.

checked by us previously: both unconjugated and conjugated steroids are recovered quantitatively [4].

The disposable anion-exchange method described earlier [6] works well for serum, urine and amniotic fluid. Total conjugated steroid profiles found were similar to those published using the more elaborate Sephadex LH20 or Sephadex based lipophilic anion-exchange methods [7–10]. The glucuronide and

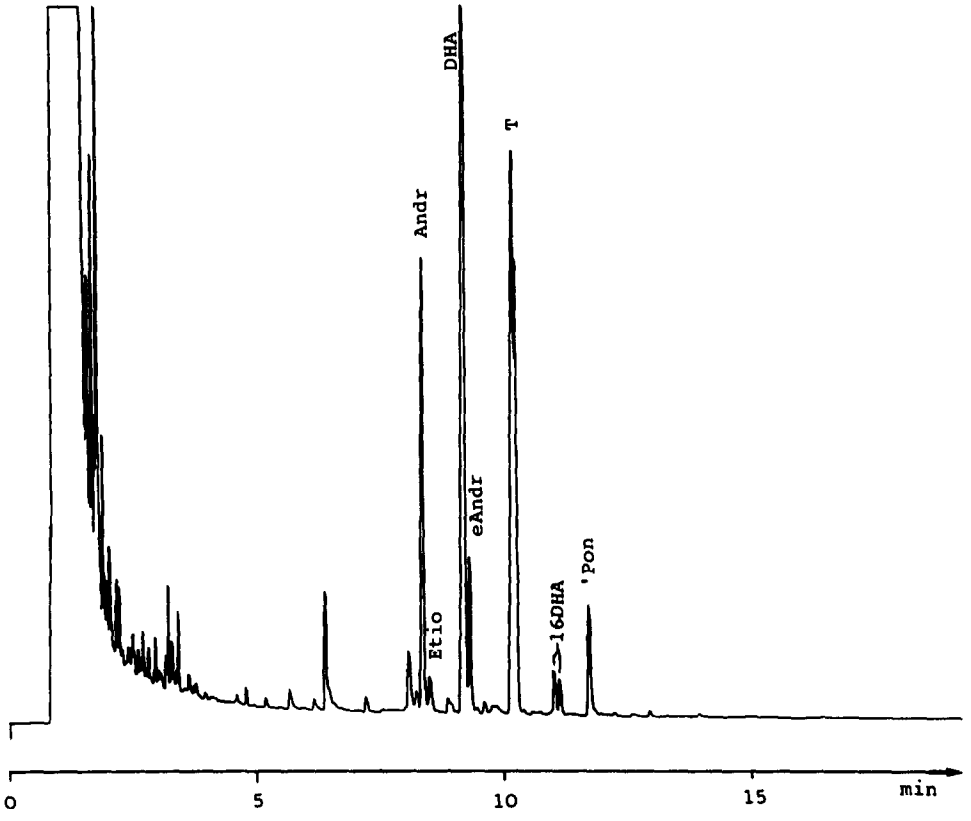

Fig. 5. Chromatogram of the assumed steroid sulphate fraction of a pool of follicular fluid (nitrogen specific thermionic detection).

