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RECENT ADVANCES
IN DOPING ANALYSIS
(5)

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The Interpretation of Female Steroid Profiles
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The Interpretation of Female Steroid Profiles

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Abstract

Some characteristic parameters of the female urinary steroid profile, analysed for dope control purposes, are very stable. The most stable steroid profile parameters for female are the ratios androsterone/etiocholanolone (A/E) and 5α -androstan- $3\alpha,17\beta$ -diol/ 5β -androstan- $3\alpha,17\beta$ -diol (Adiol/Bdiol). The in male individual very stable ratio of testosterone/epitestosterone (T/epiT), often shows high variation as concentrations of these steroids are near the detection limit and often coeluting with other endogenous substances.

These parameters can be influenced by the application of oral contraceptives and ethanol as well as bacterial activities in the urine. Pregnancy and special female diseases (adrenal syndrom, polycystic ovarial syndrom) often lead to characteristic steroidprofile patterns and problems of analytical evaluation.

For judging single urine samples or endocrinological studies it is important to take those factors into consideration.

Introduction

Steroidprofiling is a well known method of clinical endocrinology to detect enzyme deficiencies (1,2). This method was introduced in dope analysis by Donike et. al. (3,4) to detect misuse of exogenous testosterone. Several studies have shown, that the steroid profile parameters, especially the steroid ratios used for dope control purposes are very stable. These ratios are not influenced by training or severe physical endurance performance (5,15), menstrual cycle (6,7), circadian rhythm (8), or circannual rhythm (9).

Factors which lead to obvious changes of these parameters are going to be explained and therefore should be taken into account when interpreting female steroidprofiles.

Experimental

Sample preparation (10)

2 ml of urine and 20 µl of an internal standard mixture (17 α -methyltestosterone 50ppm, [2,2,4,4,-²H₄]-etiocholanolone 50ppm, [16,16,17,-²H₃]-testosterone 2ppm, [2,2,4,4,²H₄]-11 β -hydroxyandrosterone 14ppm) are added to a Amberlite XAD-2 column. The column (pasteur pipette, closed with glass pearl, bed height 2 cm) is washed with 2 ml of bidestilled water and the absorbed fraction is eluted with 2 ml of methanol. The methanolic eluate is evaporated to dryness and the residue is dissolved in 1 ml of 0.2 M sodium phosphate buffer pH 7.

To the buffer solution, 50 µl of beta-glucuronidase from E.coli is added and hydrolysis is performed for 1 h at 50°C. The buffered solution is alkalized with 250µl of 7% potassium carbonate solution to pH 9-10 and the steroids are extracted with 5 ml of tert.-butylmethylether on a mechanical shaker for 5 minutes. After centrifugation the ethereal layer is transferred and evaporated to dryness under vacuo.

Derivatisation

The dry residue is derivatised with 100 µl of MSTFA/NH₄I/ethanethiol 1000:2:6 (v:w:v) and heated for 15 min at 60°C.

3 µl of the solution are injected into the GC/MS.

GC/MS parameters

GC/MS: HP 5890/HP 5971A (Hewlett Packard)

electron impact: 70 eV

column: HP Ultra I (OV-1), 17m, 0.2mm i.d., 0.11 µm film thickness

carrier gas: 1ml helium at 180°C, split 1:10

temperature programm: 180°C, 3°C per min, 229°C, 40°C per min, 320°C

GC/MS quantitation: (11,19)

Urine samples

Application of ethanol (12): Two grams of ethanol per kg body weight was orally applied (application form: wodka) to six female healthy volunteers within four hours. Urine samples were taken every two hours.

Influence of oral contraceptives (13): Four healthy female volunteers collected morning urine samples for two female menstrual cycles each; one female menstrual cycle under the influence of oral contraceptives and one without.

Influence of pregnancy: Three pregnant volunteers collected morning urine samples one time a week until delivery.

Influence of polycystic ovarian syndrom: 20 urine samples collected in hospitals (Frankfurt/Main and Mönchengladbach)

Other factors influencing steroid profiles were examined in urine samples taken from routine analysis samples.

Results and Discussion

Bacterial activities (11)

Bacterial activity in urine samples is indicated by an elevated pH-value.

Female urines in doping control have a higher incidence of bacterial activity than male urines. The possibility to find urines with bacterial activity in summer is much higher than in winter, as transport conditions of the urine samples may be compromised.

The main change in steroid profiles of such urines are the evaluation of 5α -androstandion and 5β -androstandion (Fig1) resulting from bacterial deconjugation of androsterone- and etiocholanolone-glucuronide and a following bacterial 3-hydroxy-steroid-dehydrogenase activity. Due to bacterial deconjugation, high amounts of non-conjugated steroids can be found. These are normally excreted as conjugates (androsterone and etiocholanolone).

Another rare effect of bacterial activity on steroidprofile is the increased testosterone concentration leading to elevated testosterone/epitestosterone ratios

(Fig 2). Elevated testosterone levels may result from bacterial hydrolysis of 5-androsten- $3\beta,17\beta$ -diol-sulphate and a following 3β -hydroxy- Δ^5 steroid-dehydrogenase and steroid- Δ -isomerase activity. Testosterone derived from bacterial activity is non-conjugated and can be well separated from the testosterone-glucuronide by an ether extraction before hydrolysis. It is recommended for routine analysis to boil the buffer-solution before using for hydrolysis. For confirmation analysis it is important to separate the free fraction with ether before hydrolysis.

Application of ethanol (12)

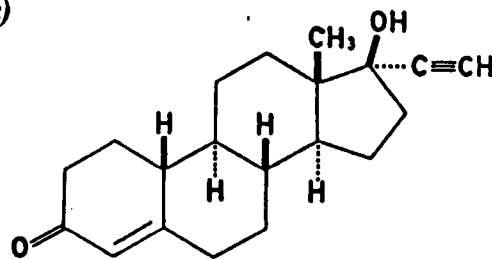
Ethanol application may lead to a significant increase of the ratio T/epiT and decrease of the ratio A/T, depending on an increased testosterone excretion and a decrease of androsterone excretion.

Changes in steroid profile parameters depending on alcohol consumption are always connected with the presence of ethanol in the same urine sample (Fig 3).

These results have always to be taken into account if high T/epiT-ratios are found in dope control samples, especially in "out of competition" controls.

To avoid false positive results, ethanol should be analysed additionally in this urine sample (e.g. by Headspace/GC).

Norethisterone (17 α -ethinyl-19-nor-testosterone)



Snf: $C_{20}H_{26}O_2$

Tab.1: Norethisteron containing oral contraceptives (Germany)

Micronovum	Noristat	Etalontin
Neorlest	Non-Ovlon	Orlest
Ovysmen	Sinovula	Stediril
Ortho-Novum	Sequostat	Synphasec
Tri-Novum		

Norethisterone is frequently used as gestogen-compound in several oral contraceptives (Tab1). Two metabolites of norethisterone are detectable in the screening chromatogramm for anabolic steroids: Norandrosterone and H4-norethisterone (Fig 4).

