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RECENT ADVANCES IN DOPING ANALYSIS (5)

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M. MACHNIK, P. DELAHAUT, S. HORNING, W. SCHÄNZER:
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Preparation of Immuno-Affinity-Columns

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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 and other banned substances. 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 subsequent physico-chemical detection (GC-MS, Elisa, FID).

A method to overcome the difficulties related to these problems is immuno affinity chromatography (IAC) [2,3].

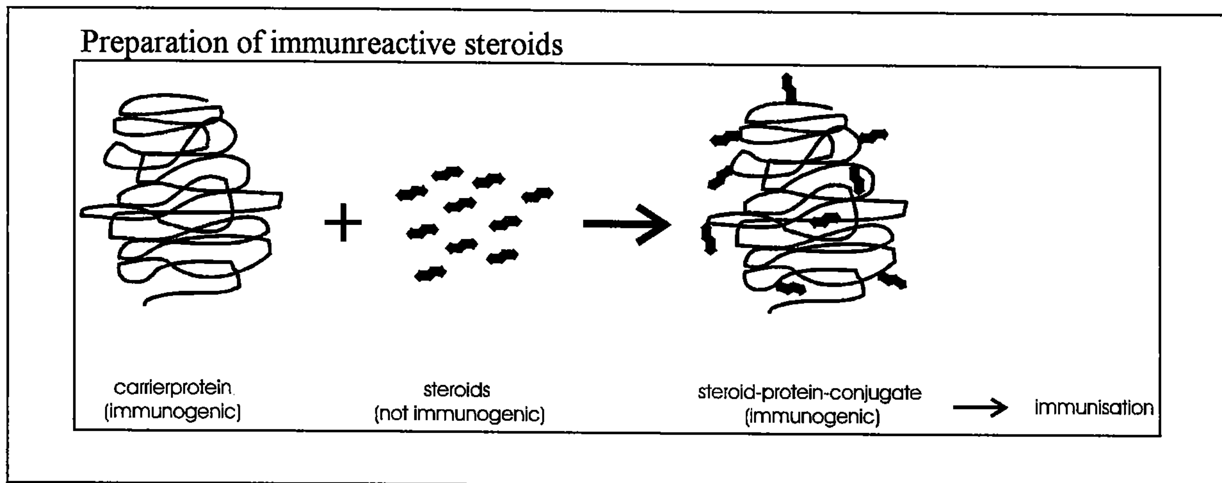
In immuno affinity chromatography the molecule to be purified (antigen) is reversibly adsorbed by a complementary ligand (antibody) covalently attached to an insoluble support (immunosorbent). That means IAC combines the retention and therefore the resolution of an analyte in a chromatographic system with the high affinity and specificity of the antigen-antibody-interaction.

The preparation of chromatographic immunoaffinity columns includes the following steps:

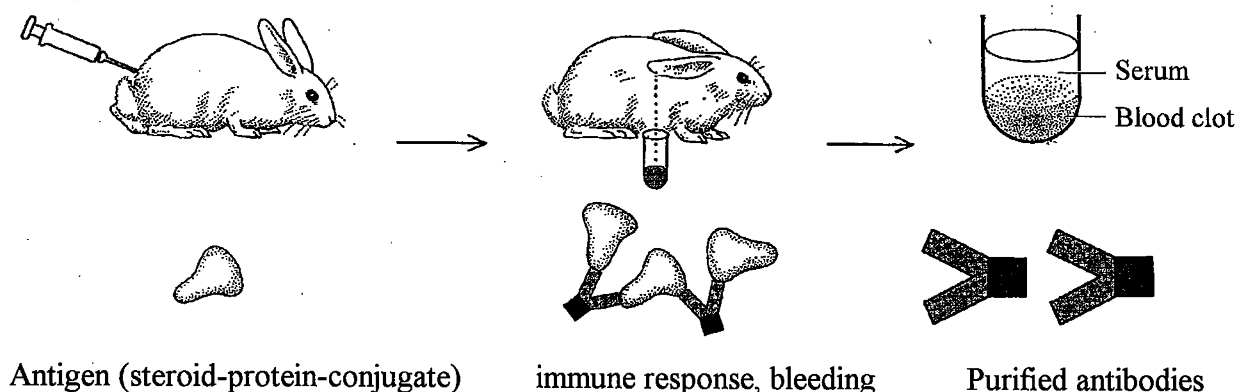
1. Production of the immunogens (antigens)
2. Production of the antibodies
3. Activation of the solid support and immobilisation of the antibodies to the reactive matrix
4. Application into proper columns

