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Urinary steroid sulphates: Sample preparation, reference values and investigations in biosynthesis and metabolism

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1. Introduction

Identification, characterization and quantitative analysis of sulphoconjugates as metabolic intermediates or phase-II-metabolites of endogenous steroids can yield fundamental knowledge about biosynthesis, metabolism and urinary excretion of steroids.

The urinary sample preparation method for the complete separation of steroid sulphates is applied to a representative reference population of male and one of female athlete samples. Detailed statistical calculations of endogenous steroid sulphates and steroid glucuronides gives rise to reference ranges which can be of significance in evaluating single urinary steroid profiles. Furthermore statistical analysis may simplify the complex system of urinary steroid concentrations. The relations of urinary steroid sulphates in any individual can be described with only a few independent parameters and metabolic relationships between steroid metabolites are made clear.

Abbreviations:

5Aendl: Androst-5-ene-3β,17β-diol, Adiol: 5α-Androstane-3α,17β-diol, And: Androsterone, Bdiol: 5β-Androstane-3α,17β-diol, conc: concentration, D_3epiT: [16,16,17-$^2H_3$]-Epitestosterone, DHEA: Dehydroepiandrosterone, DHT: Dihydrotestosterone, extr.: extraction, G: glucuronide, OHA: 5α-Androstane-3α,11β-diol-17-one, OHE: 5β-Androstane-3α,11β-diol-17-one, Pdiol: 5β-Pregnane-3α,20α-diol, rel: relative, S: sulphate, T: Testosterone, Thco: Tetrahydrocortisol

2. Sample preparation

2 ml urine + 0.75 ml phosphate buffer (0.8 M, pH 7)

↓

Internal Standard: [2,2,3,4,4-$^2H_5$]-Androsterone,17ß-D-Glucuronide;

[2,2,4,4-$^2H_5$]-Androsterone,17ß-D-sulphate; [2,2,4,4-$^2H_4$]-Etiocholanolone

↓

Enzymatic hydrolysis: + 25 µl ß-Glucuronidase E.coli, 50 °C, 60 min

↓

+ 250 µl K_2CO_3/$KHCO_3$ (20 %), extract with 5 ml tert-butyl-methylether

↓

Organic phase (free steroids and glucuronides):

Evaporation, derivatization with 100 µl MSTFA-NH_4I-ethanthiol (15 min 60 °C),

transfer in vials ⇒ GC/MS

↓

aqueous phase (steroid sulphates): remove ether residue, + 1 ml sodium acetate buffer (1 M, pH 4,9), centrifuge, decant on C_{18}-column; 2 washsteps (H_2O; n-heptane)

elution (1 ml Methanol) + [2,2,4,4-$^2H_4$]-Etiocholanolone

↓

Solvolysis: + 5 ml ethylacetate/H_2SO_4 (250 ml/200 mg), 55 °C, 60 min

↓

+ 0.25 ml KOH (1M), evaporate ethylacetate, extract with 5 ml tert-butyl-methylether (pH 9)