If in female urine Norandrosterone is detected, the urine is suspicious for application of Nortestosterone. If additionally the norethisterone-metabolite H4-norethisterone is detected, one must consider that both substances may be metabolites of norethisterone.

In this case the urine sample is not positive for application of nandrolone.

Oral contraceptives (13)

The main impact of oral contraceptives is seen in the level of epitestosterone and pregnandiol. Application of oral contraceptives may lead to an increase of the ratio T/epiT due to a suppression of the epitestosterone-excretion (Fig 5).

After withdrawl of oral contraceptives the excretion of epiT will increase, the ratio T/epiT will decrease and the variation of the ratio T/epiT will be more stable due to the higher concentration of epiT (Fig 6).

During application of oral contraceptives the excretion of pregnandiol is a stable intraindividual parameter.

After withdrawal a strong increase of the pregnandiol-excretion can be observed in the second part of the female menstrual cycle (Fig 7) due to the progesterone production of the corpus luteum with consecutive metabolism of progesterone to pregnandiol.

In the first part of the female menstrual cycle the follicle is stimulated by FSH. While growing the follicle produces estradiol. Due to a high level of estradiol the production of FSH is suppressed. After the ovulation the follicle transforms to a corpus luteum. The corpus luteum is stimulated by LH and produces progesterone which itself suppresses the LH-production via negative feed-back. With decreasing of the LH levels the progesterone-production stops and menstruation starts.

Oral contraceptives often consist of two compounds. The first compound is an estradiol-derivative most often ethinylestradiol. It suppresses the FSH-production in the first part of the female menstrual cycle and hinders ovulation.

The second compound is a progesterone-derivative. Levonorgestrel or norethisterone is frequently used to both, suppression of LH- and progesterone-production.

In the second part of the female menstrual cycle the constant application of progesterone-derivative leads to a stable excretion of the main metabolite pregnandiol.

For judging steroid profiles or endocrinological studies in female it is important to know, that changes in steroid profile parameters (most important: T/epiT) can be due to application or withdrawal oral contraceptives.

Pregnancy

There are three characteristic changes in female steroid profiles during pregnancy (Fig 8):

1. The excretion of pregnandiol increases significantly due to missing feed back mechanism. The popular based reference ranges of pregnandiol for female (14) are situated between 80 and 3000 ng/ml. In the first part of pregnancy between 5000 and 10.000 ng/ml pregnandiol was detected, decreasing to more than 20.000 ng/ml short time before delivery.

2. Also due to the missing feed back mechanism estrogens are excreted in very high amounts. In the course of pregnancy the excretion increases significantly.

One of the estrogens is coeluted with 17α -methyltestosterone, which is frequently used as internal standard. In this case a correct calculation of endogenous steroids is not possible. In the present method 17α -methyltestosterone is only used for setting the windows in the screening printout, because 17α -methyltestosterone is eluted in the middle of the analytical run. For calibration deuterated etiocholanolone ($[2,2,4,4\text{-}^2\text{H}_4]$ -etiocholanolone) is used, which is eluted in an area with low biological underground. This internal standard works well for most urines of pregnant women. Only in few cases there was a coelution with estrogenous steroids (mostly in the end of pregnancy; such urines are rarely found in dope control).

3. Norandrosterone can be detected in the later course of pregnancy. It was possible to detect norandrosterone from the 14th week of pregnancy. The calculated concentrations are between 1 and 23 ng/ml. Explanation for the norandrosterone production is possible transformation from the high amounts of estrogens.

The best method to confirm pregnancy is the determination of B-HCG.

Adrenal syndrom (16,17,18)

The adrenal syndrom is an enzyme deficiency disease of the 21-hydroxylase (Fig.9)

This enzyme is needed for transformation of 17α -OH-progesterone into corticosteroids. If this pathway is not possible, 17α -OH-progesterone is transformed into androstendion and testosterone. Due to the lack of corticosteroids there is no feed back suppression of ACTH and precursors of corticosteroids. Precursors of corticosteroids are produced abundantly and consequently transformed into androgenous steroids.

Clinical appearance of adrenal syndrom is virilisation. The steroid profile of a women with adrenal syndrom has a typical pattern: concentrations of endogenous steroids are situated outside of the popular based reference range and low corticosteroid concentrations.

Therapy of adrenal syndrom is treatment with corticosteroids for suppression of ACTH or treatment with antiandrogens. Occurrence of adrenal syndrom is very low.

Polycystic ovarian syndrom (16,17,18)

Polycystic ovarian syndrom has been estimated to involve 3,5-7% of the female population. The ovaries are enlarged and spotted with cystic follicles. Symptoms of the disease are adipositas, virilisation and sterility. Due to an increased activity of 5α -reductase in skin and liver the production of dihydrotestosterone is increased (Fig. 10). LH, DHEA-S, androstendion and testosterone levels in serum are also increased.

Therapy: Women who do not wish to become pregnant are treated with oral contraceptives with antiandrogen gestogen component (e.g. Diane: Cyproteronacetat + Ethinylestradiol) or combination from Cyproteronacetat (e.g. Androcur) with Diane.

Women who wish to become pregnant are treated with corticosteroids (Dexamethason) in combination with antiestrogens (Clomifen) to start ovulation.

The therapy of PCO patients with Clomiphen induces ovulation in more than 80% and pregnancy in about 40% of all treated patients.

For judging steroid profiles one has to know wether there are also high levels of testosterone, androstendion, dihydrotestosterone and DHEA detectable in the urines.

The typical steroidprofile of a women with polycystic ovarian syndrom shows concentrations of endogenous steroids in levels near the upper boarderline of the popular based reference ranges. The concentrations of testosterone and dihydrotestosterone in urine are not situated outside of the reference range (Fig. 11, 12).

DHEA-sulphate is not hydrolysed by β -glucuronidase from E.Coli. As a matter of fact it is not possible to calculate the real concentration of DHEA. The calculated concentration of DHEA was situated within the popular based reference range.

Furthermore detection of norandrosterone in those urines is not possible.

It is not possible to detect polycystic ovarian syndrom with steroid profile analysis.