Synthesis of antigens and complementary antibodies

The synthesis of specific antibodies towards the respective molecules envolves a cascade of preparation and reaction steps and is limited by the immune response of a biological system (host animal, cell culture). One criterion for inducing a reasonable immune response is a certain size of the antigen that must be recognized as foreign particle by the infected organism. Steroids themselves are to small to stimulate B-cells to produce antibodies. They can be rendered immunogenic by attaching them in sufficient number to a protein carrier for example to BSA, RSA, HSA or KLH [4,5] according to the scheme:



Once a suitable antigen is available a number of steps are neccessary [6,7]:
1. Immunisation procedure: Multiple injections of antigen solution into animals, e.g. rabbits, 2. Test bleeds: Deciding to boost again or to fuse, 3. Blood taking, 4. Isolation and purification of the IgG-fraction, 5. Characterisation of the antibodies (titer, affinity, crossreactivity)



Antigen (steroid-protein-conjugate) immune response, bleeding Purified antibodies

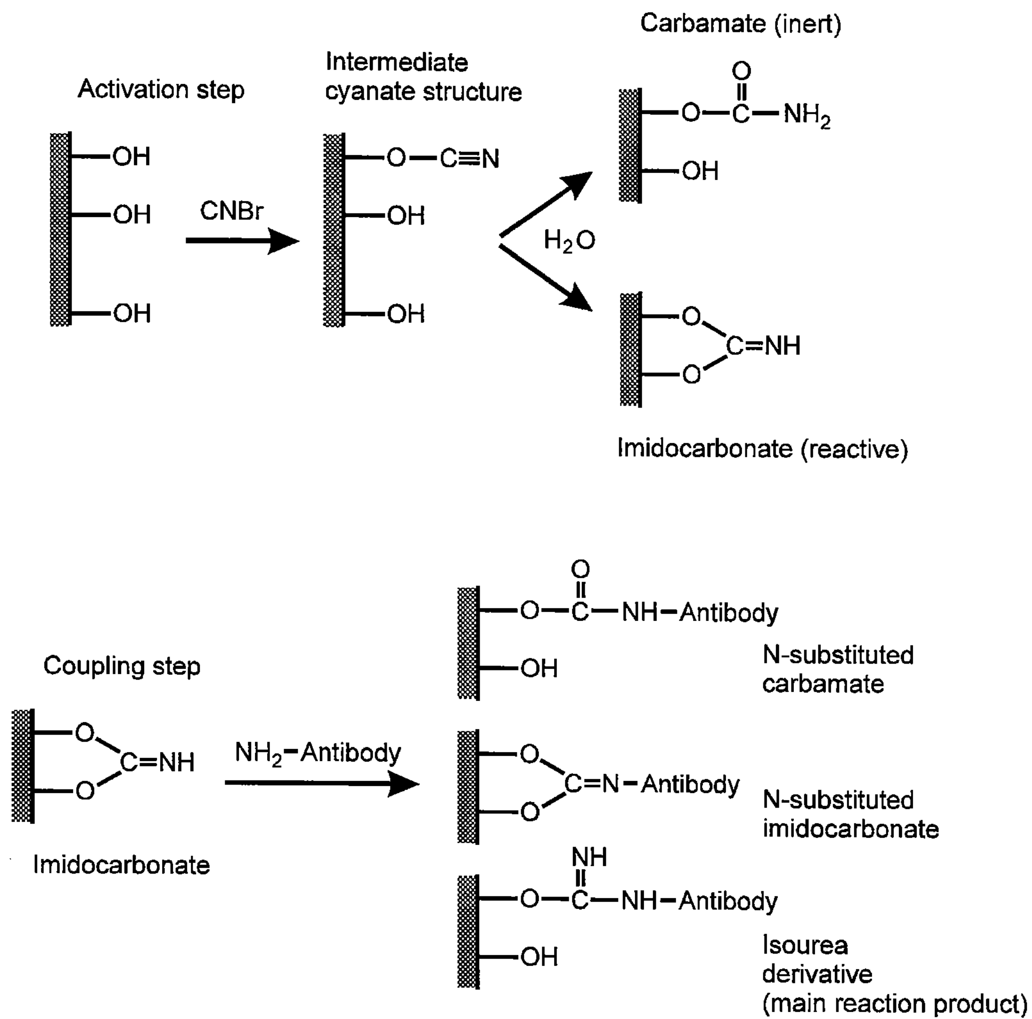
Immobilisation procedure

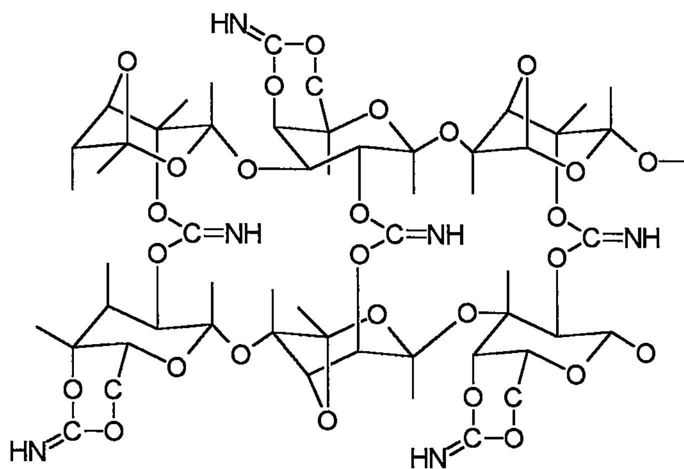
For the immobilisation of the purified antibodies numerous efforts have been made [8,9]. Many supports and modes of coupling procedures have been used [10]. Among these widespread application have nowadays polysaccharide matrices, e.g. cellulose, the crosslinked dextrans and agarose.
In contrast to the polysaccharide gels entirely synthetic gels such as polyacrylamide or methacrylate gels, ceramics and porous glass supports (with or without protein coating) have been developed.

Ideal characteristics of a solid support:

- insolubility
- sufficient permeability and a large specific area
- rigidy and robustness against physical stress
- chemical inertness, no nonspecific adsorption
- capability of functionalisation
- resistance to chemical, microbial and enzymatic attack

