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STEROID PROFILES OF BODY FLUIDS OTHER THAN URINE, OBTAINED BY CAPILLARY GAS CHROMATOGRAPHY

E. VANLUCHENE* and D. VANDEKERCKHOVE
Gynecological Clinic, State University of Ghent, Academic Hospital, De Pintelaan 185, B-9000 Ghent (Belgium)
and
J. JONCKHEERE and A. DE LEENHEER
Laboratory of Medical Biochemistry and Clinical Analysis, State University of Ghent, Academic Hospital, De Pintelaan 185, B-9000 Ghent (Belgium)

SUMMARY

Steroid profiling by capillary gas chromatography has been applied to the analysis of free and conjugated steroids in serum, ovarian follicles, lutein cyst and peritoneal fluids. Sep-Pak C_{18} octadecylsilica was used for extraction, whereas the separation of free and different conjugates was accomplished by successive hydrolysis and liquid extraction steps.

INTRODUCTION

Profiling of steroids has frequently been used to demonstrate the separation power and the column inertness obtainable with capillary gas chromatography (GC). These profiles or multi-component steroid analyses were, however, nearly always restricted to urinary metabolites. This paper describes an extension of the profiling technique to other body fluids, such as serum or plasma, ovarian follicle, lutein cyst and peritoneal fluids.

EXPERIMENTAL

For the clean-up of the samples, containing various amounts of protein or lipid material, an octadecylsilica (Sep-Pak C_{18}; Waters) extraction step is used, essentially as described by Heikkinen *et al.*[1]. This step enables both free and sulphated and glucuro-conjugated steroids to be concentrated. These three classes are then further separated from each other by successive extractions with ethyl acetate: free steroids are recovered first; after solvolysis of the water layer, sulphated steroids are recovered and, finally, after β-glucuronidase enzymatic hydrolysis, steroid glucuronides and mixed conjugates are obtained.

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Extraction procedure

To the biological fluid (from 50 μl to 13 ml) 1250 ng of tetrahydrocortisol are added as an internal standard. Levonorgestrel (2000 ng) is added to minimize protein binding of estrogens. After 30 min, potassium acetate buffer of pH 5, 0.5 *M* triethylammonium sulphate (TEAS) and water are added so that the final concentration of the acetate buffer reaches 0.15 *M* and that of the TEAS 0.125 *M*. After 20 min, the sample is passed through a Sep-Pak C_{18} cartridge at an elution rate of two drops per second. The cartridge is washed with 10 ml of 0.15 *M* acetate buffer (pH 5) and, finally, the steroids are eluted with 3 ml of pesticide-free methanol. After addition of 1250 ng of 5β-cholestan-3α-ol as a second internal standard, the methanol is evaporated and the residue is dissolved in 5 ml of 8% sodium carbonate solution. Free steroids are extracted with 15 ml of ethyl acetate. The water layer is transferred to another glass tube. The ethyl acetate layer is washed with 5 ml of water and finally dried over anhydrous sodium sulphate. To the combined water layers, 1250 ng of both internal standards, 2 g of sodium chloride, ten drops of concentrated sulphuric acid and 25 ml of ethyl acetate are added. The conjugates are transferred to the ethyl acetate layer by shaking for 15 min and are then solvolysed by incubating for 16 h at 37°C. The solvolysed steroid sulphates are purified by washing the ethyl acetate layer with 5 ml of sodium hydrogen carbonate and 5 ml of water.

To the combined water phases 1250 ng of 5β-cholestan-3α-ol are added. After evaporation of the remaining ethyl acetate by concentrating to *ca.* 3 ml, 2 ml of 2 *M* acetic acid are added and the mixture is diluted with water to a final volume of 9 ml. A 1-ml volume of 2 *M* acetate buffer (pH 6.2) is then added, together with two drops of bacterial β-glucuronidase (Institut Pasteur) containing 200 I.U./ml, and the mixture is incubated for 2 days at 37°C. Extraction as above with 20 ml of ethyl acetate yields separate fractions of free and hydrolysed steroids, which are converted into methoximes, whereafter a persilylation reaction is performed with trimethylsilylimidazole. After purification by Lipidex 5000 (Packard) chromatography, the sample is concentrated to 100 μl and stored in the frozen state.