↓

Decant organic phase, evaporate, derivatize with 100 µl MSTFA-NH_4I-Ethanthiol

(15 min 60 °C), transfer in vials ⇒ GC/MS

3. Results of optimising the method

a) Yield of extraction

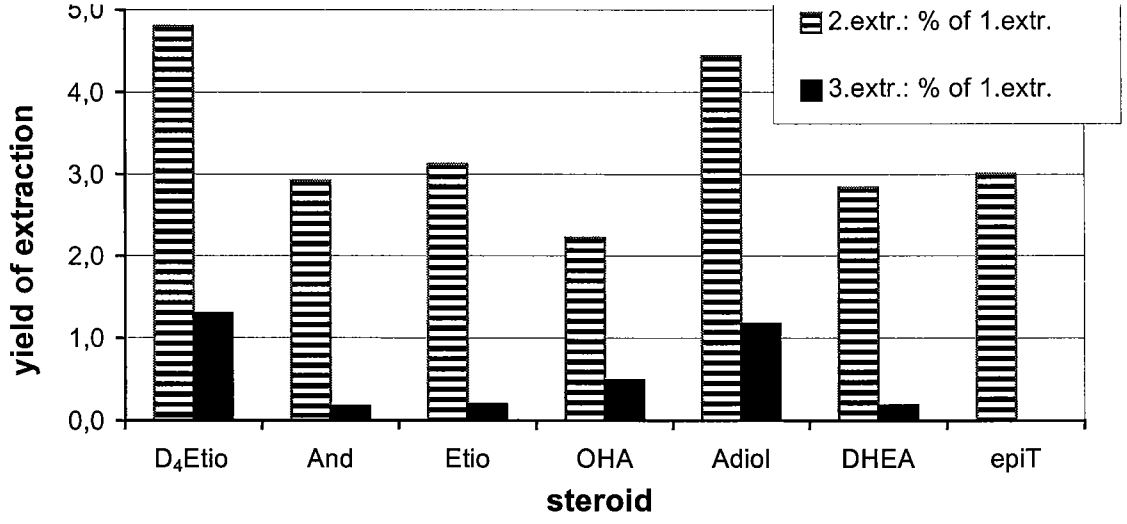

Fig. 1: Repeated extraction of hydrolysed steroids with tert-butyl-methylether

The yield of the 1st extraction is set 100 %, 2nd and 3rd extraction amount to only 2 – 6 %. This is to be reminded when analysing steroid sulphates with only one previous extraction.

b) Time and conditions of chemical cleavage

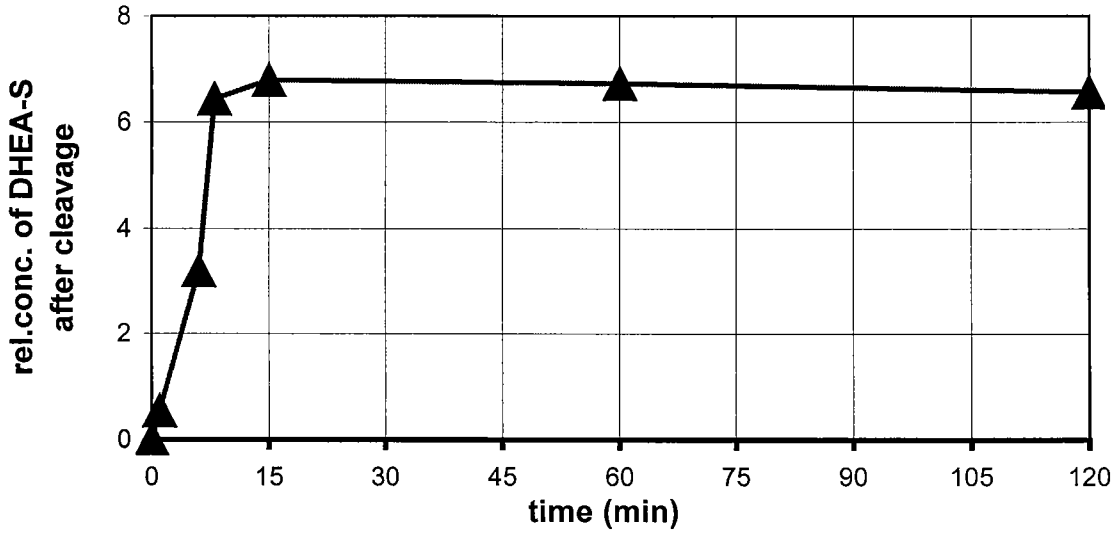

Fig. 2: Kinetic of chemical cleavage with ethylacetate/methanol/ H_2SO_4

The reaction is stopped with 0,5 ml 20 % $K_2CO_3/KHCO_3$ (1 : 1). Solvolytic cleavage with the solvolytic reaction mixture as outlined in the sample procedure is accomplished in 15 min. The kinetics are confirmed with various standard sulphoconjugates and matrices.

