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Estrogens as additional parameters of steroid profile analysis in doping control

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Introduction:

Steroid profile analysis is a well established mean of detecting application of endogenous steroids and medicaments with influence to parameters of the steroid profile. Estrogens as their major metabolic excretion products estrogen glucuronides and estrogen sulphates as well as steroid sulphates in general are hitherto neglected in routine quantification of urinary steroid concentrations [1]. Screening results of this class of steroids may give additional or missing data to the steroid profile especially in women [2, 3]. Presented data are the result of case studies and should be confirmed with more women participating.

Analytical method:

A screening method based on the sample preparation for anabolic steroid conjugates is introduced. Sample preparation of 2 ml urine matrix includes the cleavage of steroid glucuronides with E.coli enzyme and their subsequent basic extraction. A solid phase extraction step isolates steroid sulphates in the aqueous residue, followed by solvolytic cleavage in a mixture of methanol, ethylacetate and H_2SO_4 and a basic extraction step. Internal standard mixture is added in two steps to glucuronide fraction and the sulphate fraction prior to cleavage and contains both glucuronides and sulphates for control of cleavage of steroid conjugates and 17α-Ethinyl-19-nortestosterone as a separate calibration standard for estrogens (apart from [2,2,4,4-$^2H_4$]- Etiocholanolone).

The screening for steroid glucuronides and sulphates in human urine in detail is presented in the following flow scheme:

2 ml urine + 0.75 ml (17.3 g Na_2HPO_4 + 8.8 g $NaH_2PO_4*H_2O$ + 203.9 g H_2O)

add 40 µl internal standard mixture 1 :

Norethisterone 12.5 ppm; 17α-Methyltestosterone 25 ppm; [2,2,4,4-$^2H_4$]- Etiocholanolone 25 ppm; [2,2,3,4,4-$^2H_5$]-Androsterone,17ß-D-Glucuronide 25 ppm; [16,16,17-$^2H_3$]-Testosterone 4.5 ppm; [16,16,17-$^2H_3$]-Epitestosterone 0.75 ppm

↓

Add 25 µl ß-Glucuronidase E.coli, 50°C 60 min;

add 250 µl $K_2CO_3/KHCO_3$ (20 %);

add 5 ml tert.-Butylmethylether, shake 5 min, centrifugate

↓ ↓

1. Decant organic phase, evaporate, derivatise etherresidue with 100µl, MSTFA-NH_4I-TMS-S-Ethanol 60°C 15 min, transfer in vials → GC/MS of free and glucuronated steroids

2. 3ml aqueous layer, remove add 0.75 ml sodiumacetat (1M pH 4.9) centrifugate, extract on C_{18} column wash step 2ml H_2O

↓

Elution with 2 ml Methanol, evaporation, + 0.5 ml Methanol

..... add 40 µl internal standard 2 :

Norethisterone 12.5 ppm; 17α-Methyltestosterone 25 ppm; [2,2,4,4-$^2H_4$]- Etiocholanolone 25 ppm; [2,2,4,4-$^2H_5$]-Androsterone,17ß-D-Sulphate 25 ppm; [16,16,17-$^2H_3$]-Epitestosteron-Sulfat 25 ppm; [16,16,17-$^2H_3$]-Testosterone 4.5 ppm

↓

5 ml Ethylacetate/H_2SO_4 (250 ml/200mg) 55°C, 30 min;

add 0.25 ml KOH 1M, evaporate Ethylacetate;

add 1ml (17.3 g Na_2HPO_4 + 8.8 g $NaH_2PO_4*H_2O$ + 203.9 g H_2O) +0.25 ml $K_2CO_3/KHCO_3$ 20 %;

add 5 ml tert.-Butylmethylether, shake 5 min, centrifugate; and handle like glucuronated fraction (see above) →GC/MS of sulphated steroids

Results of spiked urines and validation:

Steroid	RT [min]	Calibration factor	Two ions in single mode	Recovery [%]	Coefficient of variation
Estrone	11,63	0,2661	414, 399	80	13,6
17α-Estradiol	11,34	0,9737	416, 285	78	15,2
17ß-Estradiol	12,05	0,6446	416, 285	78	14,3
Norethisterone	13,56	1	442, 427		
16-Ketoestradiol	17,10	0,027	502, 487	79	11,0
16α-Hydroxyestrone	16,84	1,7248	487, 502	76	7,4
2-Hydroxyestrone	15,02	0,0154	502, 487	97	7,7
Estriol	16,73	0,5028	504, 489	71	7,1
16α-Hydroxyestriol	17,33	0,4826	504, 489	66	8,6
HP 6890/5973, column HP Ultra-1, 17 m, 0.2 mm i.D., 0.11 µm, programm : 180°C; 3°C/min up to 230°C; 30°C/min up to 310°C					

Table 1: Data concerning the validation of sample preparation and methods (validation of recovery and coefficient of variation with 250 ng of estrogens) and GC/MS-conditions and properties of O-Trimethyl-ethers of additionally quantified steroids (data refers to the major products of per-TMS-silylation)

Concerning validation (linearity, recovery and coefficient of variation in repeated sample preparation), the monitored estrogens fulfil the requirements for proper quantification within the validated range 25 - 1250 ng/ml in SIM mode. With the employed sample preparation no problems are arising from oxidation and degradation of labile estrogens, such as 2-Hydroxy-estrone (see recovery). Because of enolisation 16α-Hydroxyestron and 16-Ketoestradiol share the same minor derivatisation product. Additional minor products can be detected.