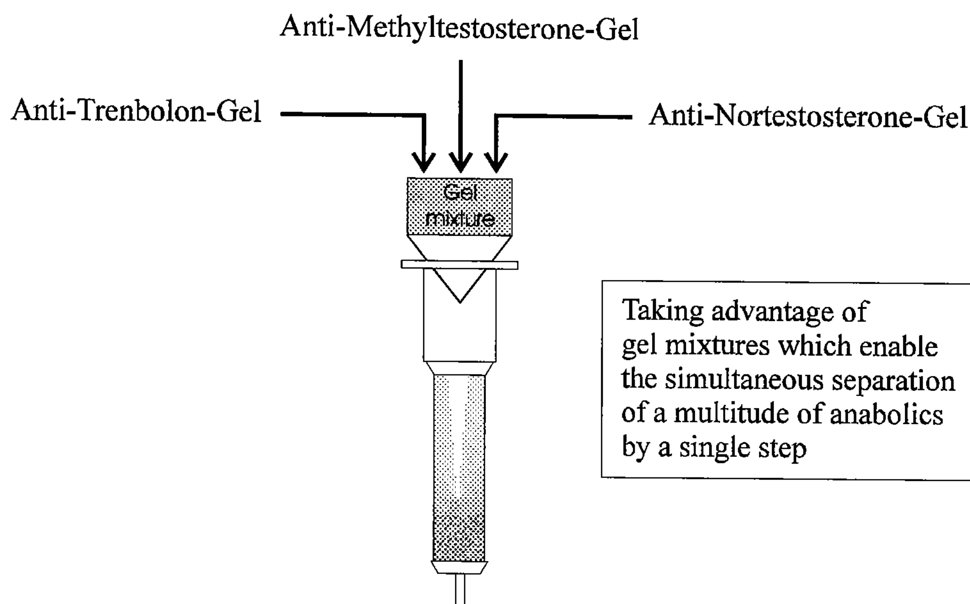
For attaching ligands to the hydroxylic groups of the gel an activation step is neccessary in order to make them more reactive towards nucleophilic groups e.g. free amino groups of lysine residues present on the ligand. The most general technique is the CNBr-method. The activation of polysaccharides with CNBr was introduced to affinity chromatography by Axen et al. (1967) [11]. Nowadays ready to use gels are commercially available. Pharmacia markets a number of CNBr activated Sepharose gels in a freeze-dried state which can be swollen in HCl/H_2O for direct immobilisation of ligands.





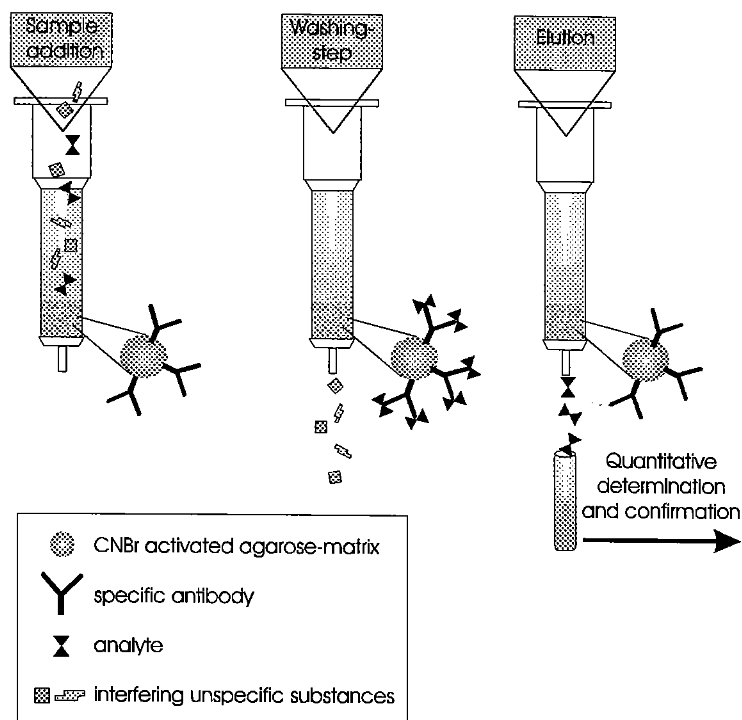
Hypothetic structure of Sepharose 4B. Reproduced from [12].

Preparation of "Gel-Cocktails"