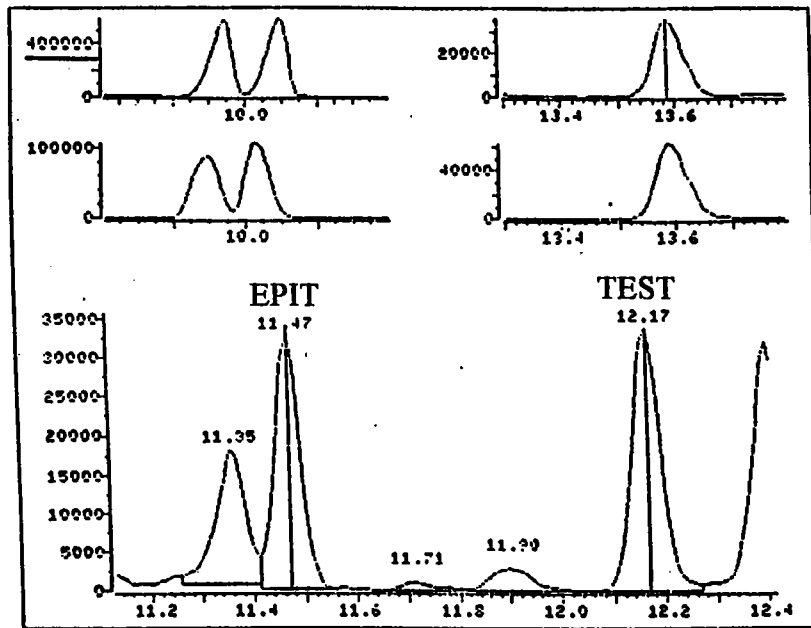
Acknowledgements

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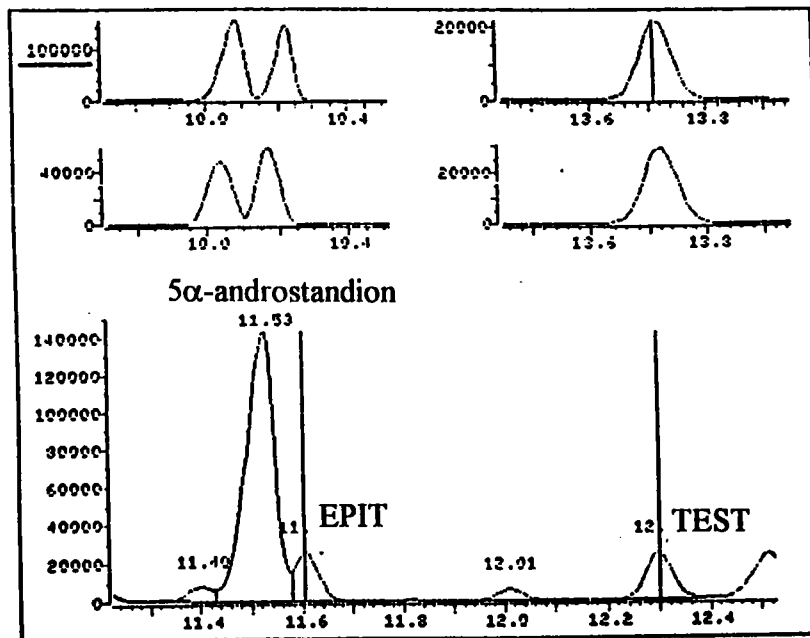
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pH 6



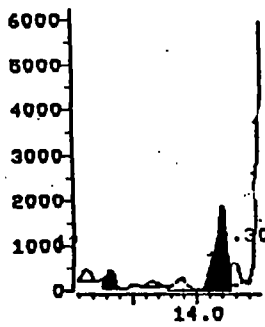
48 h 30°C

pH 7.5

Fig.1: Changes of the steroidprofile by bacterial activities.

10 ml of a quality control urine were spiked with 0.5 ml bacteria urine.
After 48 hours at 30°C high amounts of 5α-androstandion were found.

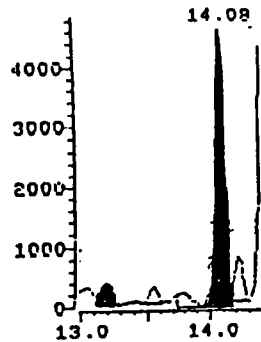
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pH 5

combined fraction
Q T/E = 5.3

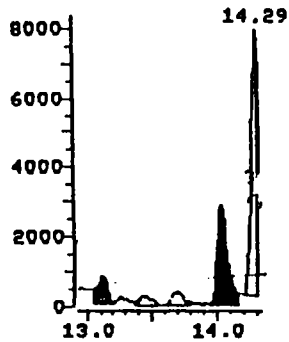
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storage 6 months + 4°C
pH 8.9

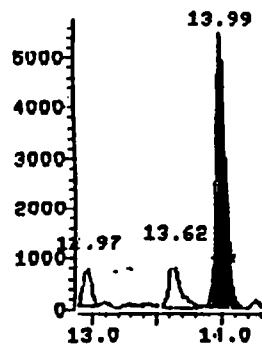
combined fraction
Q T/E = 9.8

File: >21017 431.7-432.7



conjugated fraction
Q T/E = 4.9

File: >21015 431.7-432.7



free fraction
with testosterone

Fig.2: Formation of testosterone and increase of the ratio testosterone/epitestosterone (T/epiT) by bacterial activities in a female urine.
High amounts of testosterone were found in the free form.