c) Temperature of chemical cleavage

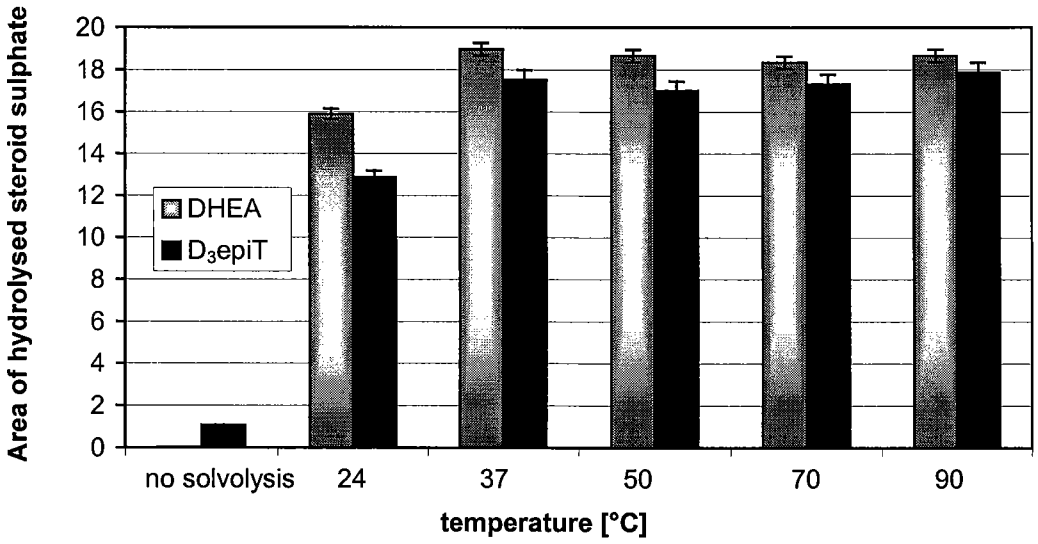

Fig. 3: Chemical cleavage of steroid sulphates (1 µg each) at different temperatures

There is incomplete cleavage at 24 °C, consequently 55 – 60 °C is employed. Sulphuric acid catalyses the chemical hydrolysis (data not shown). 0,025 g H_2SO_4/100 ml ethylacetate will be sufficient for a complete cleavage of sulphoconjugates. Cleavage of steroid glucuronides is not observed under the conditions employed and described in the sample preparation.

4. Validation data

GC-System:	HP 5890	Data in 4.a – c) is valid for the steroids
MSD:	HP 5971	And, Etio, T, OHA, Adiol, DHEA,
Aquisition mode:	SIM	Aendiol, D_3epiT–(Sulphate),

a) Recovery: 8 urines spiked with 25 or 250 ng/ml were analysed. Recovery ranges from 62,4 – 83,4 % .

b) Linearity: The method is linear over the concentration range 2,5 – 250 ng/ml with samples prepared and analysed in duplicate.

c) Reproducibility: Data was obtained after sample preparation and analysis of 8 urines. The coefficient of variation as validated with endogenous steroid sulphates and spiked D_3-Epitestosterone-sulphate ranges between 4,3 and 10,7 %.

5. Population-based studies

Reference population	141 male , 61 female athlete's urines out of Cologne doping controls 2000 and 2001 with a specific gravity ≥ 1,01 g/cm^3
Analysis of steroid parameter	13 steroids in glucuronidated and sulphated fraction (And, Etio, T, epiT, OHA, DHEA, OHE, Adiol, Bdiol, 5Aendiol, Pdiol, Thco, DHT)
Evaluation	Software-program "R"
Individual values: Parametric transformation	Boxcox-transformation into a Gaussian distribution (Lognormal)
Testing the distribution	Graphic evaluation (e.g. bimodal is not accepted), Shapiro-Wilks-Test (P-values ≥ 0,1)
Non-parametric distribution	Percentile ranks are assigned to concentrations if a parametric distribution can not be obtained
Practical use	95 % Confidence interval of concentrations

