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Purification and Concentration of Anabolic Steroids by Immuno Affinity Chromatography  
(IAC)  
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## **Purification and Concentration of Anabolic Steroids by Immuno Affinity Chromatography (IAC)**

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### *Abstract*

Purification and concentration of individual substances in biological fluids (e.g. anabolic steroids) and a reduction of negative background effects can be achieved by means of immuno affinity chromatography.

Nowadays the group of the 17 $\alpha$ - methylated steroids is still one of the most abused anabolics. Therefore an antibody towards 17 $\alpha$ -methyltestosterone via its 3-carboxymethyloxime derivative was prepared and coupled to an agarose gel which was filled in special columns. The immuno columns were tested for their capacity and crossreactivities to related anabolic and endogenous steroids. Comparisons between XAD-solid phase-extraction and immuno affinity chromatography have shown that immuno affinity chromatography is a worthwhile tool to isolate and concentrate anabolic steroids from complex matrices such as urine and serum.

### *Introduction*

The commonly established sample preparation steps in dope analysis [1] are often not adequate to give unequivocal evidence of the abuse of anabolic steroids.

The reasons for that are the following:

1. The urine concentration of the substances in question is very low (often near the detection limit)
2. Biological samples prove to be such a complex matrix that interferences of the biological background affect strongly the GC/MS-detection.

The method of choice to overcome the difficulties related to these problems is immuno affinity chromatography because it is the only technique which enables purification of a biomolecule on the basis of its individual molecular structure or biological function.

Affinity chromatography was first introduced by Cuatrecasas 1968 [2], for general considerations see Wilchek et al. 1984 [3].

Immuno affinity techniques have already been used to a major extent in the field of clinical chemistry and biotechnology for the characterisation and identification of macromolecules in biological fluids [4].

Since the work of Landsteiner [5], who has shown that against almost every molecule specific antibodies can be prepared, developments in the area of immunochemical applications have made drastic progresses. The application of immuno technology in the field of steroid chemistry was first described by Erlanger, Liebermann et al. in 1957, when they developed coupling proceedings for the formation of BSA-testosterone and BSA-cortisone-conjugates ready for the immunisation of animals [6].

### *Immunological aspects*

Particles (macromolecules, microorganisms, etc.) which enter the body of vertebrates are recognized as foreign substances and induce an immune response [7].

An early step in this complex cascade of reactions is the production of specific antibodies that bind to the surface of the foreign particle. This marking enables that the immunogenic invader will be eliminated or excreted by subsequent mechanisms of the immunological system.

Antibodies are large proteins representing a Y-shape with molecular weights of approximately 150,000 D (see fig. 1). They consist of 4 peptide chains: 2 so called light and 2 heavy chains. Each pair is made up of an identical composition of amino acids. The linkage between single chains is by disulfide-bonds in different amounts and positions.

The two domains that carry the antigen binding sites are known as  $F_{AB}$ -fragments (named for the *f*ragment having the *a*ntigen *b*inding site), and the protein domain that is involved in the immune regulation is termed the  $F_C$ -fragment (for the *f*ragment that *c*rystallises).

Antibodies are divided into five classes, IgG, IgM, IgA, IgE and IgD, on the basis of the number of Y-like units and the type of heavy chain polypeptide they contain.

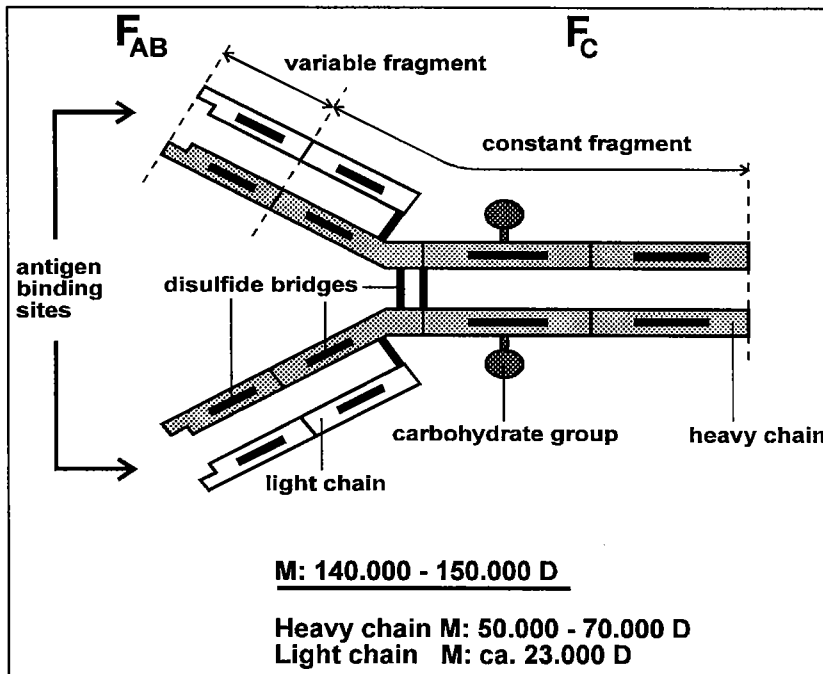


Fig. 1: Schematic of a mouse antibody of the IgG<sub>2A</sub> subclass.

The reversible binding between antibody and antigen acts like a key-lock system: only the one key that fits will be bound (fig. 2). Immunological methods are based on this highly specific binding between an antigen, i.e. the steroid molecule, and an antibody raised against it. This is why immunoaffinity enables individual molecules such as steroids to be recognized sensitively and reliably in complex matrices.

