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IMMUNOASSAYS AND IMMUNOAFFINITY CHROMATOGRAPHY FOR THE DETECTION OF DRUG RESIDUES

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INTRODUCTION

Modern immunoassay techniques have been used to measure the concentration of several hundreds of compounds. The techniques used depend on the measurement of binding between antibodies and antigens. Immunoassays are classified according to the label which is attached either to the antigen or to the antibody.
The label can be a radioactive atom in radioimmunoassays (RIA) or an enzyme in enzyme immunoassays (EIA). These techniques have been used most widely in endocrinology where the measurement of low concentrations in biological material has presented a problem.
However, the techniques have been recently applied to other molecules including drugs. Immunoassays are also used to measure drug residues in athletes, race horses and also in farm animals.
In traces analysis, the critical step is the extraction. One of the most successfull techniques developped during the past few years for extract purification is immunoaffinity chromatography (IAC). This technique combines the selectivity of immunochemistry with the easy use of simple chromatographic cartridges.

HISTORY

The development of immunoassays is linked to the discovery of antibodies.
In 1896, Erhlich postulated that there were cells in the body which are producing antibodies when they come into contact with an antigen. He compared the specificity of the antigen-antibody reaction with the key-lock principle. Rapidly, it was demonstrated that this reaction also occured *in vitro*, in a test tube for instance.
This was the beginning of the development of a large number of immunoassays.
In 1959, trying to understand diabetes, Mirsky, Yalow and Berson set up a radioimmunological assay for plasma insulin.
We had to wait till 1971 when Engvall, Perlmann, van Weemen and Schuurs replaced radiolabelled tracers by molecules conjugated to enzymes. The enzyme immunoassay was at its beginning and was going to progressively replace RIA in many fields.

Nowadays, the sophistication in this field is so high that we see the appearance of systems such as dipsticks (1). This system allows to measure substances which are present, in biological fluids, at a concentration of approximately 1 ppb with a little band on which an antibody is absorbed. Unfortunately, the system is still not available for all molecules.
For IAC we had to wait for the eighties.

IMMUNOASSAYS

1. PRINCIPLE

Immunoassays are based on the high binding specificity of the antibody-antigen reactions. They are extremely sensitive because certain labelling techniques for molecules allow the measurement of very small quantities (pg) (2,3).
In order to detect low molecular mass compounds the competitive assay format is obligatory. The competitive method is based on the competition between free antigen (Ag) and labelled antigen (Ag^*) for a limited number of antibody combining sites (Ab). The immunochemical principle can be presented as follow:

$$Ag + Ag^* + Ab \rightleftharpoons AgAb + Ag^*Ab$$

In most cases, the assay response represents the bound labelled antigen which is inversely proportional to the concentration of analyte. In order to make a distinction between unreacting and reacting complex, most assays use either antibody or antigen bound to a solid phase. Thus all the reagents that are not bound by the antibody can be easily removed by washing the solid phase. The typical assay for quantitative evaluation is the microtitre plate assay.
The potential sensivity is directly related to the affinity of the antibody. Incubation time and temperature have also an influence on the assay sensitivity. Another requirement that has to be met is that the affinity of the antibody for free and labelled antigen must be equal.

2. PREPARATION OF LABELLED LIGAND

a. Radiolabelled ligand

The labelled ligand must have a sufficient labelling for accurate measurement. Most of the radiolabelled tracers are produced by commercial companies (Amersham, NEN) but enzymatic tracers can be synthetized in a laboratory. Radioactive tracers can be prepared by incorporating tritium (^3H) or iodine (^{125}I).
Tritium emits low energy b radiations with a half-life of more than 12 years.
If it is easy to find tracers radiolabelled with tritium for most endogenous steroïds (testosterone, 17 β oestradiol, oestriol, oestrone .....), companies do not put tracers for xenobiotics

(nortestosterone, methyltestosterone, trenbolone, .) on the market. The reasons are mainly commercial.

Iodine (^{125}**I**) ligand is not very often used for small molecules. It is mainly used for protein.The efficiency of counting is higher than for tritium but one disadvantage is its short half-life (± 60 days). Many kits (glycoproteins, thyroid hormone, ...) in human medicine use this type of tracer .

b. Enzyme labels

Following the limitation of the use of isotopes and because of the easy use of this kind of technique, enzyme immunoassays are supplanting RIA.

A wide variety of enzymes have been used as labels for antigens or antibodies.

The enzyme must have a specific activity and a low non specific binding capacity to antibody. It must be stable and easy to detect.

The most common enzymes in assays are horseradish peroxydase (HRP 65%) and alkaline phosphatase. HRP is a glycoprotein with a molecular mass of 44 kD. In order to increase the detection, most authors have used chromogenic substrates which are initially colourless.They become strongly coloured during reaction.

The enzyme reaction of HRP can be stopped with H_2SO_4. It can be visually assessed or spectrometrically at 450 nm.