Table 1: Design of the study and statistical evaluation

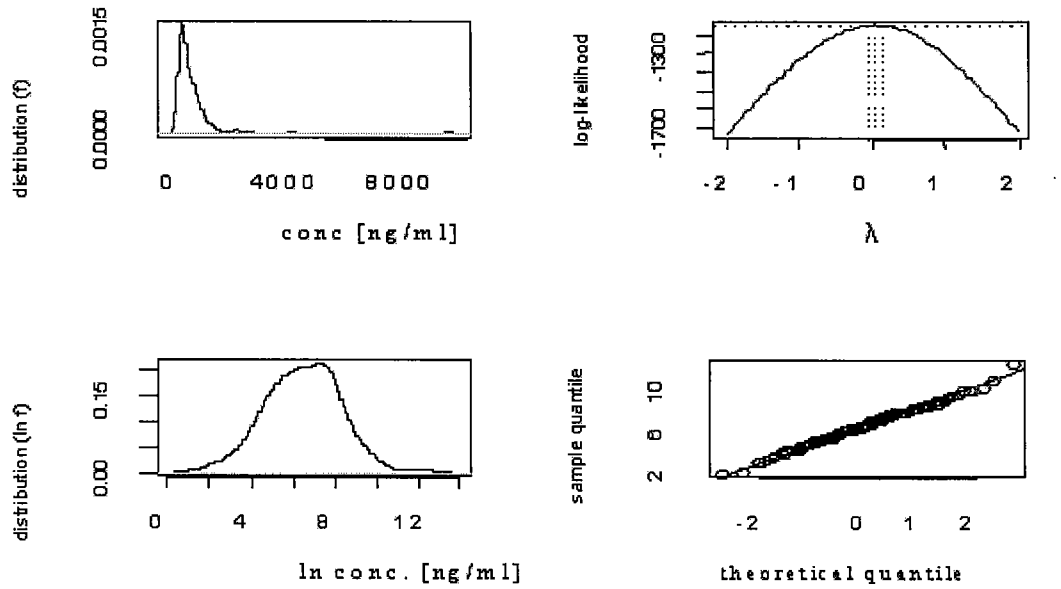

Fig. 4: Boxcox-transformation of individual concentrations of Androsterone-sulphate in 141 male athletes' samples

If a Boxcox-Transformation of individual urinary steroid concentrations fits a Gaussian distribution (Fig. 4) and yields a high P-Value in the Shapiro-Wilks-test, this transformation is accepted. Otherwise (Fig. 5) a non-parametric transformation, assigning percentile ranks to concentrations is used in order to calculate reference ranges and their confidence intervals (Table 2, Fig. 6) for the steroid glucuronides and sulphates in male and female atheletes' urines.