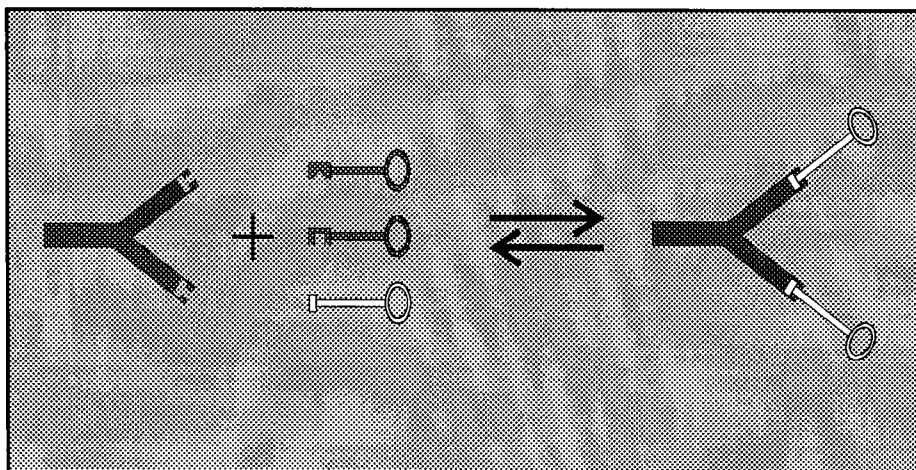


Fig. 2: The key-lock-principle of antibodies.

### Preparation of antigens

Condition for the generation of antibodies is a certain size of the antigen.

Steroids themselves are too small to induce an immune response.

They can be rendered immunogenic by attaching them in sufficient number to a protein carrier for example to BSA, RSA, HSA or keyhole limpet hemocyanin [8, 9, 10] (fig. 3).

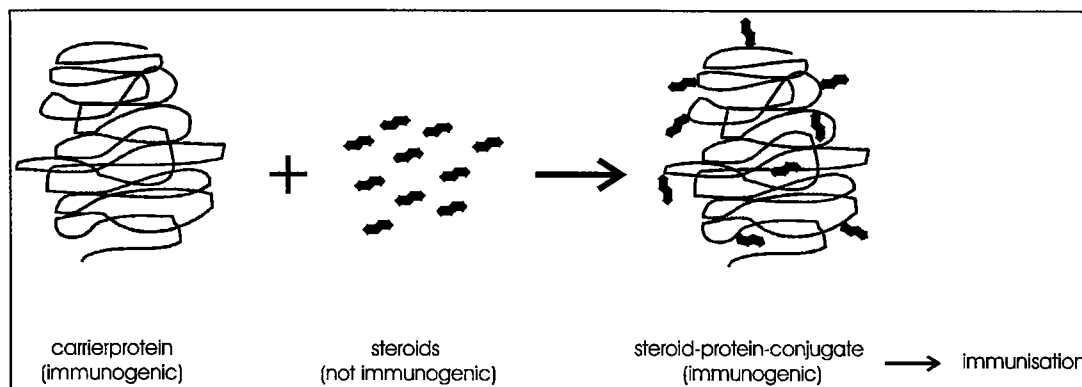
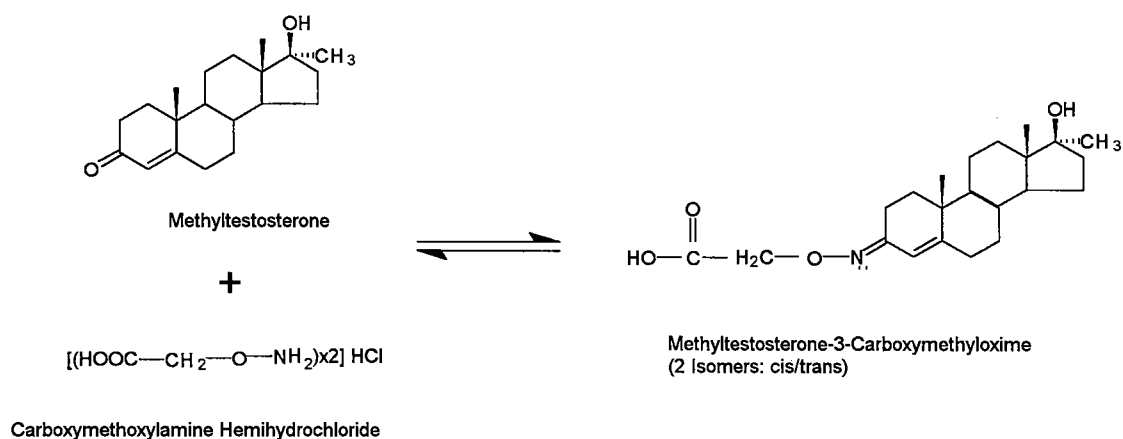
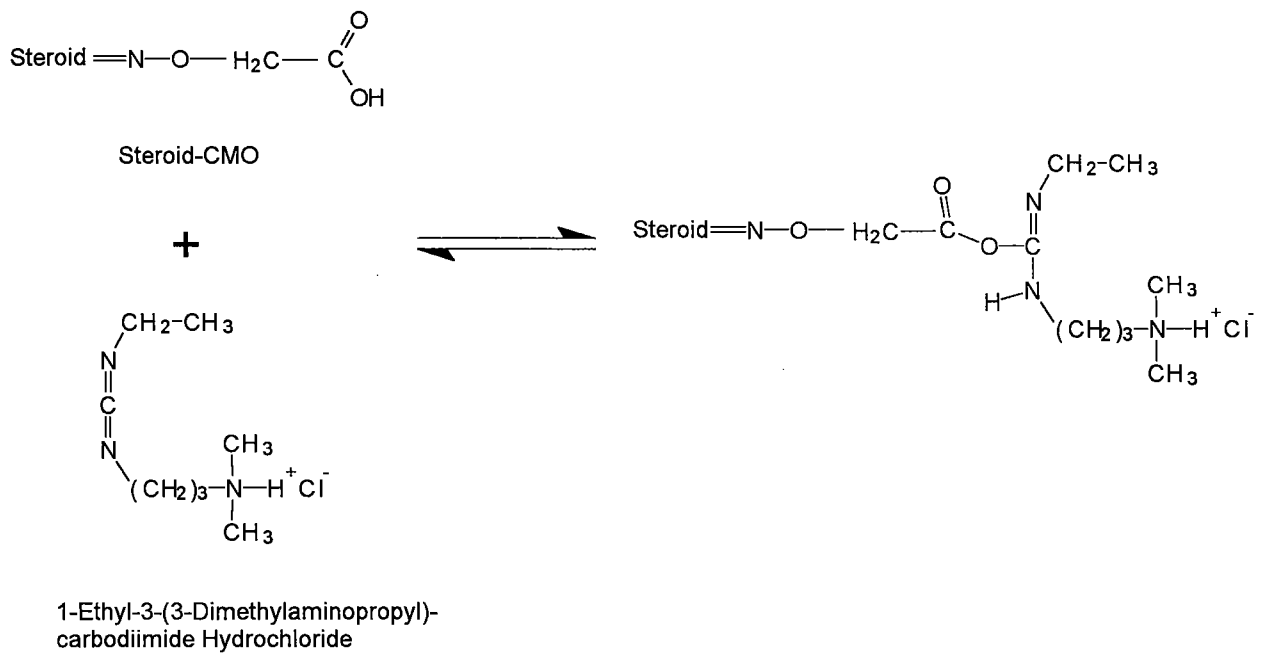