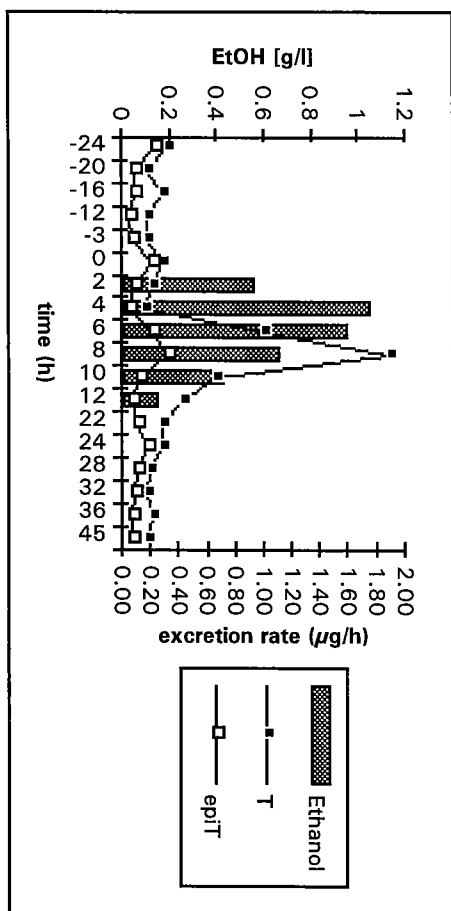
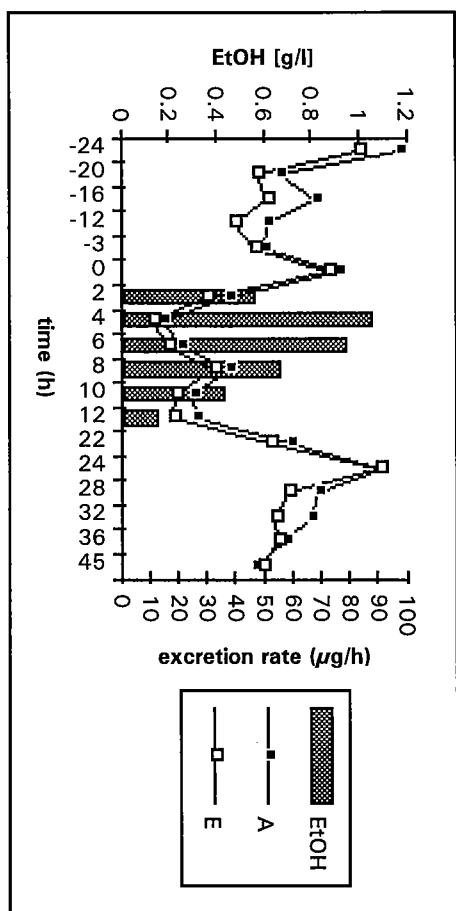
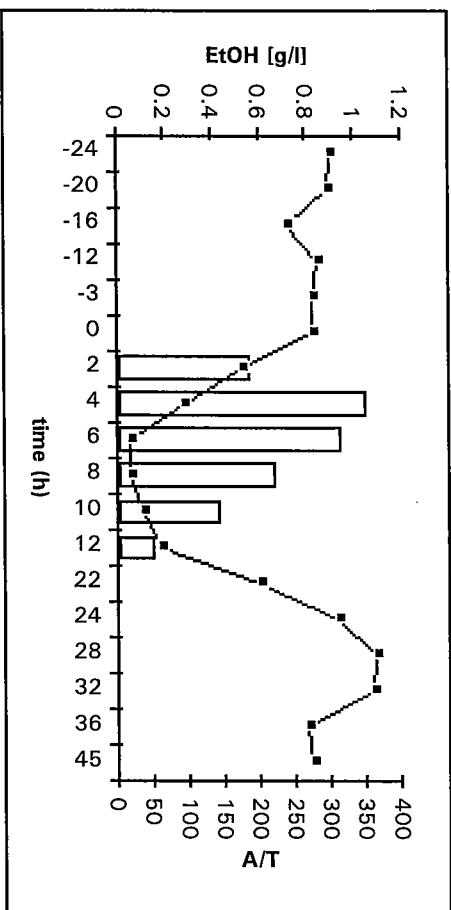
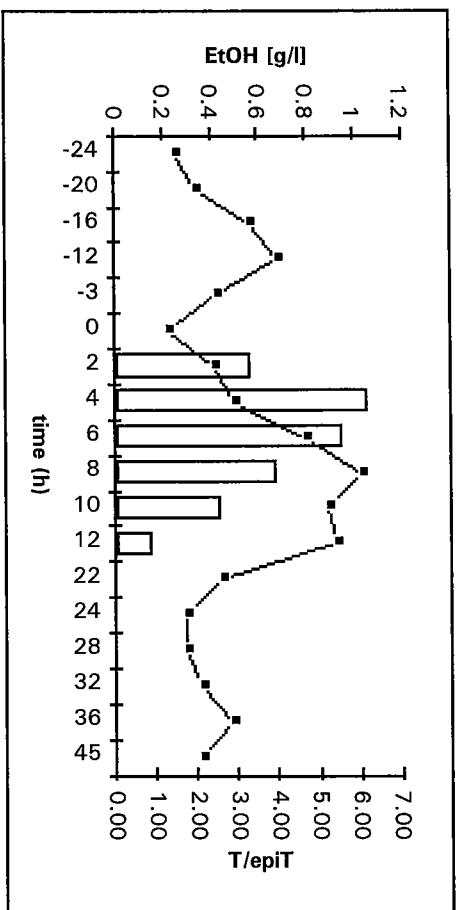
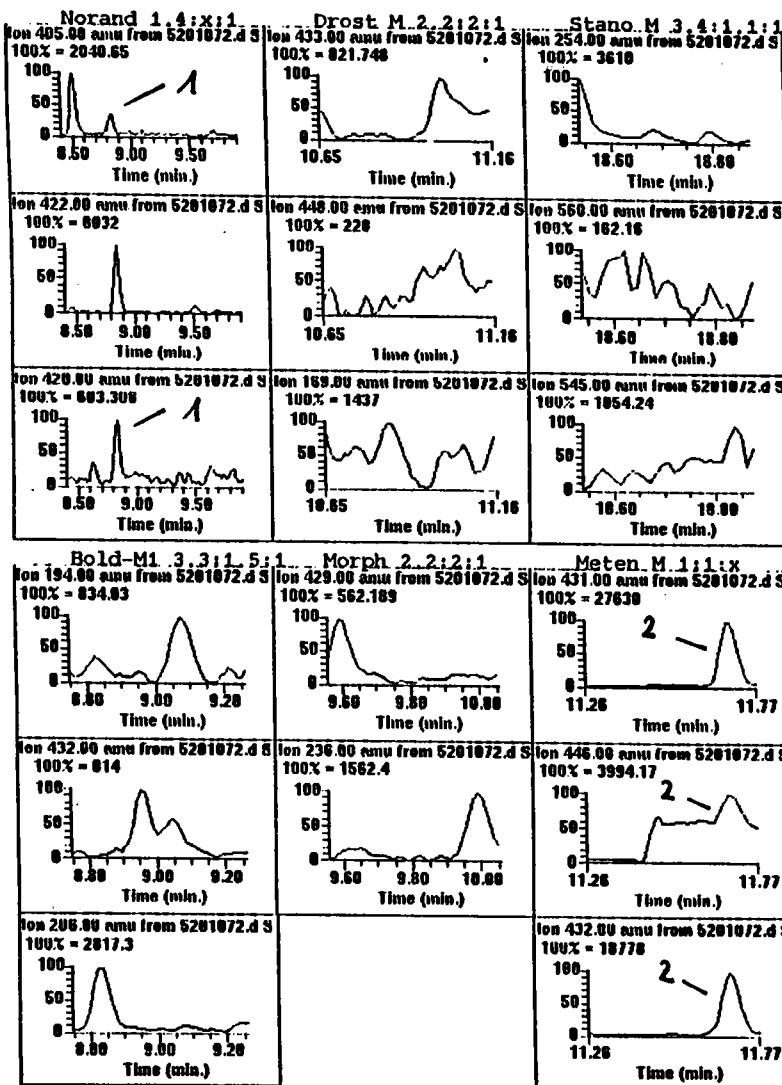


Fig 3: Influence of Ethanol on steroid profil parameters.
Changes of ratios and excretion rates of endogenous steroids during application of alcohol.
volunteer F1



1. norandrosterone-
bis-TMS

2. H4-norethisterone-
bis-TMS

Fig.4: Screening printout of anabolic steroids (combined fraction) after application of norethisterone containing oral contraceptive.