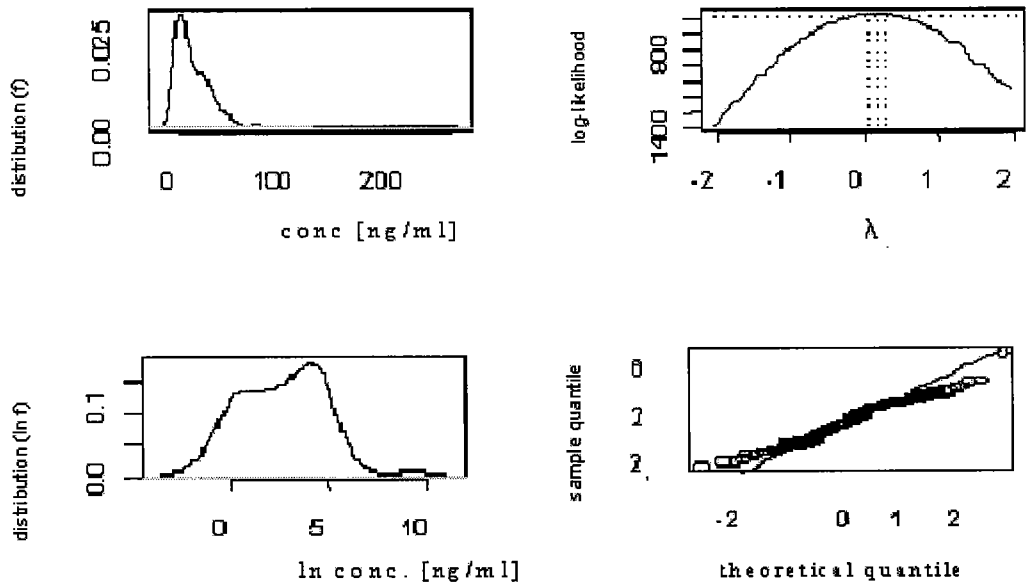

Fig. 5: Unsuccessful parametric transformation of individual concentrations of Epitestosterone-sulphate (bimodal values), 141 male athletes' samples

Concentrations [ng/ml]	And	T	OHA	Adiol	DHEA	5AenDl
median sulphates, ♂	229	3	34	7	141	91
0.025 quantile sulphates, ♂	16,4	0,3	2,0	0,6	1,7	4,7
0.975 quantile sulphates, ♂	2415	19	289	35	6004	612
median glucuronides, ♂	3032	32	729	52	41	20
0.025 quantile glucuronides, ♂	265	0,7	62	5,8	3,1	1,4
0.975 quantile glucuronides, ♂	9687	187	3887	227	212	119

Table 2: Reference ranges [ng/ml] of 6 characteristic urinary steroid sulphates and glucuronides in male athletes samples, N= 141

In general it can be shown that median, 0.975 and 0.025 quantiles of the concentrations of steroid glucuronides are roughly ten times higher than the corresponding concentrations of steroid sulphates (Table 2, Fig. 6,). Only DHEA-sulphate and Androst-5-en-diol-sulphate exceed the concentrations of the glucuronides with a factor around 5, since they are excreted mainly as sulphates. The results are evident in the reference population of male as well as female (not shown) athletes`sampleswith lower reference concentrations in the later population. Only the data of 6 representative steroids is presented. The data in the sulphate fraction can be used in addition to those of the glucuronide fraction to calculate a probability for a single steroid profile to be inside the 95 % reference ranges and whether or not a certain urine sample is suspicious.

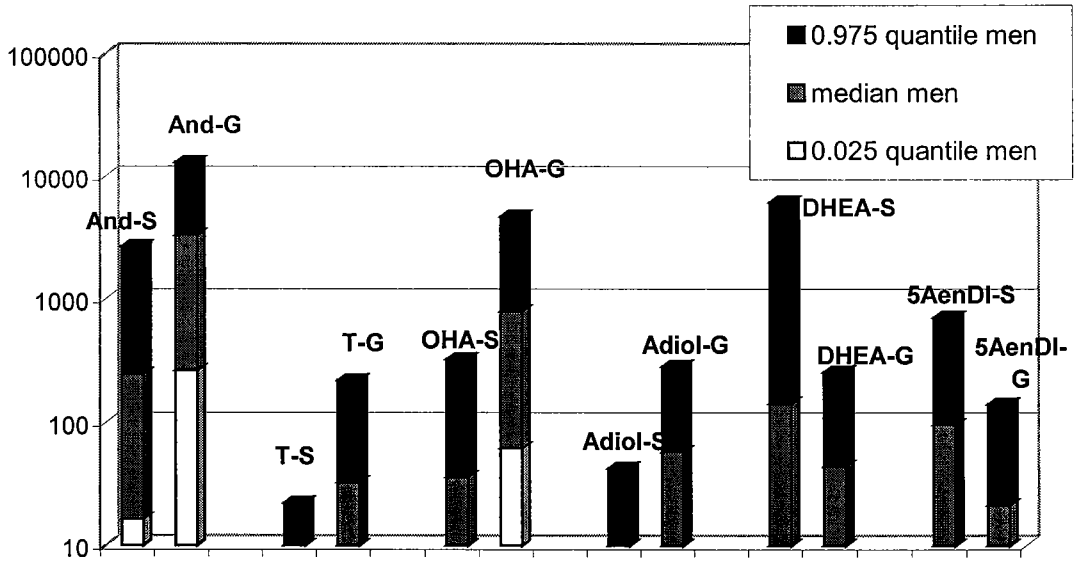

Fig. 6: Reference ranges of 6 urinary steroid sulphates and glucuronides [ng/ml] in 141 male athletes`samples