Fig. 3: Preparation of immunogenic steroids.

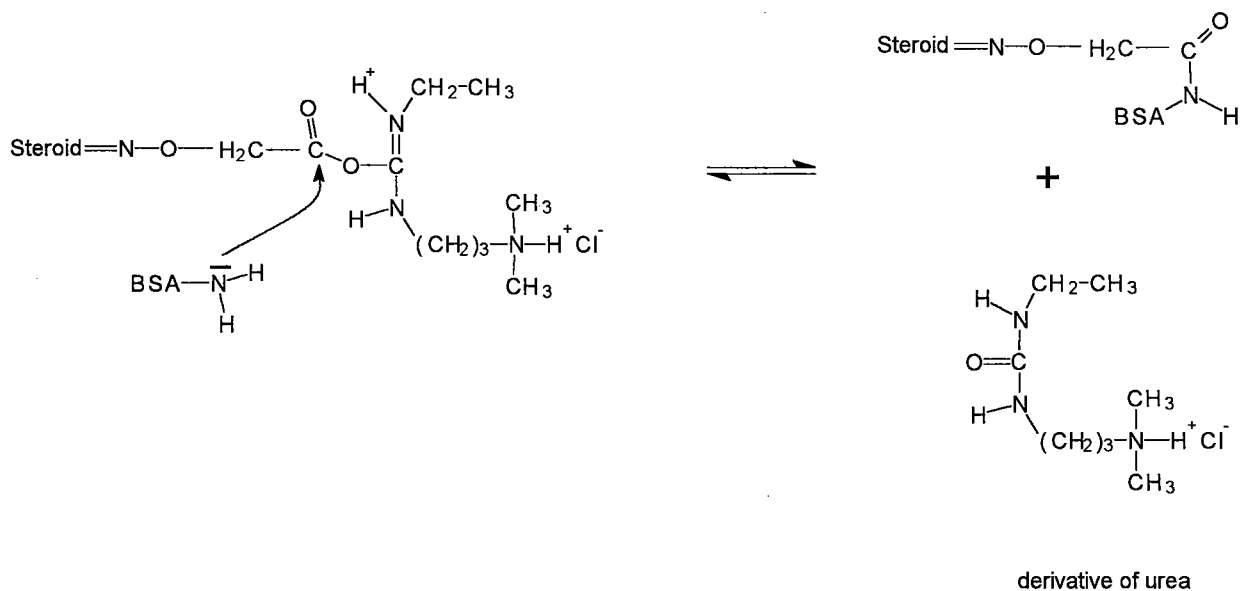
The linkage between steroid and protein is often done via the carboxymethyloxime or the hemisuccinate derivative of the steroid [11]. In our institute we acted according to the following procedure of transforming an existing keto group into a carboxyoxime group:



In the next step the carboxy group of the steroid was activated by a carbodiimide reaction to form a reactive mixed anhydride.



The free amino residues of the BSA (e.g. lysine) react with the activated carboxy group to form a peptide bond.



A conjugate with a reasonable immune response should consist of at least 10 steroid molecules.

The subsequent injection in rabbits with this immunogene will result in an antiserum that is not only directed towards the steroid but also towards BSA-determinants.

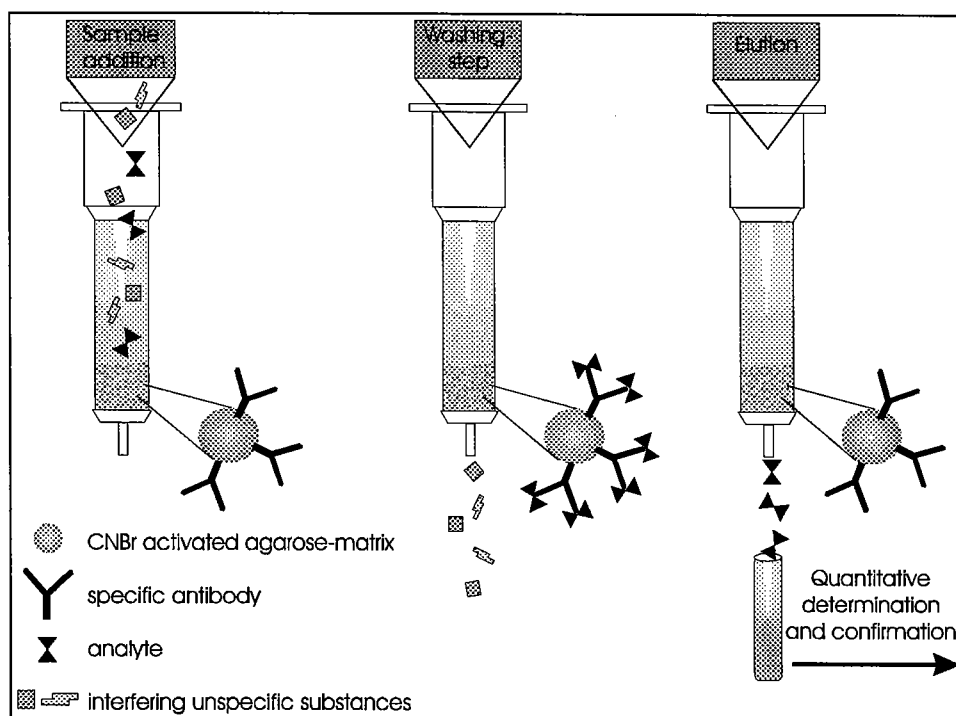
After isolation and purification of the IgG-fraction on a A Sepharose CL 4B Protein (Pharmacia) an antiserum with a high specificity for the steroid was obtained [12, 13].