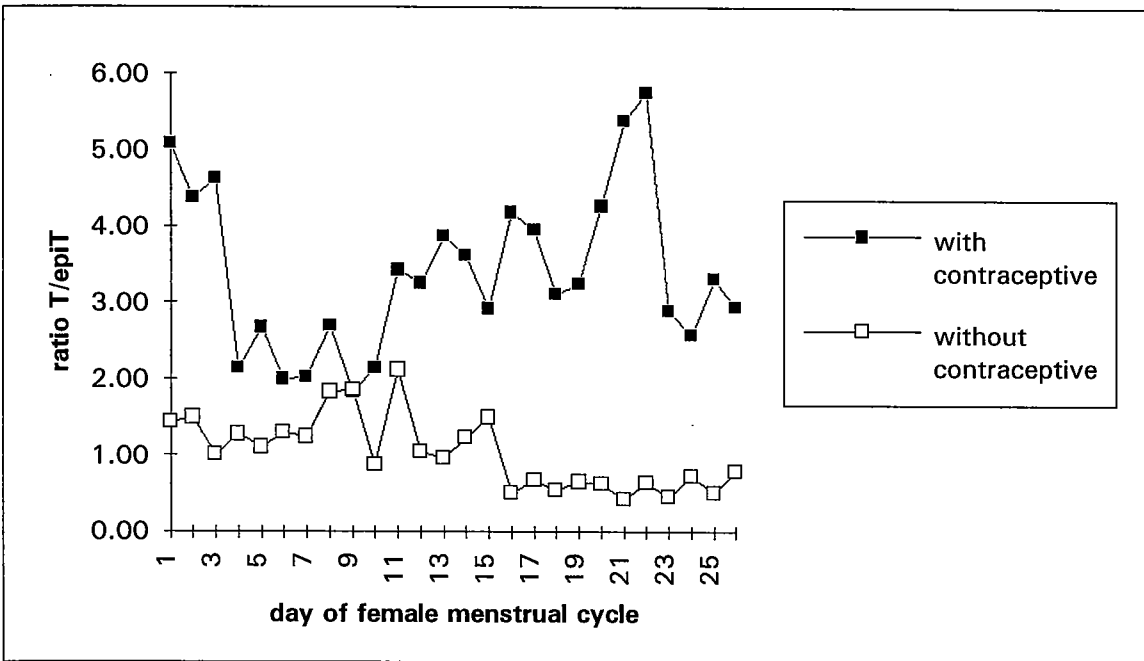


Fig.5: Influence of oral contraceptives on female steroid profiles.
Comparison of the ratio T/epiT over the course of one female menstrual cycle each with and without application of oral contraceptives.

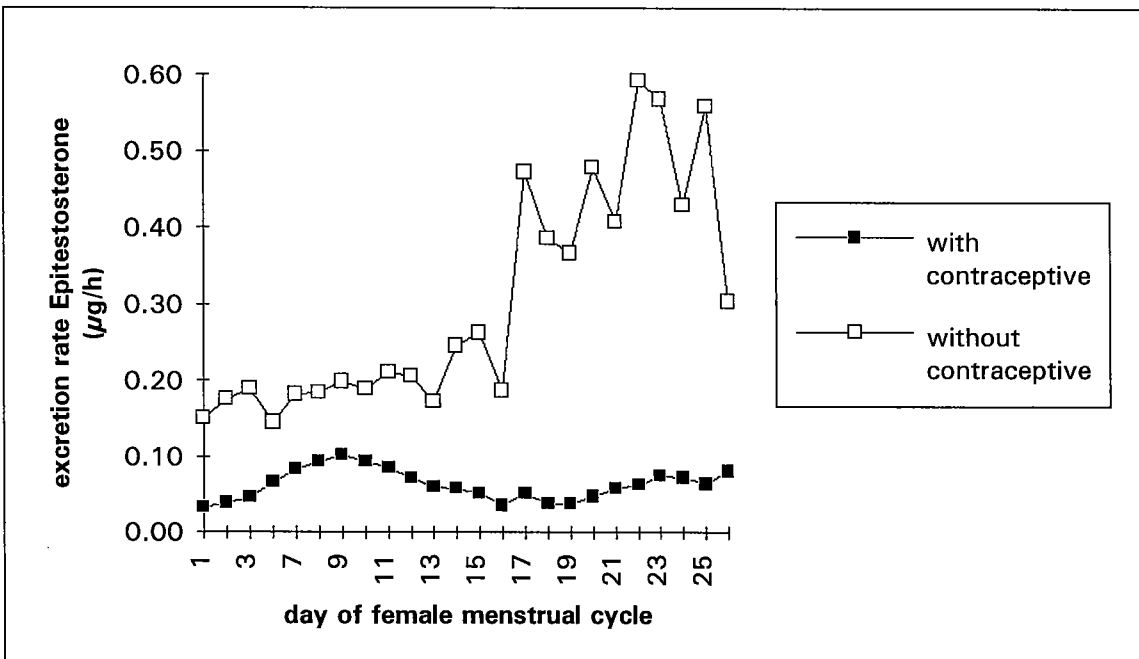


Fig.6: Influence of oral contraceptives on female steroid profiles.
Comparison of the excretion from epitestosterone over the course of one female menstrual cycle each with and without application of oral contraceptives.

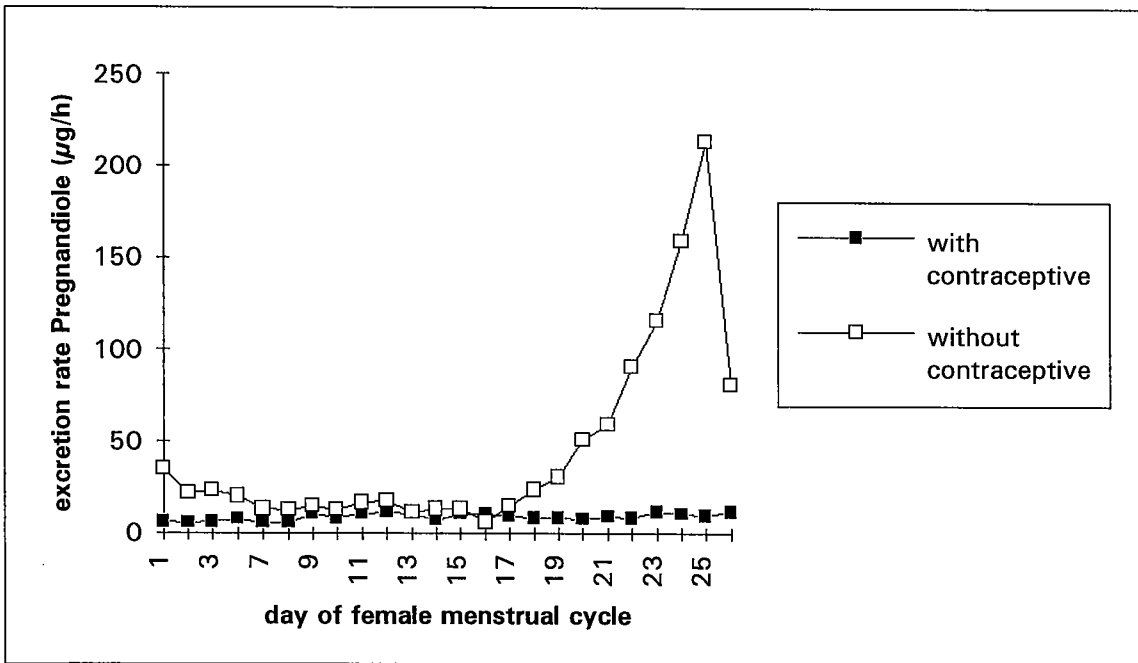


Fig.7: Influence of oral contraceptives on female steroid profiles.
Comparison of the excretion from pregnandiol over the course of one female menstrual cycle each with and without application of oral contraceptives.