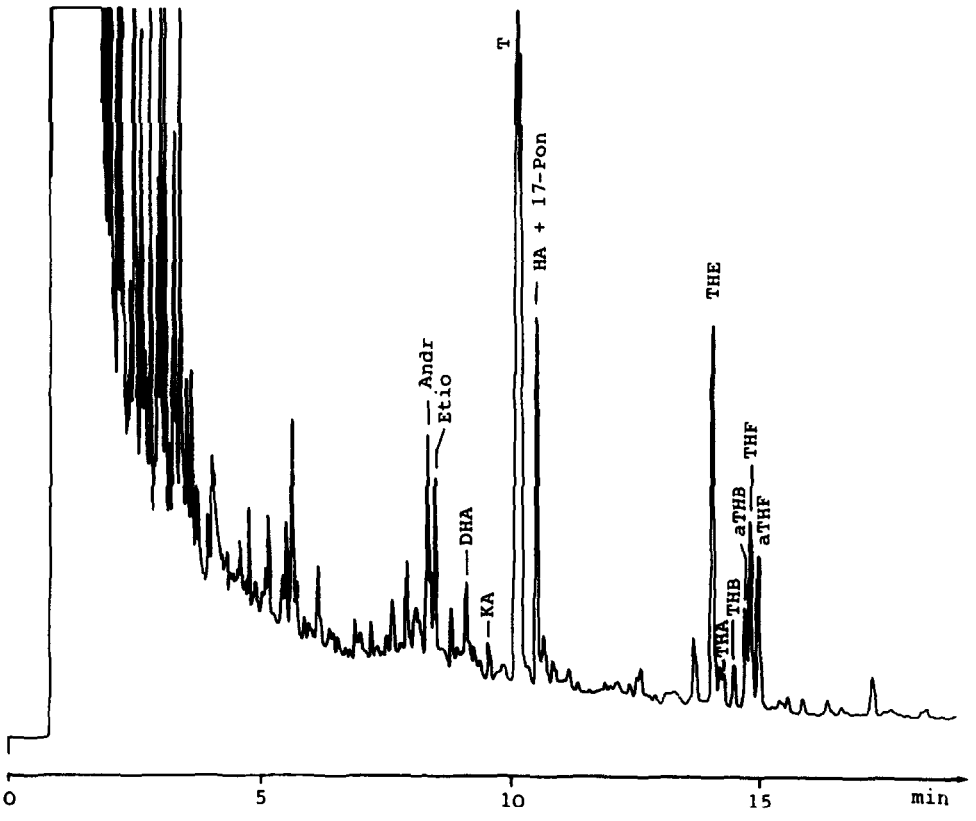

Fig. 6. Chromatogram of the assumed steroid glucuronide fraction of a pool of follicular fluid (nitrogen specific thermionic detection).

Table 3. Unconjugated steroids and steroids assumed to be sulphate and glucuronide conjugates in a pool of follicular fluid and in a serum pool on the day of oocyte retrieval

Steroid	Follicle (ng/ml)	Serum (ng/ml)	Ratio (follicle:serum)
Progesterone	12,748	1.2	10,623
Cortisol	78	169	0.46
Androsterone-S	415	447	0.93
Dehydroepiandrosterone-S	1,175	1433	0.82
16α-Hydroxydehydroepiandrosterone-S	91	46	1.98
Pregnenolone-S	147	41	3.59
3β,17α,20α-5-Pregnenetriol-S	84	111	0.76
Androsterone-G	18	42	0.43
Etiocholanolone-G	7	35	0.20
11β-Hydroxyandrosterone-G	19	56	0.34
Tetrahydrocortisone G	44	85	0.52
Tetrahydrocortisol G	20	42	0.48

(S = sulphate; G = glucuronide).

sulphate fractions should be analysed by a method not requiring prior hydrolysis of these conjugates. As long as this has not been done, comparison of follicular fluid sulphate profiles with those of serum and of glucuronide profiles with those found in urine, strongly suggests that our glucuronide and sulphate fractions do exist as true steroid glucuronides and sulphates.

Dehydroepiandrosterone sulphate levels are lower than those determined by Dehennin *et al.* [1]. The pool of FF we analysed was composed only of FF with progesterone levels above 5000 ng/ml, and this may be the reason for the discrepancy.

The FF/serum ratios in Table 3 suggest a relatively high local production of sulphates of 16α-hydroxydehydroepiandrosterone and pregnenolone. On the other hand, most of the cortisol and dehydroepiandrosterone sulphate could originate from the blood.

Acknowledgement—This study was made possible with the financial support of the Belgian NFWO.

REFERENCES

1. Dehennin L., Jondet M. and Scholler R.: Androgen and 19-norsteroid profiles in human preovulatory follicles from stimulated cycles: an isotope dilution-mass spectrometric study. *J. Steroid Biochem.* **26** (1987) 399–405.

2. Dehennin L., Nahoul K. and Scholler R.: Steroid 21-hydroxylation by human preovulatory follicles from stimulated cycles: a mass spectrometrical study of deoxycorticosterone, 21-hydroxypregnenolone and 11-deoxycortisol in follicular fluid. *J. Steroid Biochem.* **26** (1987) 337–343.
3. Vanluchene E. and Vandekerckhove D.: Group fractionation of free and conjugated steroids by means of disposable silica-based anion-exchange columns. *J. Chromat.* **456** (1988) 175–182.
4. Vanluchene E. and Vandekerckhove D.: Total steroid profiling with capillary GC. Details on sample preparation. *J. High Res. Chromat. Commun.* **8** (1985) 400–403.
5. Heikkinen R., Fotsis T. and Adlercreutz H.: Reversed-phase C18 cartridge for extraction of estrogens from urine and plasma. *Clin. Chem.* **27** (1981) 1186–1189.
6. Hill G. A., Herbert C. M., Wentz A. C. and Osteen K. G.: Use of individual human follicles to compare oocyte *in vitro* fertilization to granulosa cell *in vitro* luteinization. *Fert. Steril.* **48** (1987) 258–264.
7. Jänne O., Vihko R., Sjövall J. and Sjövall K.: Determination of steroid mono- and disulfates in human plasma. *Clin. Chim. Acta* **23** (1969) 405–412.
8. Setchell K. D. R., Almé B., Axelson M. and Sjövall J.: The multicomponent analysis of conjugates of neutral steroids in urine by lipophilic ion exchange chromatography and computerised gas chromatography-mass spectrometry. *J. Steroid Biochem.* **7** (1976) 615–629.
9. Axelson M. and Sahlberg B.-L.: Group separation and gas chromatography-mass spectrometry of conjugated steroids in plasma. *J. Steroid Biochem.* **18** (1983) 313–321.
10. Homoki J., Roitman E. and Shackleton C. H. L.: Characterization of the major steroids present in amniotic fluid obtained between the 15th and 17th weeks of gestation. *J. Steroid Biochem.* **19** (1983) 1061–1068.