Results of case study 1: Longitudinal study of pregnancy morning urines

Estrogens and androgens as their glucuronides and sulphates were quantified. Estrogens show substantial increase during pregnancy with peak concentrations between the 35th to 40th week up to 1000 times the levels measured at pregnancy onset. A following decrease until delivery is obvious, presumably a result of decreasing concentrations of precursor hormones [4]. Highest concentrations are observed for the steroid glucuronides of Pregnanediol (80000 ng/ml) and Estriol (13000 ng/ml). 17ß-Estradiol, Estrone and 16α-Hydroxyestriol are also excreted in significant amounts [3].

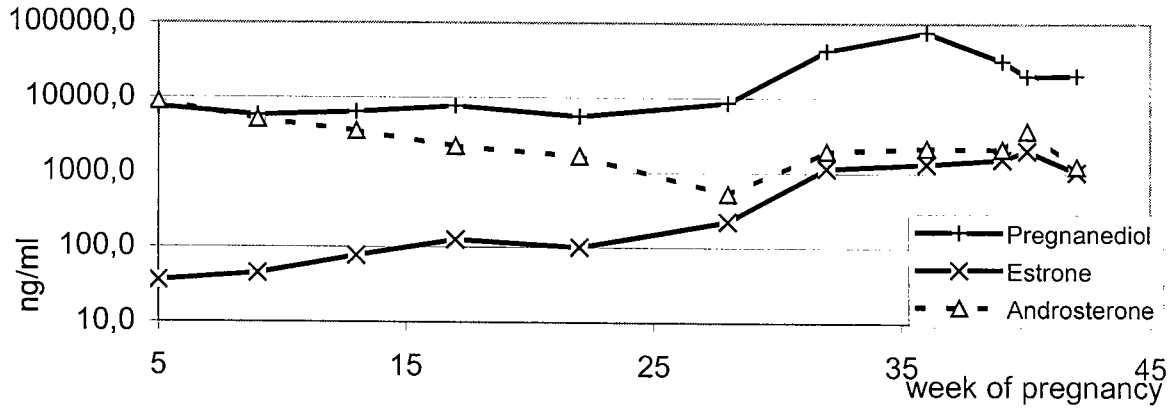

Fig 1: Concentrations of some urinary glucuronides during pregnancy

Estrogens in pregnancy are mainly synthesized from the feto-placental unit, the excretion reflects biosynthesis and metabolism of this group of steroids [2, 3].
Rising concentrations of Testosterone, Etiocholanolone, Androsterone and DHEA at mid-pregnancy can be explained by a consecutive stronger production of steroids by the fetal adrenal gland. Maximum concentrations of androgen glucuronides exceed those measured during the menstrual cycle (Androsterone and Etiocholanolone at 5000 to 8500 ng/ml and Epitestosterone at 103 ng/ml).
Steroid sulphates are in general only 10 – 20 % of glucuronide concentrations, contamination from the glucuronides can be excluded. 16α-Hydroxyestradiol-Sulphate and 17α-Estradiol with up to 100 ng/ml exceed the concentrations of the glucuronides, with the latter not being detected as glucuronide. The urinary concentrations below 72 ng/ml do not reflect the significant role of DHEA-Sulphate as a precursor hormone. This can be an indicator that synthesis and metabolism of DHEA-Sulphate may be faster than excretion [3].
Development in pregnancy can be well represented by steroid sulphates, since maximum concentrations of estrogens are reached early and androgens show an earlier increase in mid-pregnancy than glucuronides. Different ratios give information to the development of pregnancy. Some ratios show little variation throughout pregnancy (e.g. Estriol versus Estriol-Sulphate), other ratios show a typical progress throughout (Estriol versus Androsterone), or only in the beginning of pregnancy (Estriol versus Estrone).