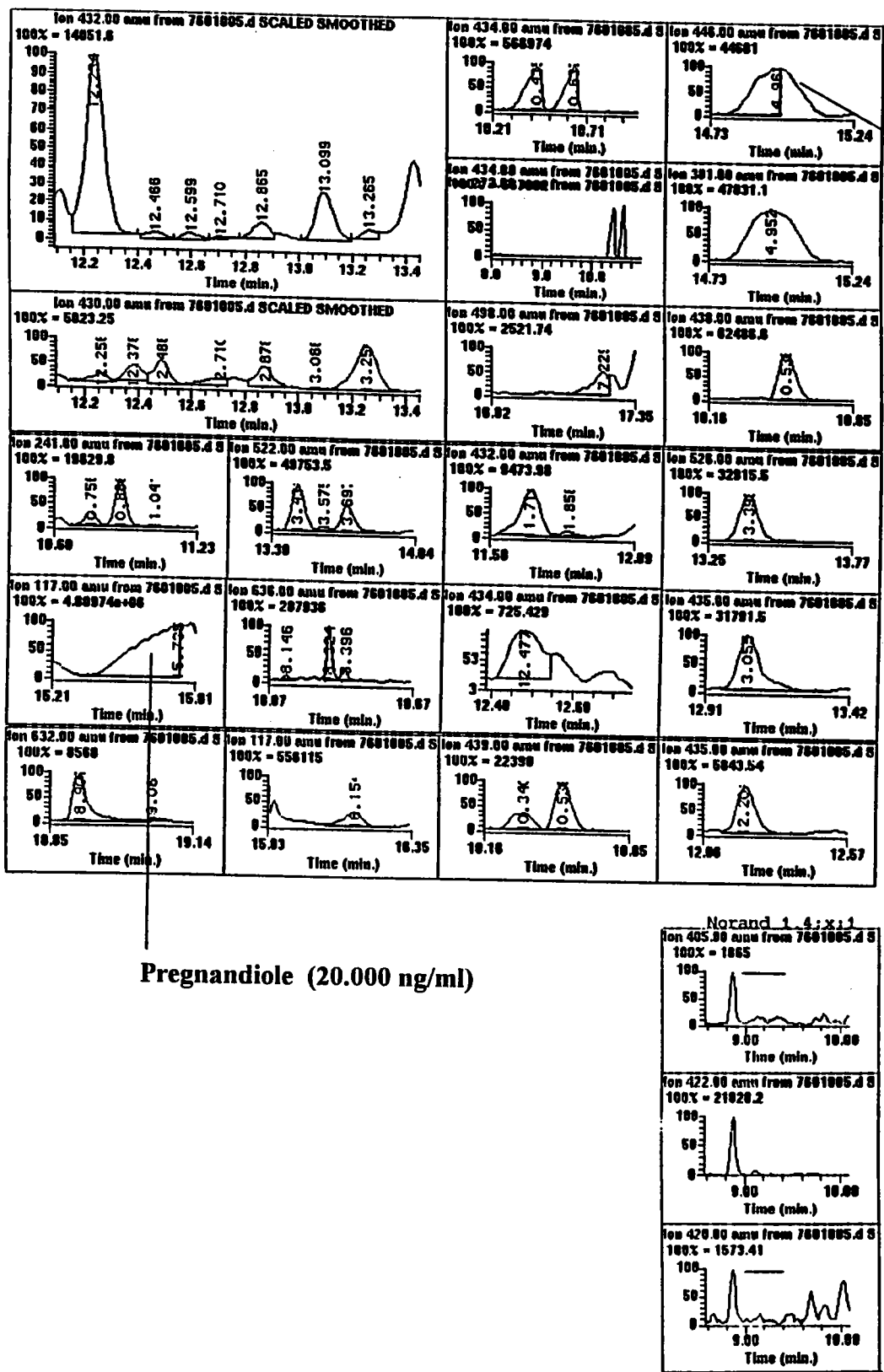


Fig.8: Screening printout: Steroid profile of a pregnant women.

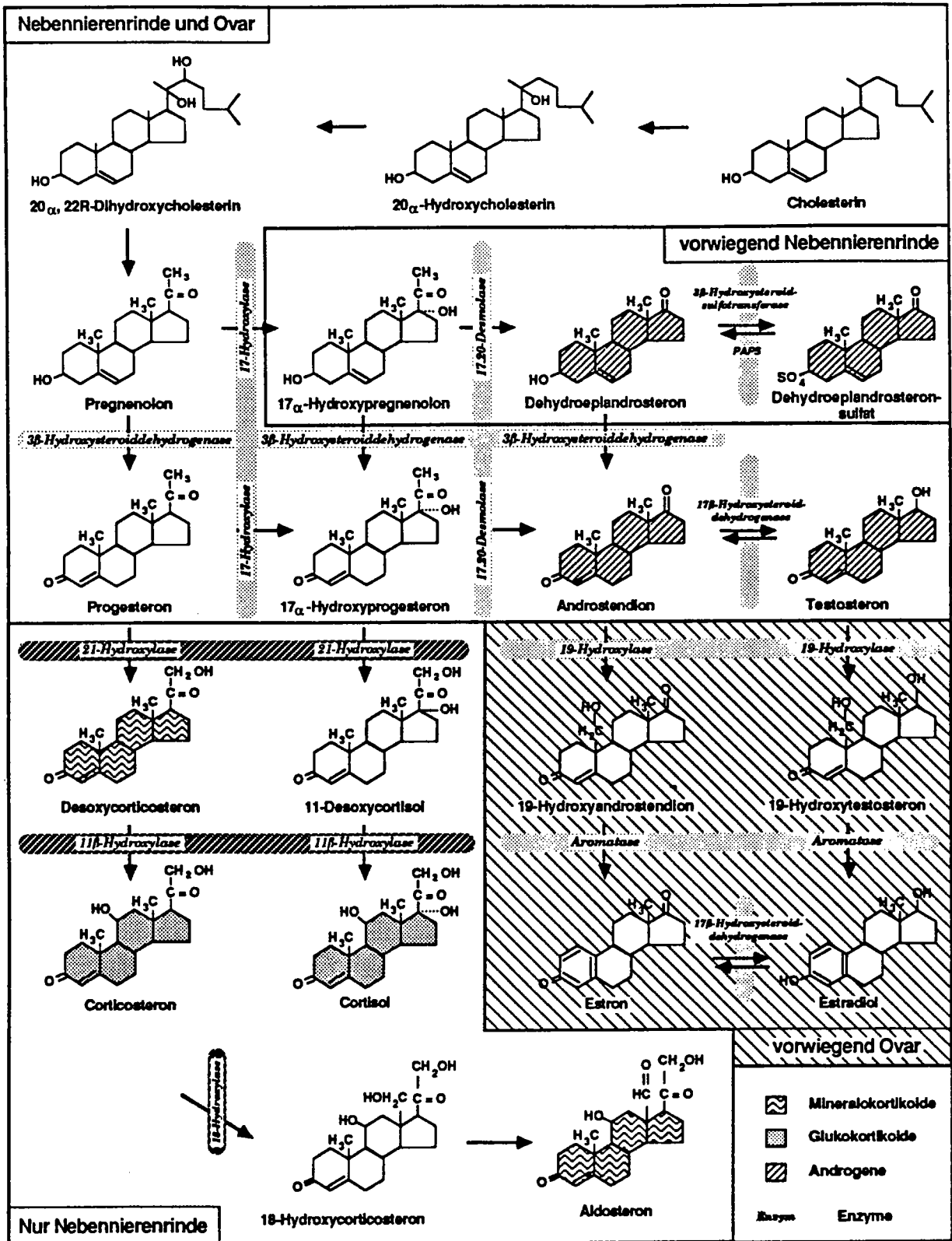


Fig.9: Biosynthesis of steroid hormones in the adrenal gland:
Localisation of enzyme deficiencies (18)