GC and mass spectrometric (GC–MS) procedures

Capillary GC was performed on commercial polydimethylsiloxane fused-silica open tubular columns (25 m × 0.32 mm I.D.) with hydrogen as the carrier gas. An all-glass moving-needle injector and a flame-ionization detector used. The temperature was increased 5°C/min from 190 to 280°C.

Quantitation was obtained by relating peak heights (or areas, especially with leading peaks at higher concentrations) to the peak height (area) of the internal standard (tetrahydrocortisol for free and sulphated steroids and 3α-cholestan-5β-ol for glucuronides). Relative response coefficients for each steroid were taken into account. For absolute identification, a Hewlett-Packard 5992B quadrupole mass spectrometer was used, coupled to the same capillary column by an open-split interface. Spectra were obtained under electron-impact conditions (70 eV) at a scan speed of 690 a.m.u./sec.

RESULTS

The profiling with FID allows the quantitation in serum samples of only cor-

tisol, cortisone and, in certain instances, progesterone and 17-hydroxyprogesterone. This limitation applies to the non-pregnant condition. In advanced pregnancy serum or plasma, free estriol and estradiol can also measured. Fig. 1 shows a chromatogram of free steroids present in an ovulatory follicle on day 14 of the menstrual cycle. Estradiol and estrone, pregnenolone, progesterone and its metabolites, 17-hydroxyprogesterone, 16α-hydroxyprogesterone and 20α-hydroxy-4-pregnen-3-one are de-