### *Sample preparation*

For our application the antibodies were immobilised on a chemically modified agarose gel (CNBr-activated Sepharose 4B, Pharmacia) of which 1 ml is filled in columns.

The purification includes 5 steps (refer to fig. 4):

1. Applying of the standard solution or the hydrolysed urine sample (ether extract dissolved in 5 ml PBS, 10 mmol pH 7.4, after first dissolving the dry residue in 100  $\mu$ l methanol)
2. Washing with 15ml of a methanol-water solution (15 %, by weight)
3. Elution of the bound fraction with 3 ml of a methanol-water solution (65 %)
4. Rinsing the column with 5 ml of the 65 % methanol to ensure that any residue of the preceding sample gets eluted from the immuno gel
5. Equilibration of the column with 15 ml of PBS-buffer for storage or reuse.



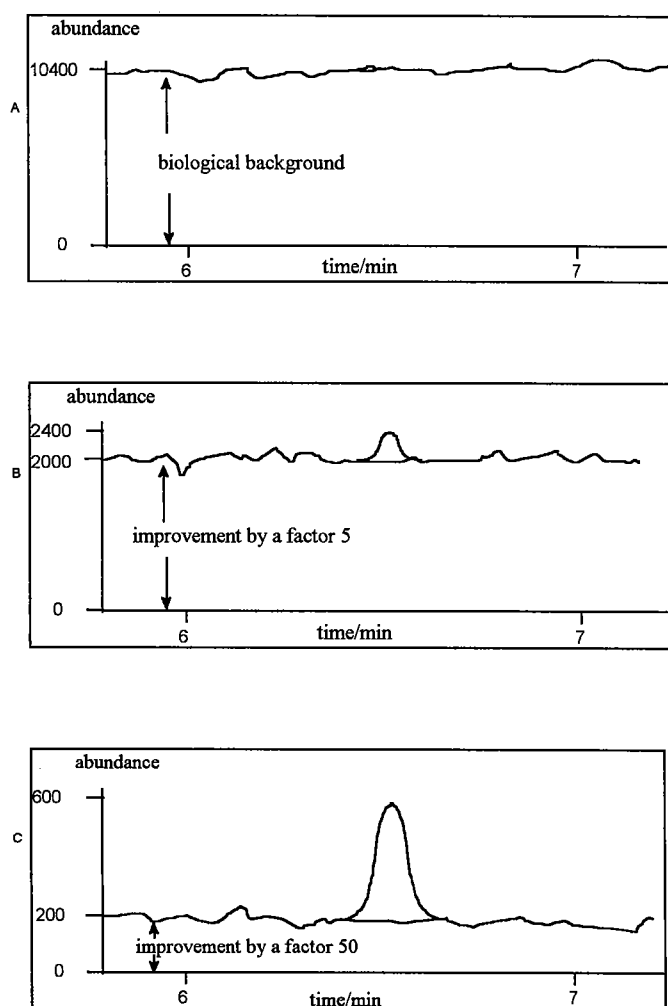
**Fig. 4:** Sample treatment for immuno affinity chromatography.

For analytical details of the derivatisation procedure of the dried eluate and the GC-MS-parameters see elsewhere [1,14].

## Results

A typical pattern of a urine sample before and after purification by immunoaffinity chromatography (IAC) is given in fig. 5.

As can be seen the absolute signal intensity remains constant but the background is reduced and one obtains an improved signal/noise ratio.



**Fig. 5:** Influence of the biological background on the GC/MS-detection:

- A: The actual signal is masked by the biological background.
- B: Reduction of the biological background by a factor 5 after using a more proper organic solvent for extraction.
- C: 50-fold improvement after IAC.



The same effect is illustrated by a GC-FID-chromatogram of 10 ml hydrolysed urine in fig. 6. The passing through and washing fractions contain many interfering substances, whereas the elution fraction of this sample is liberated from negative background influences.