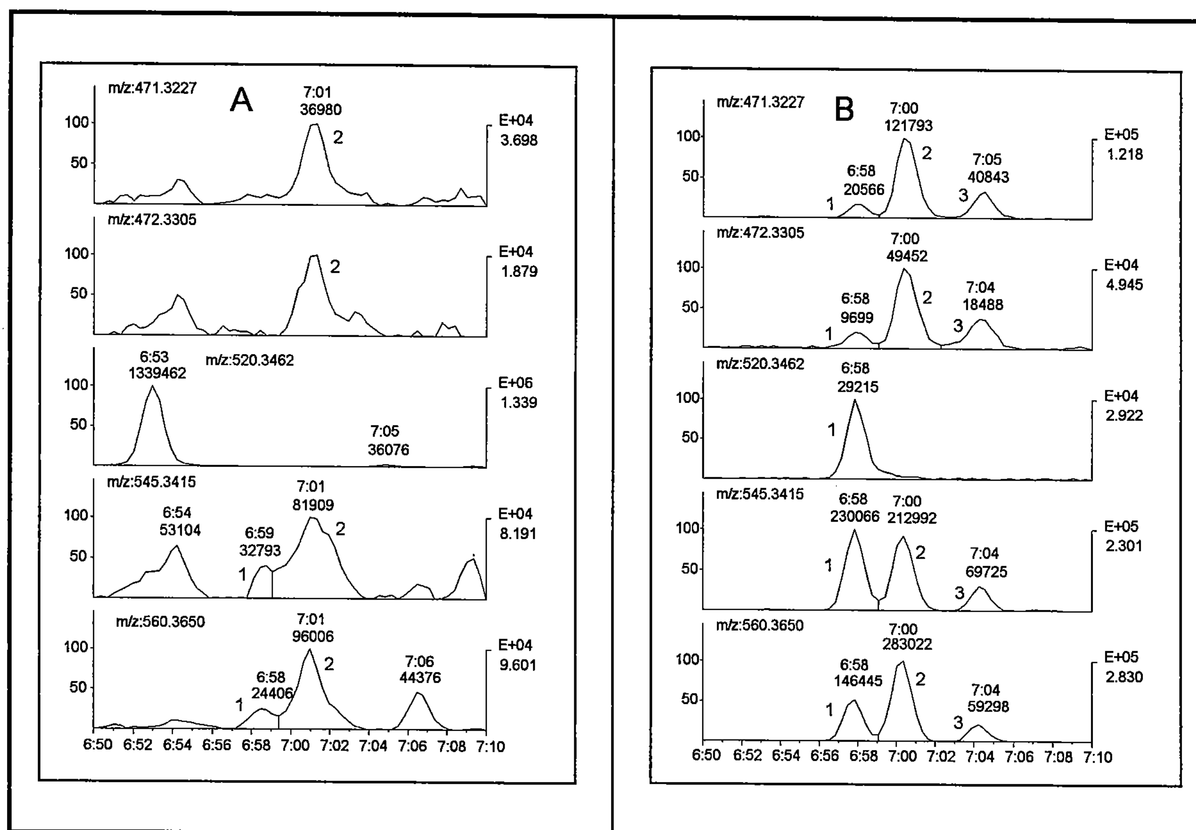


Taking advantage of gel mixtures which enable the simultaneous separation of a multitude of anabolics by a single step

Sample treatment



The sample is applied under conditions which favor its specific binding to the immobilised antibody. Unbound substances are washed away and the substance of interest can be recovered by changing the conditions to those which favor its desorption; here increasing the concentration of organic solvent content is successful [13]. The flow is adjusted by the hydrostatic pressure of the fluids.



A: HRMS analysis of a stanozolol positive sample monitored after routine procedure for anabolic steroids. B: HRMS analysis of the same sample obtained after additional IAC-application. Acquisition was run in SIM mode according to the indicated ion traces. Peak 1 refers to 3'-OH stanozolol, peak 2 refers to 4β-OH stanozolol and 4α-OH-stanozolol is used as internal standard in a concentration of 0.5ng/ml urine (peak 3).

Conclusions

- The immobilisation of antibodies by the CNBr method forms stable covalent bonds between matrix and ligands
- The immobilisation has little effect on the activity and interaction between antibody and antigen
- Possibility of using "gel cocktails" for multi residue analysis
- IAC has a concentrating effect which enables convenient processing of large sample volumes
- It has become possible to utilise the advantages of HRMS to the full (lower detection limits, better confirmation results)

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