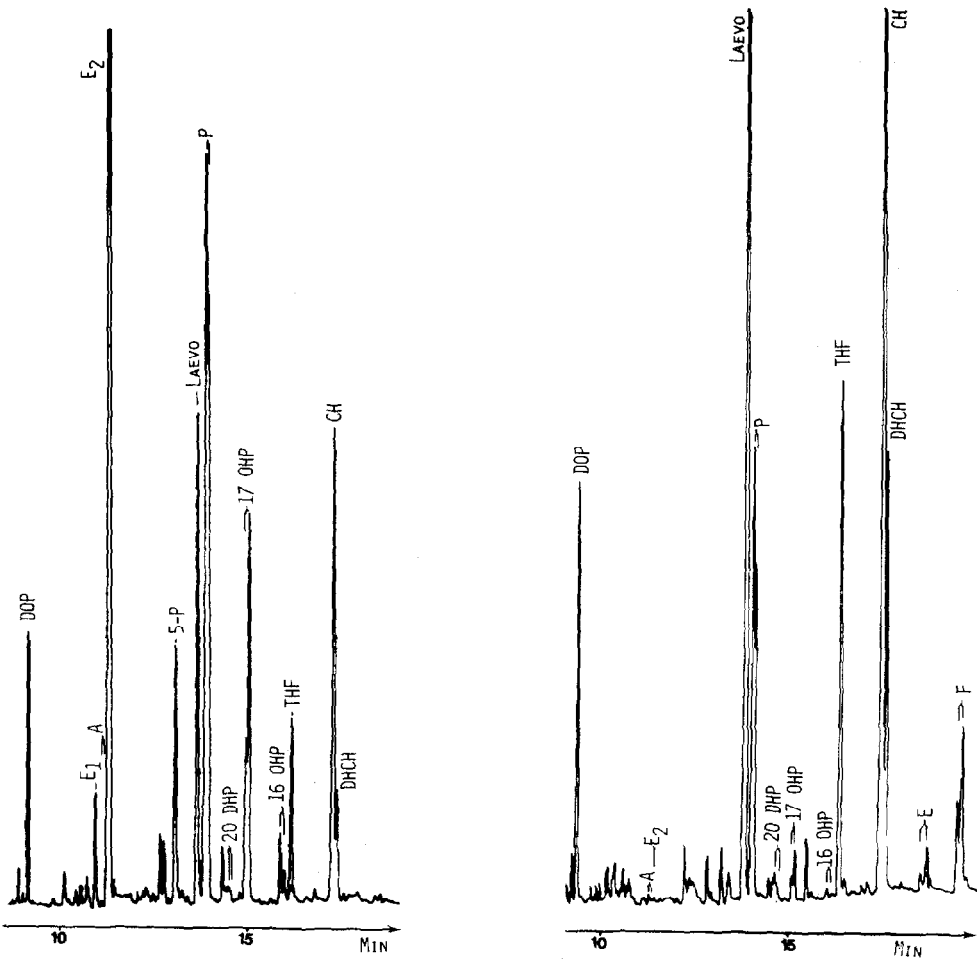

Fig. 1. Chromatogram of free steroids present in ovarian follicle fluid of day 14 of the menstrual cycle. Abbreviations: DOP = dioctyl phthalate; E_1 = estrone; A = androstenedione; E_2 = estradiol; T = testosterone; ααP = 3α-hydroxy-5α-pregnan-20-one; 5-P = pregnenolone; Laevo = levonorgestrel; P = progesterone; 20 DHP = 20α-hydroxy-4-pregnen-3-one; 17 OHP = 17-hydroxyprogesterone; 16 OHP = 16α-hydroxyprogesterone; CH = cholesterol; DHCH = 5β-cholestan-3α-ol; Andr = androsterone; Etio = etiocholanolone; DHA = dehydroepiandrosterone; eAndr = epiandrosterone; ββ'Ad = 5-androsten-3β,17β-diol; 16 DHA = 16α-hydroxydehydroepiandrosterone; 'Pd = 5-pregnene-3β,20α-diol; βα Pd = 5α-pregnane-3β,20α-diol; 'Pt = 5-pregnene-3β,17α,20α-triol; Pd = pregnanediol; THE = tetrahydrocortisone; THA = tetrahydrodehydrocorticosterone; THB = tetrahydrocorticosterone; THF = tetrahydrocortisol; αCn = α-cortolone; αCl = α-cortol; F = cortisol.

Fig. 2. Free steroids present in the peritoneal fluid of a patient on day 17 of the menstrual cycle.

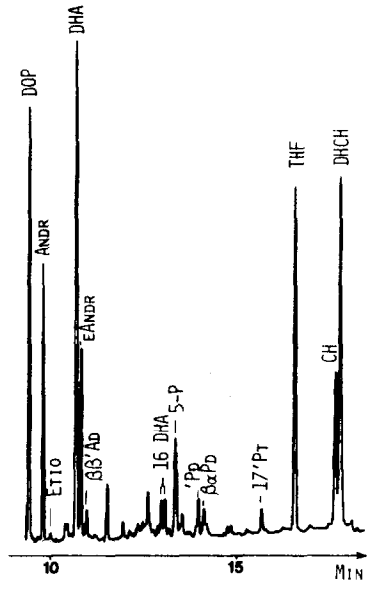

Fig. 3. Steroid sulphates in serum.

Fig. 4. Steroid sulphates in an ovarian lutein cyst.

tectable. Follicles harvested earlier in the cycle also contain androstenedione. Follicles obtained after luteinization, *i.e.*, on day 15, just prior to ovulation, contain only minute amounts of estradiol, whereas the progesterone concentration has increased in comparison with preceding days of the cycle. In atretic follicles only androstenedione and 17-hydroxyprogesterone can be detected. Fig. 2 shows free steroids in the peritoneal fluid of a patient on day 17 of an ovulatory menstrual cycle. Progesterone and some of its metabolites are present, together with cortisol and cortisone.