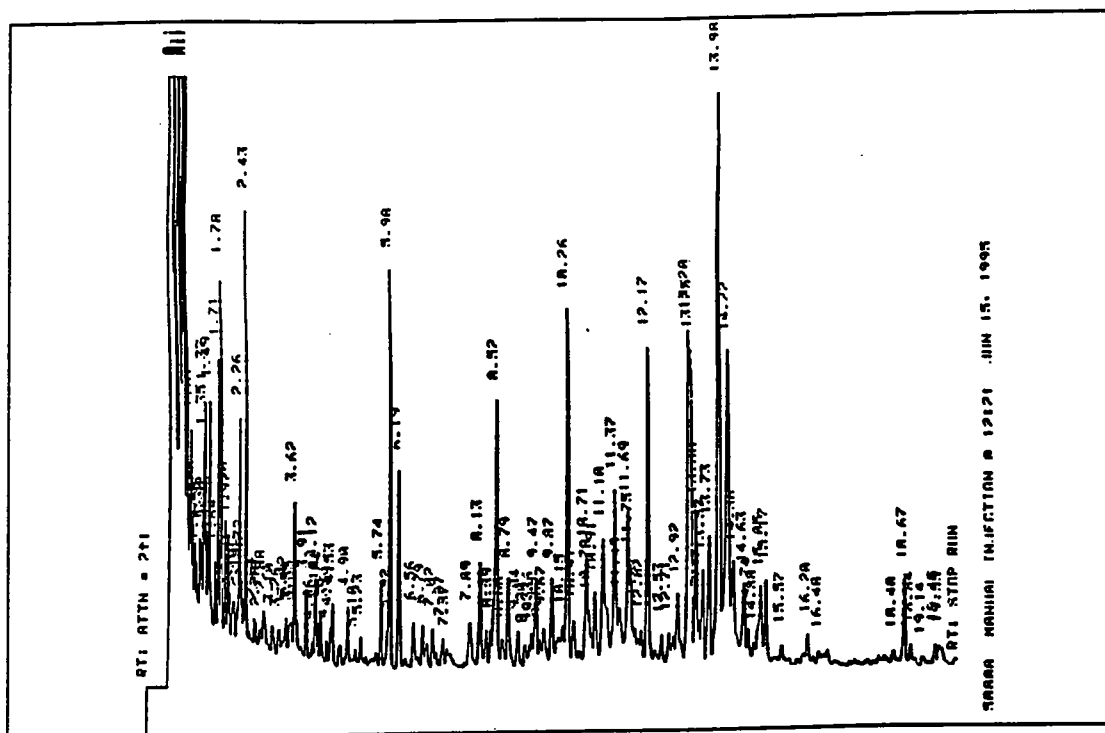


Fig. 6A: GC/FID-chromatogram of the passing through and washing fractions of sample 2387/1995.

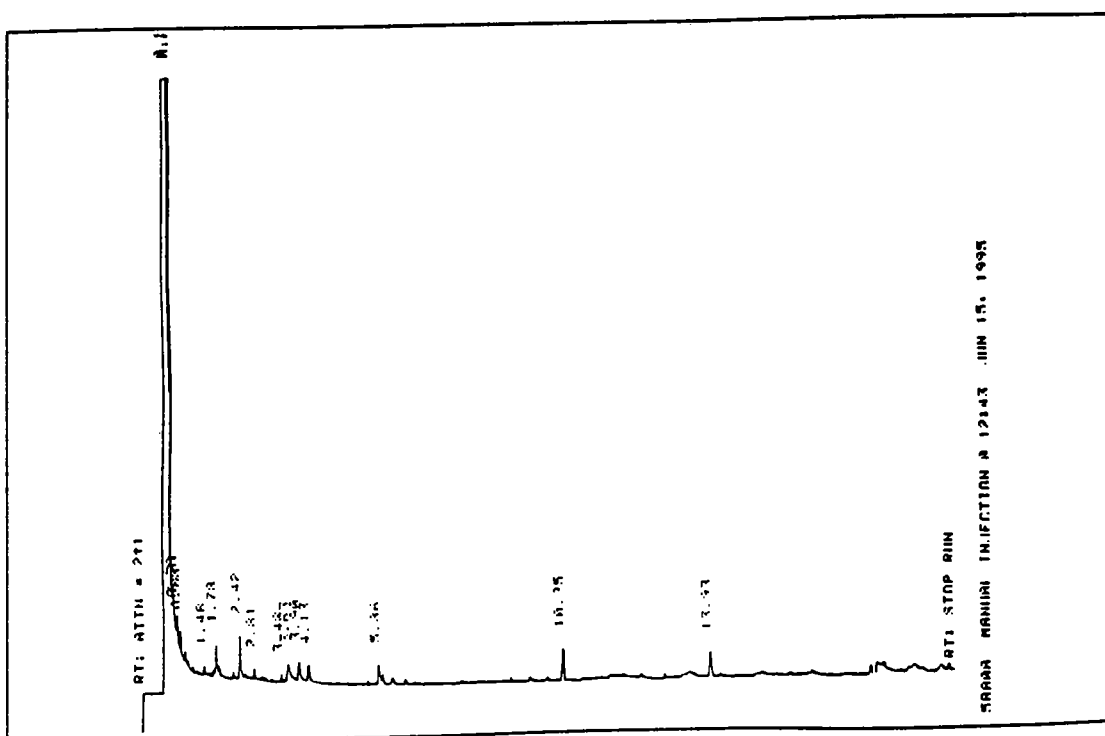
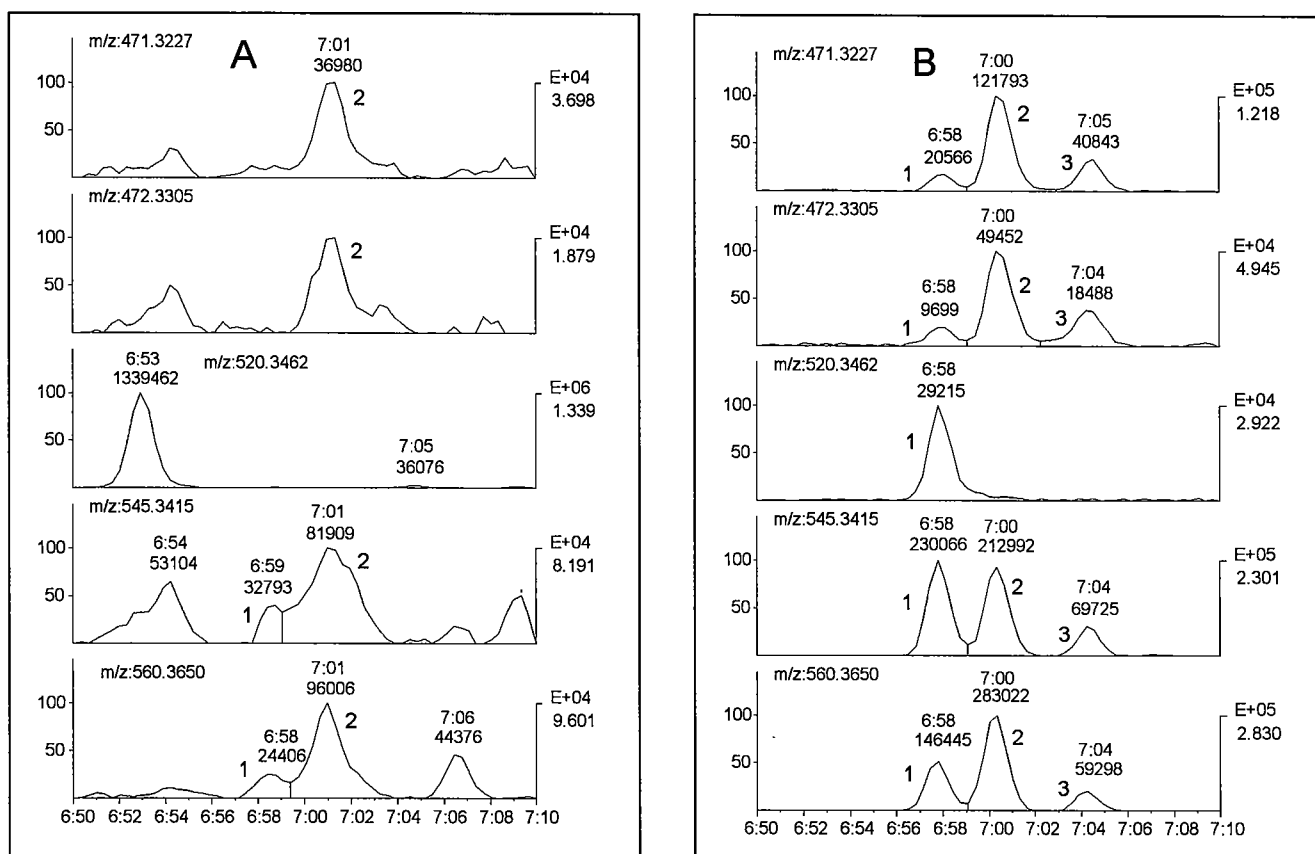


