In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
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Simple Purification of Urine Samples for Improved Detection of Anabolic and Endogenous Steroids

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Introduction

A prerequisite for the identification and quantitation of anabolic and endogenous steroids by gas chromatography/mass spectrometry is a pure chromatographic signal. However, often coeluting substances lead to asymmetrical chromatographic signals and impure mass spectra of the substances of interest.
Possible methods for separating coeluting substances are: e.g., the change of the gas-chromatographic temperature program, the use of columns with different polarities, the use of other derivatives and/or the addition of purification steps in the sample preparation.

In our laboratory we use a simple extraction step with n-pentane to remove many polar substances which coelute with anabolic steroids and their metabolites and endogenous steroids.

Experimental

Sample preparation - normal procedure
2 ml of urine and 20 µl of an internal standard mixture* 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 bidistilled 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 250 µl of 7% potassium carbonate solution to pH 9-10 and the steroids are extracted with 5 ml of diethyl ether or tert.-butylmethyl ether on a mechanical shaker for 5 minutes. After centrifugation the etheral layer is transferred and evaporated to dryness under vacuo.

* the internal standard mixture (methanolic solution) consists of:

17α-methyltestosterone	50 ppm
[2,2,4,4-$^2H_4$]-etiocholanolone	50 ppm
[16,16,17-$^2H_3$]-testosterone	2 ppm
[2,2,4,4,-$^2H_4$]-11ß-hydroxyandrosterone	24 ppm

Sample preparation with n-pentane extraction

The urine samples are prepared as described above, however in the last extraction step 5 ml n-pentane is used instead of ether.

Derivatisation

The dry residue is derivatised with 100 µl of MSTFA/NH_4I/ethanethiol 1000:2:3 (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)
column: HP Ultra I (OV-1), 17m , 0.2 mm i.d., 0.11 µm film thickness
carrier gas: 1 ml helium at 180° C, split 1:10
temperature program: 180° C, 3° C per min, 229° C, 40° C per min , 320° C

Results

Extraction using n-pentane leads to a significant reduction of disturbing polar compounds. This is shown in figures 1 and 2 for a urine containing trimethoprim- and sulfamethoxazol metabolites which are removed using n-pentane solvent.
The removal of coeluting substances is shown in figure 3. After n-pentane extraction testosterone can be identified by a full scan spectrum. This was not possible after extraction using ether.

The recoveries of the substances included in the screening procedure for anabolic steroids (total fraction) after ether and n-pentane extraction are presented in tables 1 and 2. For most substances the recovery after n-pentane extraction is similar to that after ether extraction. Only polar substances as 6ß-OH-metandienone, 3'-OH-stanozolol, 11ß-OH-androsterone ,11ß-OH-etiocholanolone etc. have poorer recoveries using n-pentane extraction.

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Conclusion

Extraction with n-pentane is a very simple method to improve detection of most anabolic and endogenous steroids by removing disturbing polar substances. These also include species which coelute with the substance of interest, e.g. 11-keto-etiocholanolone coeluting with epitestosterone, 11-keto-isoandrosterone coeluting with testosterone and 11ß-OH-etiocholanolone coeluting with metenolone.
This cleanup step is not suitable for polar steroids such as stanozolol-metabolites, 6ß-OH-metandienone, fluoxymesterone etc. Therefore in the screening procedure for anabolic steroids we prefer ether as an extraction solvent.
The n-pentane extraction is very useful in the confirmation analysis for most anabolic steroids and for the confirmation of high testosterone/epitestosterone ratios.

endogenous steroids	diethylether	tert.butyl-methylether	n-pentane
Androsterone	93.2	90.7	92.5
Etiocholanolone	92.8	93.3	92.5
Epitestosterone	91.1	91.1	86.6
Testosterone	91.4	90.1	86.4
11ß-Hydroxy-androsterone	91.6	88.3	37.9
11ß-Hydroxy-etiocholanolone	92.1	91.6	21.9
5α-Androstane-3α,17ß-diol	94.5	91.8	89.0
5ß-Androstane-3α,17ß-diol	91.6	89.8	85.5
Dehydroepiandrosterone	93.7	91.0	93.2
5ß-Pregnane-3α,20α-diol	91.5	87.1	87.0
Tetrahydrocortisol	87.8	92.6	00.9

Tab. 1: Recoveries (in percent) of endogenous steroids after single extraction at pH 9.6 from 1 ml water using three different solvents.