The steroid sulphates in serum consist mainly of 3β-hydroxy-5-ene steroids (dehydroepiandrosterone, 5-androstene-3β,17β-diol, 16α-hydroxydehydroepiandrosterone, pregnenolone, 5-pregnen-3β,20α-diol, and 5-pregnen-3β,17α,20α-triol), as shown in Fig. 3. Some 5α-reduced androstanes (androsterone, epiandrosterone) and pregnanes (3β-hydroxy-5α-pregnan-20-one, 5α-pregnan-3α,20α-diol, 5α-pregnane-3β,20α-diol) are also present. Ovarian follicles and lutein cysts contain the same steroid sulphates as serum. In Fig. 4 the steroid sulphates of a lutein cyst are represented.

The steroid glucuronide conjugates in serum (Fig. 5) are identical with those found in urine. Quantitatively, however, serum contains relatively less tetrahydrocortisone and more tetrahydrocorticosterone. Pregnanediol glucuronide serum concentrations vary according to the menstrual cycle, as in urines. Most steroids have

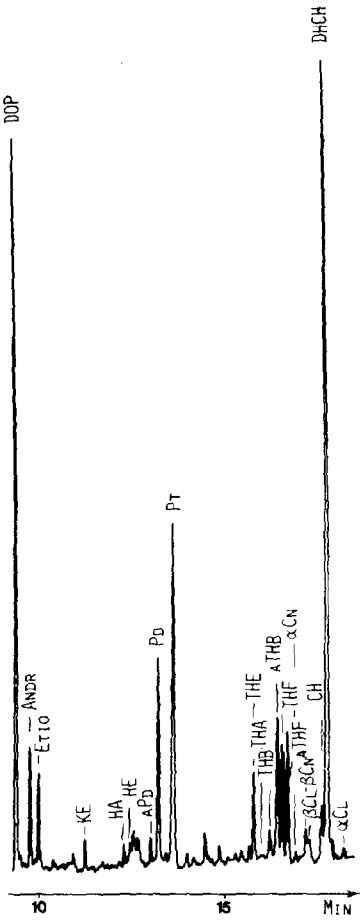

Fig. 5. Steroid glucuronide conjugates in serum.

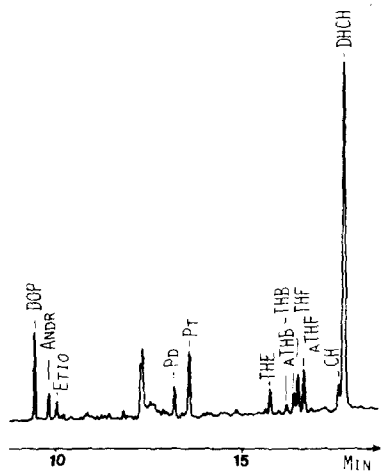

Fig. 6. Steroid glucuronides in ovarian follicle fluid.

the 5β-configuration. Ovarian structures and peritoneal fluid contain the same steroid glucuronides as occur in serum. Fig. 6 shows preovulatory follicle glucuronides.

Quantitative data are summarized in Table I. The presence of high free steroid concentrations in ovarian structures is to be expected because of their steroid-synthesizing entities. The peritoneal fluid can contain high progesterone levels, possibly of ovarian origin, on specific days of the menstrual cycle. Sulphated steroids in ovarian structures have similar concentrations to those in serum. Those of peritoneal fluids amount to 60% of the serum concentrations. Steroid glucuronide concentrations in ovarian fluids are not different from serum values.

After adding fifteen different free steroids normally present in serum to water, their recovery (capillary GC analysis after Sep-Pak c_{18} and ethyl acetate extraction) was 94–100%. Labelled progesterone and estradiol were added to some ovulatory follicle fluids and peritoneal fluids. The recovery for both steroids was 92–95% in the methanol eluate of the Sep-Pak C_{18} cartridges.