Fig. 6B: GC/FID-chromatogram of the elution fraction of sample 2387/1995.

Figure 7 reflects the results of a stanozolol positive urine sample: A after normal routine HRMS-analysis for anabolic steroids, B after additional purification over IAC-gel. In contrast to fig 7A, where the detection is accompanied by many interfering effects, the only ion traces registered following IAC-isolation (fig. 7B) are those due to 3'-OH-stanozolol and 4 $\beta$ -OH-stanozolol, the main metabolites after stanozolol intake [14].

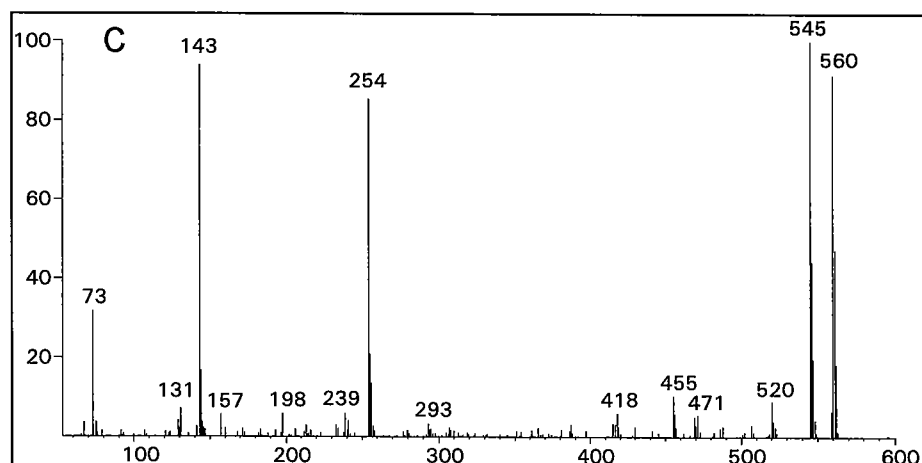
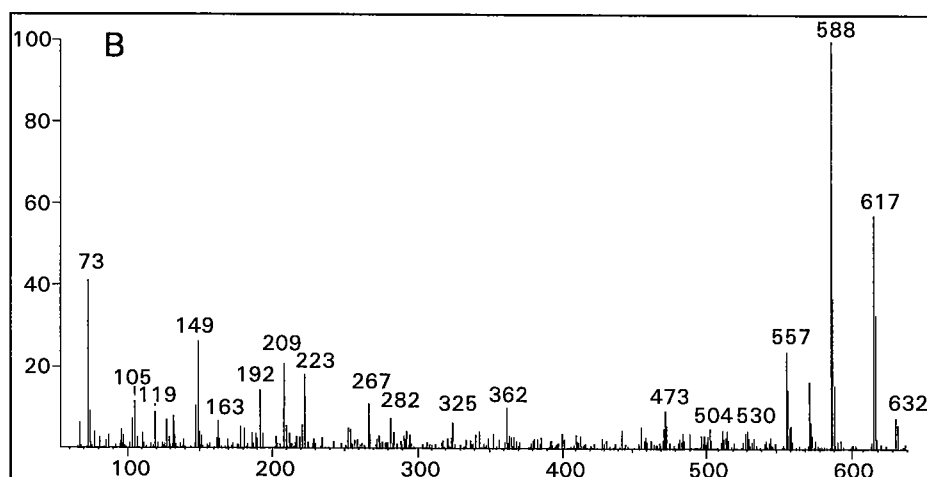
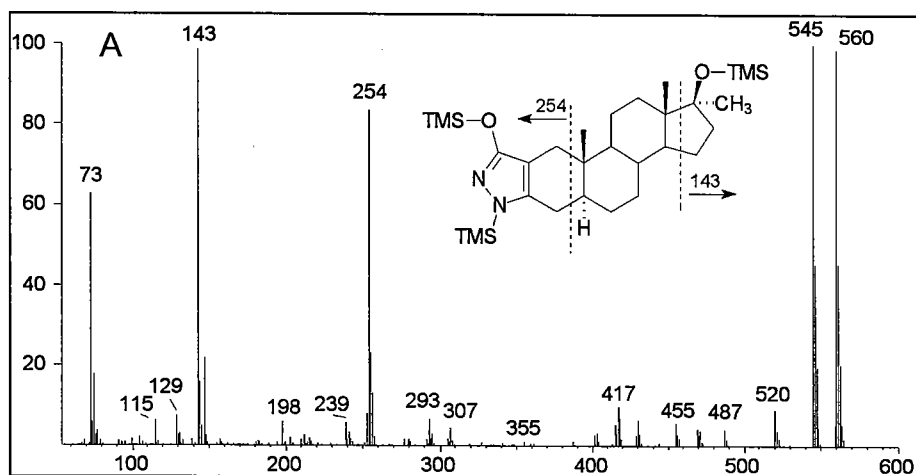