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anabolic steroids and other substances	systematic name	diethylether	tert.butyl-methylether	n-pentane
Bolasterone-M1	7α,17α-dimethyl-5ß-androstane-3α,17ß-diol	89.0	89.6	89.0
Boldenone		85.3	84.0	66.7
Boldenone-M1	5ß-androst-1-en-17ß-ol-3-one	87.7	86.6	85.1
Canrenone		70.0	64.7	62.8
4-Chlorodehydro-methyltestosterone-M1	6ß-hydroxy-4-chloro-dehydromethyltestosterone	78.8	76.0	09.2
Clenbuterol		90.5	86.1	64.2
Clostebol-M1	4-chloro-androst-4-en-3α-ol-17-one	98.4	91.8	95.1
Drostanolone -M1	2α-methyl-5α-androstan-3α-ol-17-one	82.1	80.7	84.1
Fluoxymesterone-M1	9α-fluoro-17α-methyl-androst-4-ene-3α,6ß.11ß,17ß-tetrol	26.0	47.4	00.0
Mesterolone-M1	1α-methyl-5α-androstan-3α-ol-17-one	92.7	89.4	94.3
Metandienone-M1	17α-methyl-5ß-androst-1-ene-3α,17ß-diol	90.9	92.7	81.8
Metandienone-M2	6ß-hydroxymetandienone	81.7	77.0	03.2
Metenolone-M1	1-methylene-5α-androstan-3α-ol-17-one	92.5	92.5	96.8
Methyltestosterone-M1	17α-methyl-5α-androstane-3α,17ß-diol	90.7	89.3	91.2
Methyltestosterone-M2	17α-methyl-5ß-androstane-3α,17ß-diol	90.7	89.3	91.2
Nandrolone-M1	5α-estran-3α-ol-17-one	89.6	89.6	90.7
Nandrolone-M2	5ß-estran-3α-ol-17-one	90.1	90.1	91.2
Norethandrolone-M1	17α-ethyl-5ß-estrane-3α,17ß-diol	92.4	93.0	96.5
Oxandrolone		77.4	51.6	59.7
Oxymesterone		109.0	119.5	117.6
Pemolin		38.5	44.8	00.3
Probenecid		00.4	00.7	00.1
Salbutamol		13.6	18.0	00.2
Stanozolol-M1	3'-hydroxystanozolol	89.2	93.3	06.4
Stanozolol-M2	4ß-hydroxystanozolol	85.2	88.9	06.5

Tab. 2: Recoveries (in percent) of substances included in the screening procedure for anabolic steroids (total fraction) after single extraction at pH 9.6 from 1 ml water using three different solvents. (M=metabolites)

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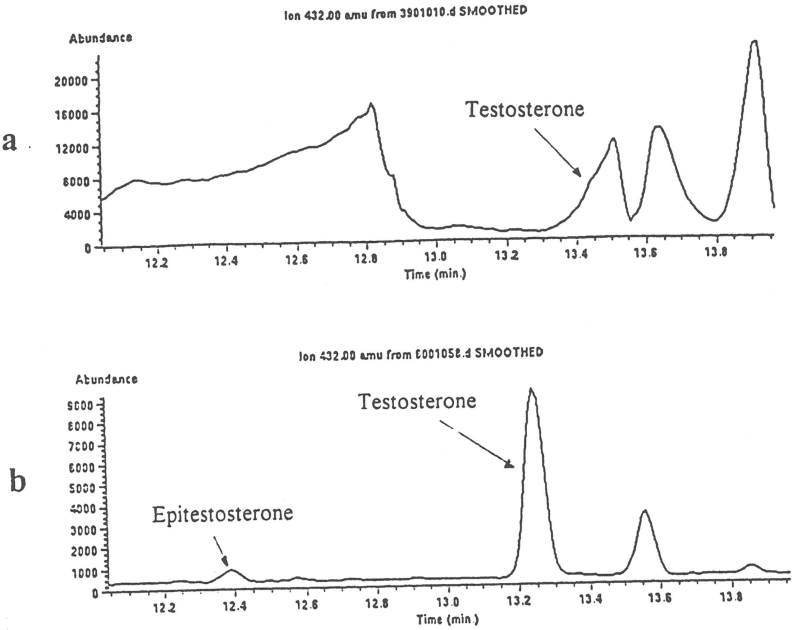

Fig. 2: Single ion chromatogram of ion m/z 432 in the region of testosterone and epitestosterone for an urine containing trimethoprim- and sulfamethoxazol metabolites a) after ether extraction b) after n-pentane extraction. The disturbing trimethoprim and sulfamethoxazol-metabolites are removed using the n-pentane solvent. Testosterone and epitestosterone show pure signals.