TABLE I

MEAN STEROID CONCENTRATIONS (ng/ml) IN DIFFERENT BODY FLUIDS

Source	E_2	*A*	*P*	*17 OHP*	*16 OHP*	*20 DHP*	*DHAS**	*AndrS**	*AndrG***	*THEG***	*F*
Preovulatory follicles (n = 14)	2714	142	4511	1641	290	116	1109	307	18	21	
Lutein cysts (n = 16)	80	127	7453	594	165	468	1297	356	18	22	
Atretic follicles (n = 8)	20	1448	20	364	20	20	1917	490			
Peritoneal fluid (n = 30)			5–700				638	179			60
Serum (n = 23)							1123	314	22	31	206

* Steroid sulphates, expressed as ng of free steroid.
** Steroid glucuronides, expressed as ng of free steroid.

TABLE II

REPRODUCIBILITY OF ANALYSIS

Coefficients of variation (C.V.) and mean levels (ng/ml) are given.

Parameter	*Steroid*			
	E_2	*5-P*	*P*	*17 OHP*
Mean (ng/ml)	318	177	856	2981
C.V. (%)	6.6	3.4	6.8	2.6

Because of the relatively low sensitivity of the method, it is important to obtain sufficient sample volumes. In some follicles only 10 μl of fluid can be aspirated, whereas large lutein cysts may contain up to 100 ml of fluid. Detectability is strongly dependent on the volume of fluid that can be used for analysis. If 10 ml of fluid are available the detection limit (FID) is 2 ng/ml. If only 1 ml is available, the detection limit increases to 20 ng/ml, and in 250 μl 80 ng/ml can hardly be detected. Fortunately, the method permits the quantitation of all detectable steroids in one analysis.

Reproducibility was tested by five replicate analyses of one sample. The coefficient of variation was 2–7% when 1 ml fluid was analysed, as shown in Table II.

DISCUSSION

The described steroid profiling technique with octadecylsilica as extractant gives much better recoveries of free steroids than our earlier, time-consuming XAD-2 extraction method. Further, much less impurities are seen in the chromatograms. Less solvents and, especially, less glassware (which causes impurity peaks) are needed in the octadecylsilica procedure. Perhaps the recovery could be further enhanced by extracting the samples at 64°C. At this temperature, further reduction of steroid-protein binding is obtained, as stated by Axelson and Sahlberg[2].

The successive solvent extraction and hydrolysis steps permit a rapid separation of the free steroids and different conjugates, present in the samples. A beter alternative would be anion-exchange chromatography, but this would make the methodology more complex. However, for more accurate determinations it would be the method of choice, especially as the partition coefficients (water–ethyl acetate) of steroids having a different number of phenolic or alcoholic hydroxyl functions may differ.

In our method, solvolysis is accomplished prior to enzymatic hydrolysis. Recent findings indicated a possible conversion of 3β-hydroxy-5-pregnenes to their corresponding 3-oxo-4-ene analogs when enzymatic hydrolysis with enzyme preparations from *Helix pomatia* are used[3].

CONCLUSION

Steroid profiling by capillary GC can be extended to biological fluids of various origins such as serum or plasma and peritoneal, amniotic, ovarian follicle and cyst fluids. Although its sensitivity for the measurement of steroids is several orders of

magnitude less than that offered by radioimmunological methods, the ability to provide a compound analysis in a single sample of all the important steroids in a steroid-rich fluid makes it an attractive method.

ACKNOWLEDGEMENT

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REFERENCES

1 R. Heikkinen, T. Fotsis and H. Adlercreutz, *Clin. Chem.*, 27 (1981) 1186–1189.
2 M. Axelson and B.-L. Sahlberg, *Anal. Lett.*, 14 (1981) 771–782.
3 E. Vanluchene, W. Eechaute and D. Vandekerckhove, *J. Steroid Biochem.*, 16 (1982) 701-703.