**Fig. 7:** A: HRMS analysis of a stanozolol positive athlete monitored after routine procedure for anabolic steroids.

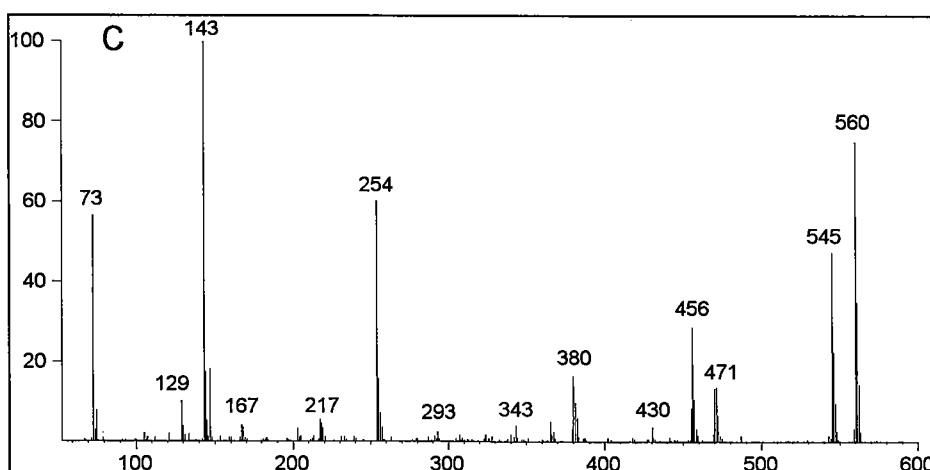
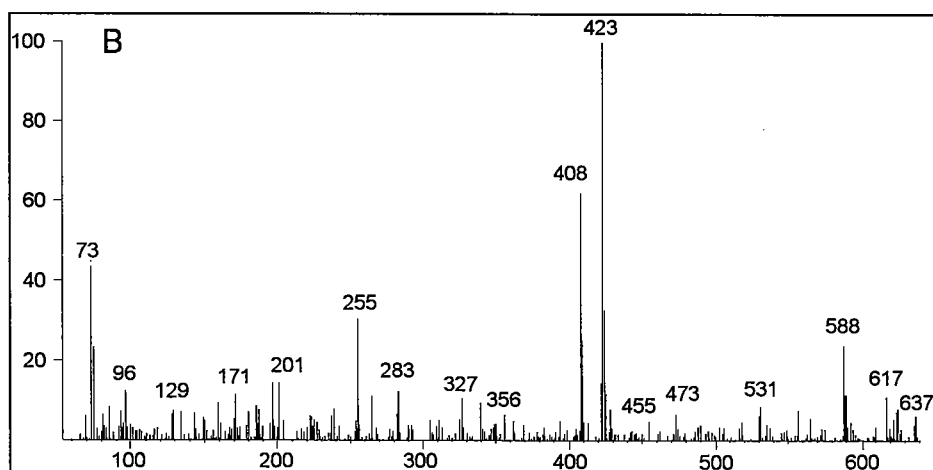
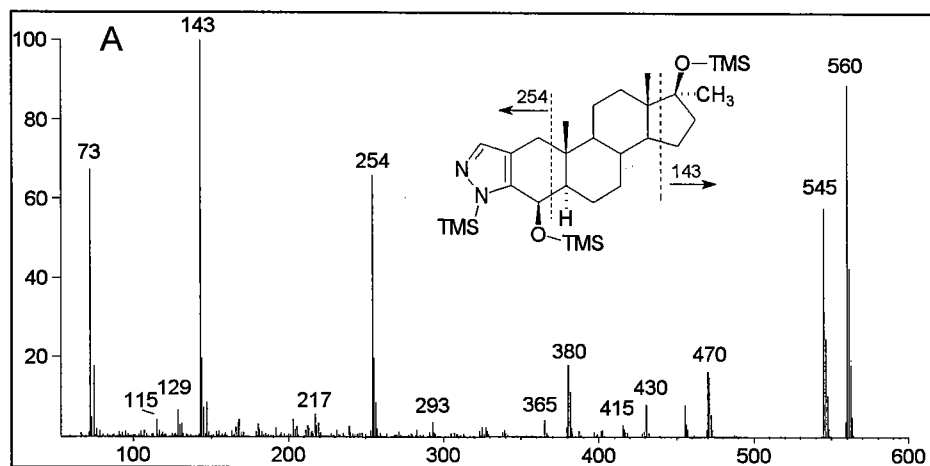
B: HRMS analysis of the identical sample obtained after additional IAC-application.

Peak 1 refers to 3'-OH stanozolol, peak 2 refers to 4 $\beta$ -OH stanozolol and 4 $\alpha$ -OH-stanozolol is used as internal standard in a concentration of 0.5ng/ml urine (peak 3).

To obtain a mass spectrum for confirmation the sample has to undergo purification by immuno affinity chromatography. Without immuno affinity application it is not possible to confirm the presence of the stanozolol metabolites because of the high level of biological background which coelutes with the stanozolol metabolites. After IAC isolation the coeluting biological background is removed and the mass spectra obtained unambiguously demonstrates the presence of the metabolites (figs. 8 and 9).



**Fig. 8:** A: Mass spectrum of a reference standard of 3'-OH-stanozolol. B: Mass spectrum obtained after normal screening procedure. C: Mass spectrum of the same sample monitored after an additional purification step by means of IAC.



**Fig. 9:** The same sequence of mass spectra for the 4β-OH-stanozolol metabolite. A the standard spectrum, B the mass spectrum after screening procedure and C after IAC isolation.

The reduction of the biological background was successful by using an antibody directed towards methyltestosterone, which shows high crossreactivity for stanozolol metabolites. This feature demonstrates that it is often of great advantage to use a low specific antibody that allows the group separation of anabolic steroids.