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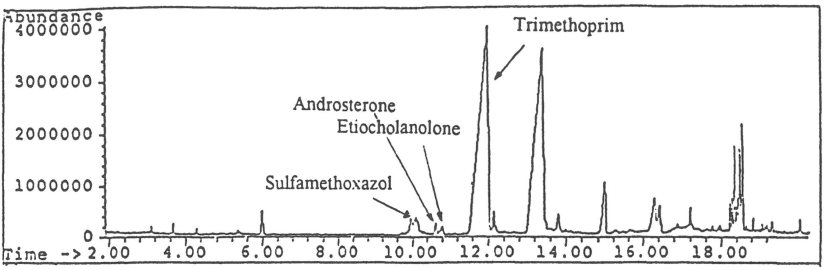
a



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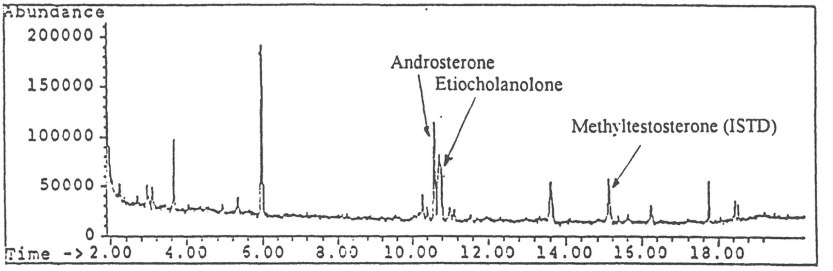

Fig. 1: Total ion chromatogram of an urine containing trimethoprim- and sulfamethoxazol metabolites a) with ether extraction b) with n-pentane extraction. The disturbing trimethoprim and sulfamethoxazol-metabolites are removed using the n-pentane solvent.

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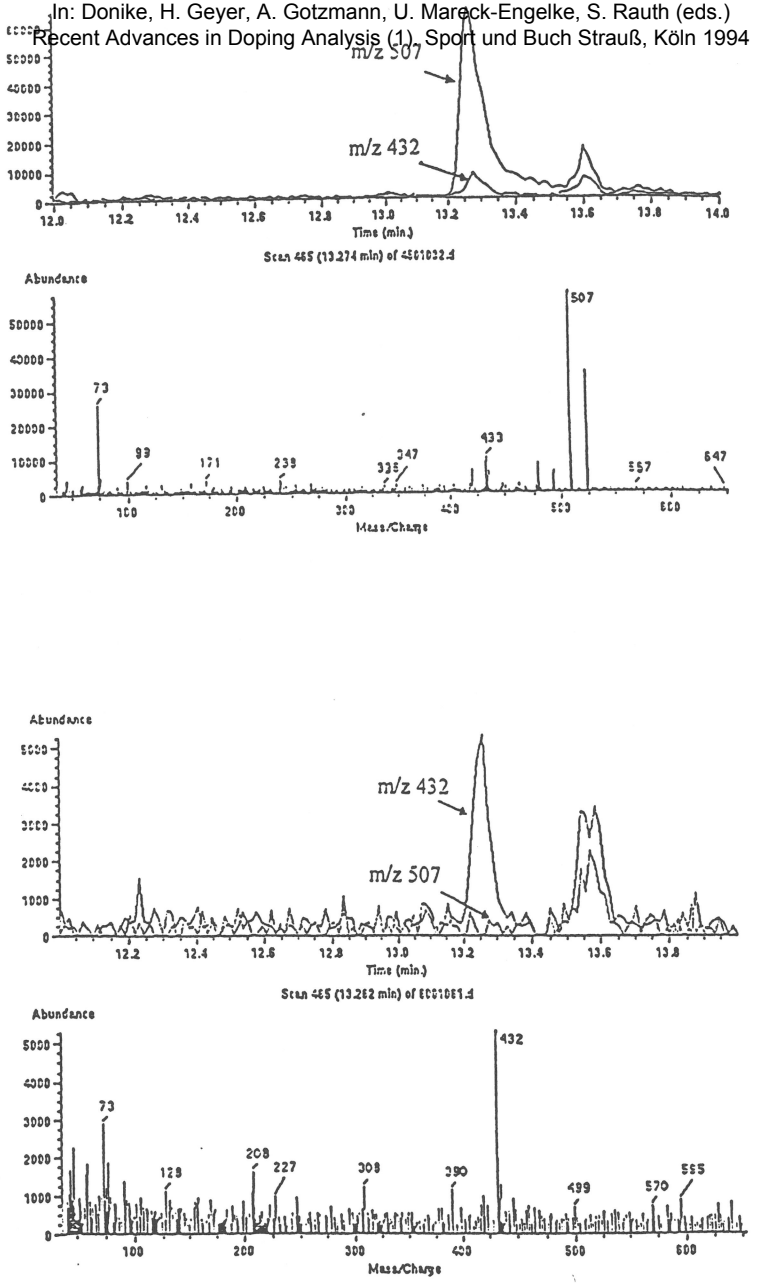

Fig. 3: Identification of testosterone. Shown are reconstructed profiles of the ions m/z 432 and 507 and mass spectra at the region where testosterone is eluted a) after ether extraction b) after n-pentane extraction. The disturbing substance with ion m/z 507 is removed using n-pentane as solvent.Testosterone can be identified by the base peak m/z 432.

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