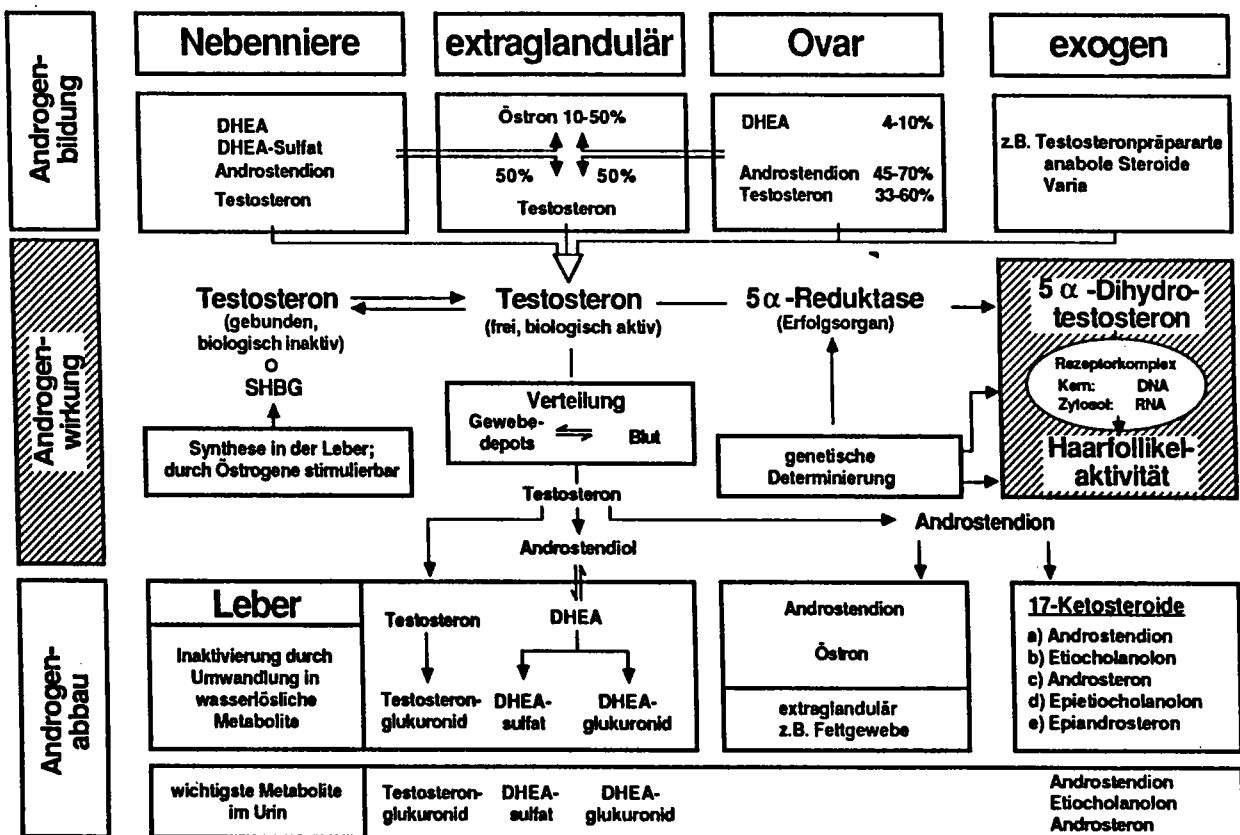
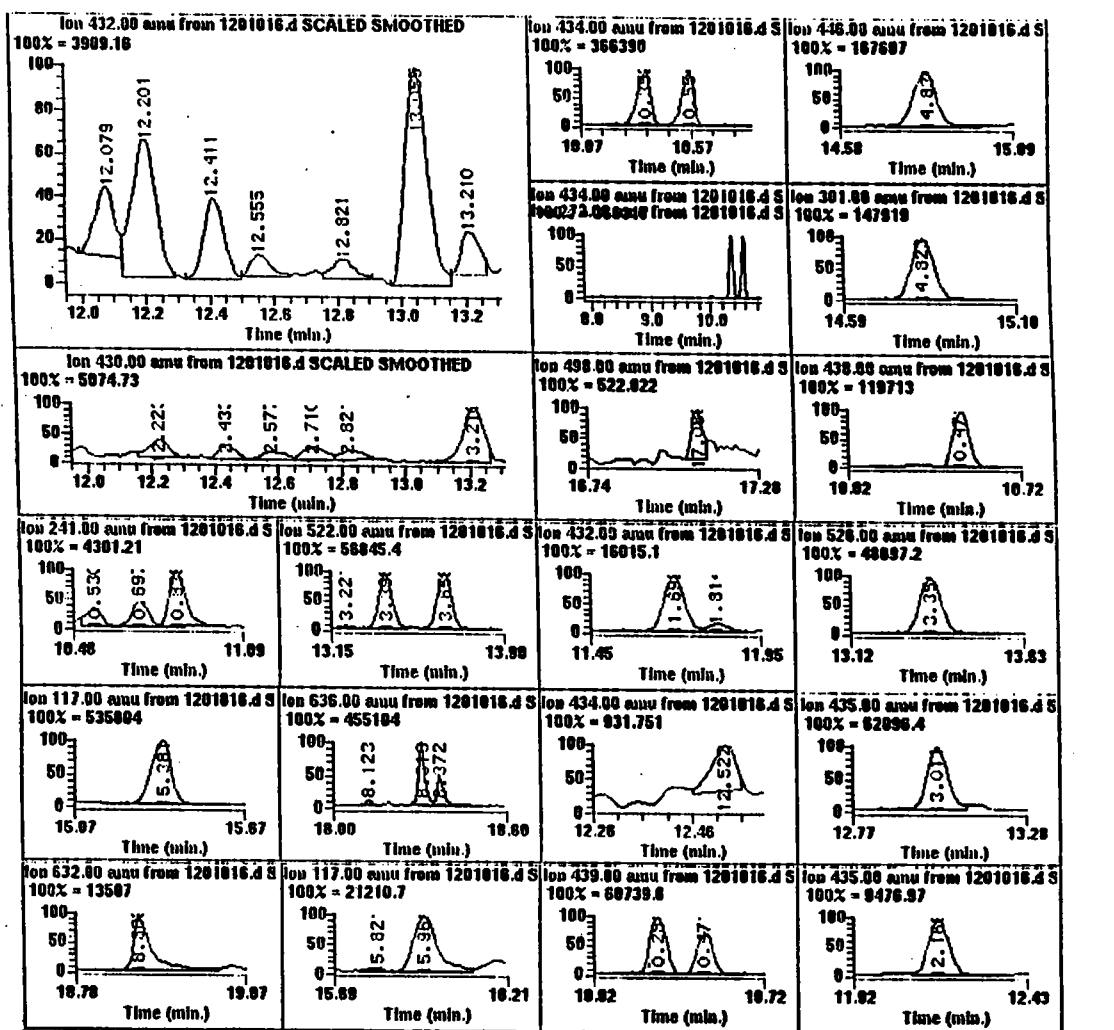
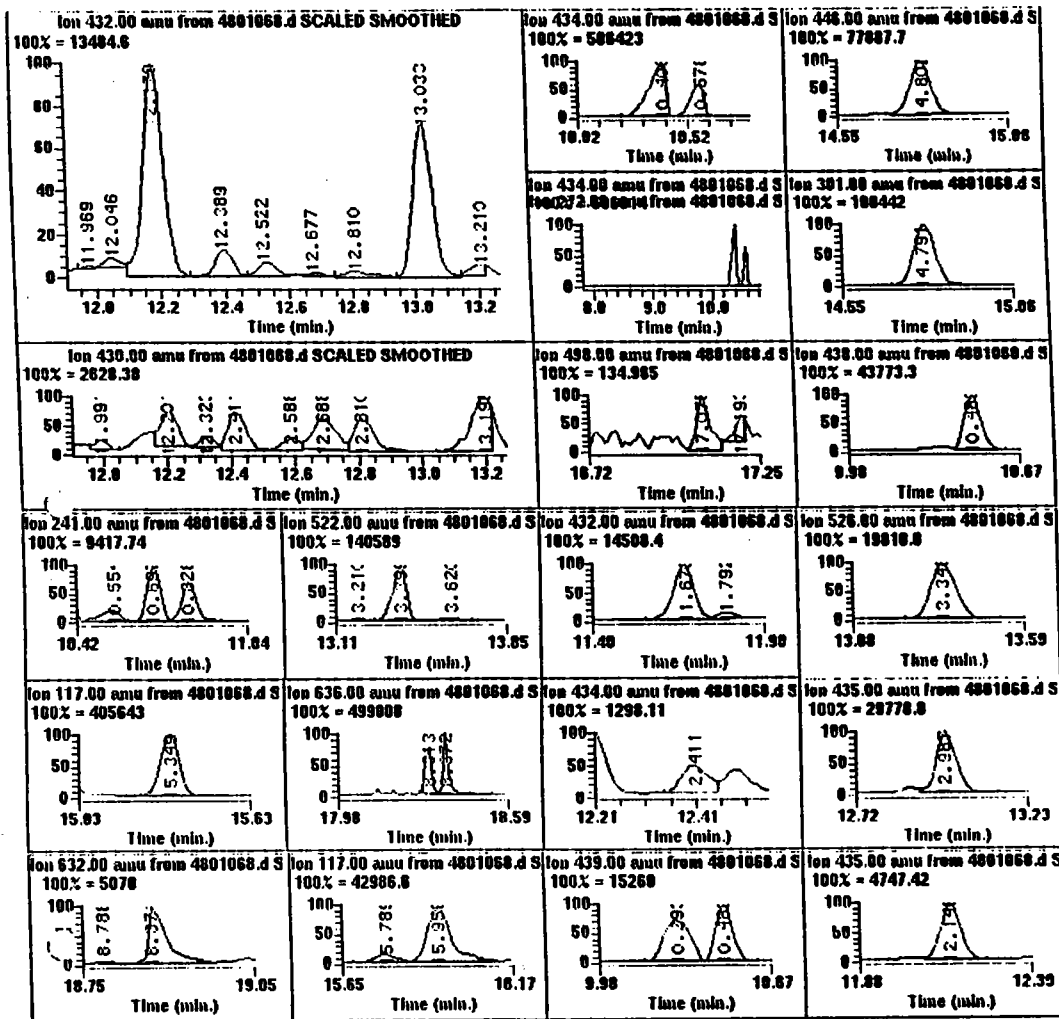