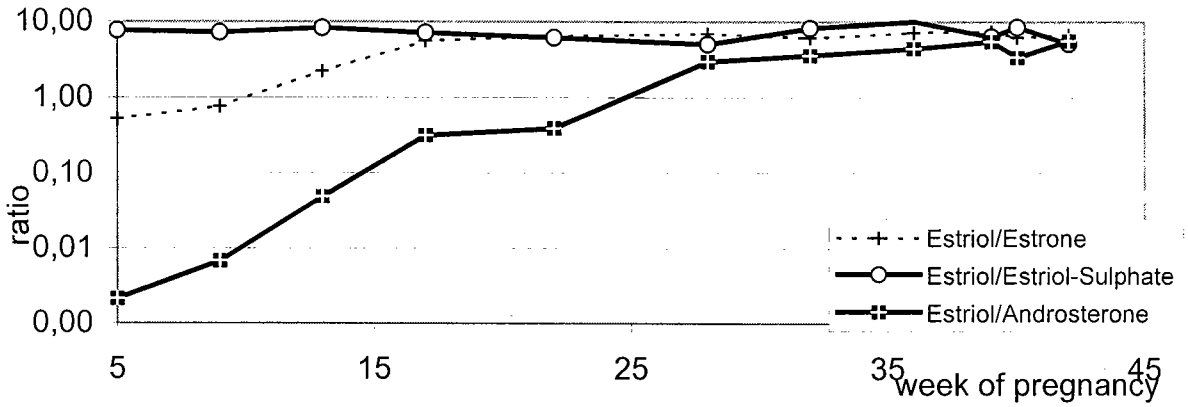

Fig. 2: Different urinary ratios during pregnancy

Results can be interesting in steroid profile analysis of women, separating pathological conditions, application of steroids and onset of pregnancy, thus being helpful in decisions upon suspicious cases with high levels of steroids. As estrogens can be incorporated into the screening procedure for anabolic androgenic steroids, a separate test for hCG can be omitted.

Longitudinal study during one menstrual cycle

Estrogen and androgen glucuronides and sulphates are quantified in morning urines of one female menstrual cycle without application of oral contraceptives. Menses is beginning at day 3, whereas the exact day of ovulation can not be determined.
Steroid glucuronides are dominant compared to the corresponding sulphates, with sulphates summing up to 15 – 20 % of glucuronide concentrations, but DHEA-sulphate surpassing the glucuronide at factor 100. Concentrations of estrogens in both fractions are low, detectable estrogens are Estriol, Estrone and 2-Hydroxyestrone in the glucuronide fraction (up to 20 ng/ml), detected only as sulphates are 17α-Estradiol and Estrone (up to 31 ng/ml).
Excretion of 5α-metabolite in the sulphate fraction is enhanced in relation to the glucuronides,

resulting in a higher ratio of 5α/5ß-metabolites.
Concentrations of steroids show strong intraindividual (and interindividual variability), so that the most stable parameters during the cycle are ratios. Androsterone versus Etiocholanolone and the ratio of their sulphates are the most stable parameters, giving evidence of common adrenal synthesis. The variation of the ratio of androgen glucuronides versus corresponding or related sulphates is higher. It is not sure, if a decrease up to day 11 and following increase till day 15 recalls a systematic alteration in succession of cyclical hormonal changes, or if it is atypical. The systematic elevation of the ratio of androgens versus estrogens in mid-cycle, e.g. Androsterone-Glucuronide versus Estrone-Glucuronide is significant. Pregnanediol is connected with the strongest variation, with maximum concentration shortly after ovulation and decline of concentration before menses. Estrogens seem to be elevated in the second half of the cycle, parallel to Pregnanediol concentration. Recent studies could not confirm a dependency of steroids from menstrual cycle, but also found Androsterone/Etiocholanolone the most stable parameter [5].

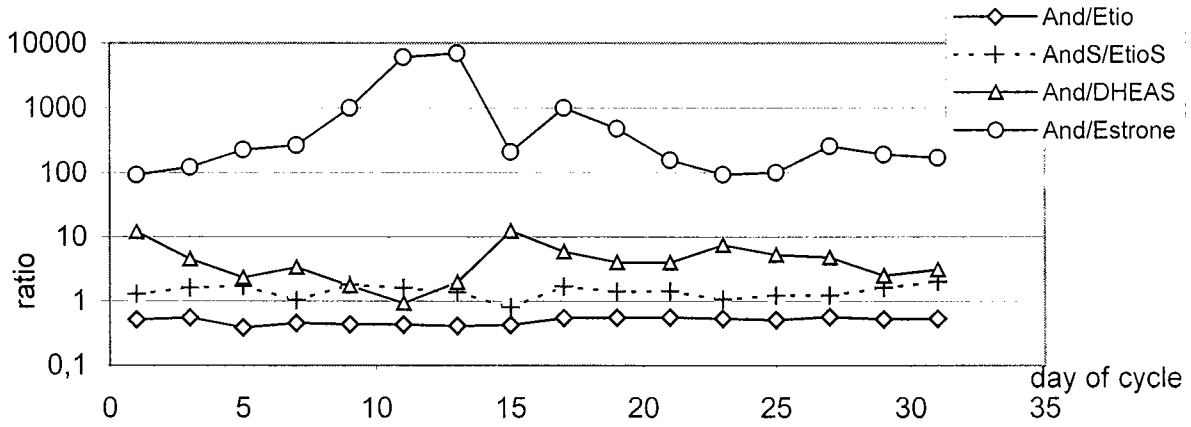

Fig. 3 . Different ratios throughout one female cycle

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