6. Paramaters of the profile of sulphoconjugated steroids

Certain endogenous steroids share biosynthetic steps with the same enzymes in organs like the gonads or adrenals or are being metabolised via the same routes in the liver or other organs.

As a hypothesis they can be united into groups not necessarily representing the classification androgens, estrogens and glucocorticoids but functional affiliation. Only the 6 most abundant steroids in the sulphate fraction were mathematically analysed.

Principal component analysis looks for a useful reduction of parameters in biological systems and can detect common correlations between biological parameters.

Principal component variables are independent from another, while the first component variables describe the main variance of the system.

	component 1	component 2	component 3	component 4	component 5	component 6
deviation	**2,97**	**1,07**	**0,81**	**0,60**	**0,50**	**0,37**
variance	**0,78**	**0,10**	**0,05**	**0,03**	**0,02**	**0,01**
cumulative variance	**0,78**	**0,88**	**(0,93)**	**0,97**	**0,99**	**1**

Table 3: Principal component analysis (PCA) of 6 steroids in the sulphate fraction in a reference population of men, N = 141

Only two independent variables can describe nearly the whole variance of the system (table 3). To assign biological parameters to those principal components, the loadings (correlation of the steroid to the independent variable) of the concentrations 6 endogenous steroid sulphates is calculated (table 4).

	component 1	component 2	component 3
Adiol	**-0,292**	**-0,436**	/
OHA	**-0,341**	**-0,579**	**-0,363**
5AenDl	**-0,359**	/	**0,842**
And	**-0,382**	/	**-0,361**
T	**-0,308**	**-0,202**	/
DHEA	**-0,654**	**0,652**	**-0,131**

Table 4: Loadings in PCA of 6 steroids in the sulphate fraction (men)

DHEA-sulphate shows the highest loadings with component variable 1 and 2 (table 4). Consequently both principal components can be interpreted as influences on pathways that participate in biogenesis of DHEA-sulphate. This could be interpreted as follows:
Component 1 can be equated with the activity of a hydroxysteroid-sulphotransferase and component 2 with the activity of the adrenals.

Further evidence for a simplification in parameters that is inherent in the biological system of steroid sulphates is the correlation of DHEA–sulphate with every other analysed steroid sulphate which is higher than the correlation of the corresponding glucuronide with DHEA-sulphate (table 5). This can be observed regardless of the steroid of interest and even in steroids that are relatively "wide apart" in their biosynthetic pathways.
Again the same results are evident in the reference population of women`s samples

Reference population of men	**T-S**	**T-G.**	**And-S.**	**And-G**	**Etio-S**	**Etio-G**	**Pdiol-S**	**Pdiol-G**
Correlation with DHEA-sulphate	**0,756**	**0,315**	**0,806**	**0,441**	**0,591**	**0,422**	**0,581**	**0,418**

Table 5: Correlation analysis of steroid sulphates and glucuronides (Pearson`s product–moment–correlation–test); urinary concentratios of male athletes

7. Conclusions and possibilities of the statistical evaluation of steroid profile parameters in the sulphate fraction

With the help of statistical calculations a number of practical applications is possible:
⇒ description of distributions of steroid concentrations in reference populations
⇒ calculation of reference ranges and possibly a discrimination of positive and negative urines
⇒ insight in relations and interactions of steroid pathways biogenesis and metabolism (PCA)
⇒ reduction in complexity and important steroid parameters (PCA, correlation analysis)
Furthermore not shown herein:
⇒ discrimination of sex with the aid of steroid sulphates
⇒ description of discrete populations within populations with cluster analysis

8. Literature

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