Fig. 10: Metabolism of androgens in female (18)



Substanz	RT	Flaeche	ng/ml	Substanz	RT	Flaeche	ng/ml
Androsteron	10.35	1490590	1535.5	Methyltest. (446)	14.83	786662	462.7
Etiocholanolon	10.55	1324059	1351.5	D4-OH-Androst.	13.35	213375	211.0
Epitestosteron	12.20	12303	4.1	D3-Epitesto.	12.17	36978	13.5
Testosteron	13.06	15305	4.6	D3-Testosteron	13.01	259984	77.7
				D4-Etioch.	10.47	546494	500.0
OH-Androsteron	13.40	224752	270.1	D5-Androsteron	10.28	283806	259.7
OH-Etiochol.	13.65	246770	291.0	Test/Epitest	1.244		
Androstandiol1	10.70	7830	10.2	d3T/d3Epit	7.031		
Androstandiol2	10.84	16790	19.9	Andr./Etio.	1.126		
DHEA	11.69	67325	55.3	Andr./Test. (c)	332.358		
Pregnan diol	15.38	2529343	393.3	D4-Etioch.	546494	196502	35.96
TH-Cortisol	18.31	599493	790.2	D4-Etioch+S	17.09	1067	0.20
allo-THC	18.37	329896	434.8	ISTD 301/446	0.865		
Dihydrotest.	12.52	2258	2.7				

Fig.11: Screening printout of a typical female steroidprofil with the ion chromatograms of the endogenous steroids and internal standard



Substanz	RT	Flaeche	ng/ml
Androsteron	10.40	2700180	7057.8
Etiocholanolon	10.58	1249429	3171.3
Epitestosteron	12.18	56975	52.2
Testosteron	13.03	38130	32.5
OH-Androsteron	13.40	569013	1946.6
OH-Etiochol.	13.62	34386	123.4
Androstandiol1	10.70	31596	94.7
Androstandiol2	10.83	24253	63.0
DHEA	11.67	60921	127.6
Pregnanliol	15.35	1803594	609.6
TH-Cortisol	18.31	545758	2460.1
allo-THC	18.37	599846	2703.9
Dihydrotest.	12.41	2164	6.9
Methyltest.(446)	14.80	342178	535.5
D4-OH-Androst.	13.34	99991	304.9
D3-Epitesto.	12.15	17617	17.6
D3-Testosteron	12.99	114974	102.3
D4-Etioch.	10.48	206809	500.0
D5-Androsteron	10.29	83494	201.9
Test/Epitest	0.669		
d3T/d3Epit	6.526		
Andr./Etio.	2.161		
Andr./Test.(c)	217.383		
D4-Etioch.	206809	71586	34.61
D4-Etioch+S	17.07	418	0.20
ISTD 301/446	1.324		

Fig. 12: Screening printout of a women suffering from polycystic ovarial syndrome with the ion chromatograms of the endogenous steroids and internal standard