*Crossreactivities of some related anabolic and endogenous steroids*

Crossreactivities of two different methyltestosterone gels towards some anabolic steroids are listed in tab. 1.

To examine the crossreactivity 500 ng of each crossreactant were dissolved in 5 ml phosphate buffer (pH 7,4) and applied on to a methyltestosterone-column. After washing the column, the amount of steroid in the elution fraction was determined by GC/MS-detection. The crossreactivity is indicated as percentage relative to the total amount of methyltestosterone bound [15].

All listed steroids have one characteristic in common. They all possess 17 $\beta$ -OH, 17 $\alpha$ -methyl configuration. It appears that this configuration is necessary for the complex formation between steroid and antibody.

Metabolite	MT-Agarose-Gel		MT-CNBr-Agarose-Gel	
	amount/ng	crossreactivity/%	amount/ng	crossreactivity/%
<b>Methyltestosterone</b>	325	100	260	100
17 $\alpha$ -Methyl-5 $\alpha$ -androstan-3 $\beta$ ,17 $\beta$ -diol	232	71	155	60
17 $\alpha$ -Methyl-5 $\beta$ -androstan-3 $\beta$ ,17 $\beta$ -diol	115	35	99	38
17 $\alpha$ -Methyl-5 $\alpha$ -androstan-3 $\alpha$ ,17 $\beta$ -diol	167	51	151	58
17 $\alpha$ -Methyl-5 $\beta$ -androstan-3 $\alpha$ ,17 $\beta$ -diol	110	34	86	33
Tetra-Hydro-Bolasterone	122	38	98	38
17 $\alpha$ -Methyl-5 $\beta$ -androstan-1en-3 $\alpha$ ,17 $\beta$ -diol	118	36	94	36
3'-OH-Stanozolol	111	34	104	40
6 $\beta$ -OH-Metandienone	99	30	111	43
4 $\beta$ -OH-Stanozolol	95	29	62	24

Tab. 1: Crossreactivities of some related anabolic steroids. Values are means of at least two separate determinations.

Steroids with epimeric 17-structure show no affinity to the antibodies (see tab 2).

Structural changes in the steroid such as additional fluor or hydroxy groups also prevent the steroid from combining with the antibody binding site. The key-lock-system does not work in these cases anymore.

Metabolite	MT-Agarose-Gel		MT-CNBr-Agarose-Gel	
	amount/ng	crossreactivity/%	amount/ng	crossreactivity/%
Epimethyltestosterone	0	0	2	<1
17-Epi-3'-OH-Stanozolol	2	<1	3	1
17 $\beta$ -Methyl-5 $\beta$ -androst-1en-3 $\alpha$ ,17 $\alpha$ -diol	0	0	0	0
3 $\alpha$ ,6 $\beta$ -Di-OH-4en-Fluoxymesterone	0	0	0	0
6 $\beta$ -OH-Fluoxymesterone	4	1	3	1

Tab. 2: Steroids without antibody affinity. Values are means of duplicates.

In another experiment 5 ml of a quality control urine are applied on to the methyltestosterone gel to see, whether the endogenous steroids compete for the antibody's binding sites.

It is obvious that only testosterone and DHT react with the antibodies. The other steroids have negligible affinity relative to their applied amount and are found in the passing through or washing fractions (tab. 3).

The results of the same experiment using the same urine but spiked with 500 ng methyltestosterone are shown in tab. 4. It is striking that the concentration of testosterone and DHT in the elution fraction is decreased when methyltestosterone is added to the urine.

This may stem from the fact that methyltestosterone displaces steroids with lower affinity from the antibody binding sites, because methyltestosterone was the steroid against which the antibodies were raised and hence should possess the highest affinity to them.

Substance	passing through	1. washing	2. washing	elution	rinsing
	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
Testosterone	1.2	1.6	0.3	70.7	0
Dihydrotestost.	13.8	7.2	0.5	9.8	0.1
5 $\alpha$ -Androstandiol	63.6	46.2	4.5	7.1	0.2
5 $\beta$ -Androstandiol	236	109	9	5.3	0.1
Androsterone	2992	1174	80.4	8.7	1.6
Etiocholanolone	2346	886	51.8	5.9	1.4
Epitestosterone	38	15.5	0.9	0.3	0
OH-Androsterone	735	231	11.7	1	0.4
OH-Etiochol.	242	76.2	3.7	0.3	0
DHEA	96.8	37.1	2.8	0.2	0
Pregnandiol	193	126	12.5	1.7	0.4
TH-Cortisol	1192	395	23.8	1.1	0
allo-THC	825	284	16.5	1.1	0.4

Tab. 3: Behavior of endogenous steroids to an anti-methyltestosterone gel.

Substance	passing through	1. + 2. washing	elution	rinsing
	ng/ml	ng/ml	ng/ml	ng/ml
Testosterone	38	42	9	0
Dihydrotestost.	9	10	1	1
5 $\alpha$ -Androstandiol	90	40	3	2
5 $\beta$ -Androstandiol	246	101	2	1
Androsterone	3325	1257	1	0
Etiocholanolone	2743	977	8	1
Epitestosterone	46	14	0	0
OH-Androsterone	681	170	1	0
OH-Etiochol.	220	7	0	1
DHEA	130	38	1	1
Pregnandiol	221	160	1	1
TH-Cortisol	1646	424	0	1
allo-THC	992	241	1	0

Methyltestost.	74	108	275	9
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Tab. 4: Crossreactivities of endogenous steroids in concurrence to methyltestosterone.

## *Conclusions*

Our investigations have proved, that immunoaffinity is a worthwhile tool to isolate and concentrate anabolic steroids from complex matrices.

When choosing appropriate antibodies it is possible to separate simultaneously many anabolics from interfering substances by a single purification step.

It has become possible to utilise the advantages of HRMS to the full (possibility of splitless injection, lower detection limits, better confirmation results).

It is now wishful to prepare antibodies for different steroids, whose detection is hindered by interfering background effects.

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