The binding of the enzyme to the molecule is done in the same way than during the preparation of the antigen for production of antibody. For haptens with carboxyl groups, mixed anhydride or carbodiimides procedures are the two direct methods that have been extensively used to prepare conjugated antigens.

3. PREPARATION OF ANTIBODIES

a. Preparation of antigen

Antibodies used in immunoassays are proteins (called IgG) which are produced as a result of the immune response system of animals to exogenous antigens.

Many drugs are not immunogenic by themselves because they are small molecules. These drugs can work as « haptens » after coupling to a large molecule (called carrier) such as BSA, KLH.

The synthesis of an antigen is done in the same way than for the production of the enzymatic conjugates. The synthesis is realized in two steps: firstly, the chemical interaction between a drug and a bridging molecule forms a hapten. The second step consists in the conjugation of this hapten to a protein (carrier). All the bridging molecules have free terminal COOH groups after they have been linked to the drug. This is necessary because the conjugate is formed by the binding of the terminal ε-amino group of a protein (AA lysine) to the COOH of the hapten.

The choice of the coupling position is very important. This position is going to determinate the specificity of the antibody.

For example, when you couple 17 β trenbolone to the carrier in position 17 β, the specifity linked to that part of the molecule is hidden. On the other hand, if the coupling is done in position 3 of the A ring of the molecule, then the specificity is maximum (table 1).

	17-HMS-β trenbolone	3-CMO-β trenbolone
β trenbolone	100	100
α trenbolone	50	< 0.025
trendione	152	0.14

Table 1: cross-reactivity (%) of two different trenbolone antibodies

In the case of residues detection, it is very often interesting to prepare antibodies which are also able to recognize the metabolites of a parent drug. For example, in animals, 19 nortestosterone is eliminated as the epimerised form (α form). In order to prepare that antibody, we have also coupled the carrier in position 17 to hide the specificity at that level. The antibody obtained allows to recognize about 50% of α nortestosterone.

b. Immunization

Most of the antibodies used in our field are polyclonal antibodies even if the production depends on the animal.

Polyclonal antibodies are an heterogenous population of antibodies produced by an animal in response to an antigen.

In our laboratory, the classical procedure consists in intradermal multisite injections of 100 µg of antigen emulsified in an equal volume of Freund's adjuvant. The addition of adjuvant allows to increase the non specific immunity response. Injections are done as follows: 3 injections the first month and after, 1 injection every month. The first blood samplings are done 10 days after the fourth injection.

c. Characterization of the antibody

The first parameter of selection of an antibody is its **titre**. The titre is determined by carrying out different successive dilutions of the antiserum which must be incubated with constant quantities of the tracer. The following figure (figure 1) shows results obtained for the production of a clenbuterol antiserum. It must be noticed that the titre increases regularly from the third month to reach a plateau at the sixth month. The affinity and the specificity are not necessarily proportionnal to the titre. However, the antisera with the highest titre are usually the ones with the best affinity.

Another important parameter is the **specificity**. It is determined by incubating successive dilutions of substances able to interfere in the assay, with a constant quantity of conjugate and antibody.

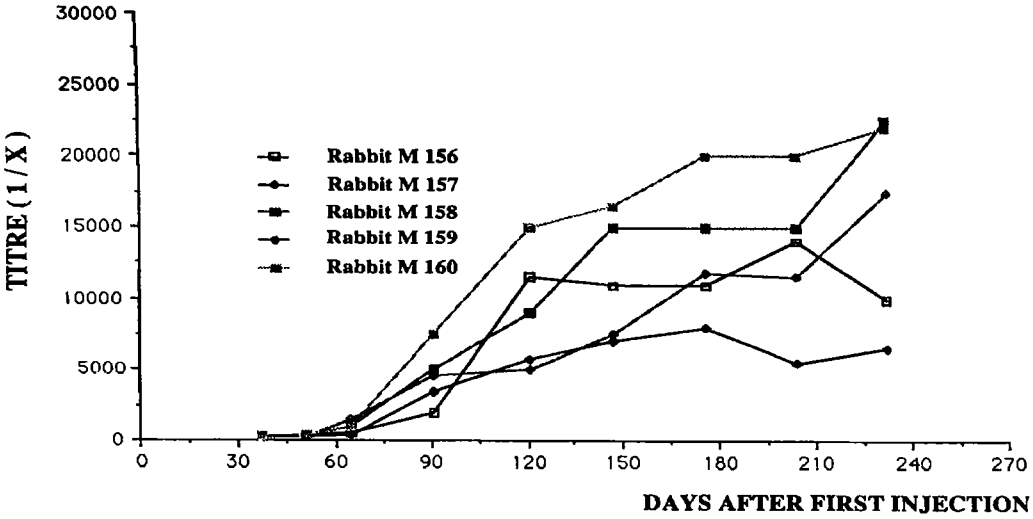

Figure 1: Evolution of the titre in five rabbits immunized with diazotized clenbuterol/human serum albumin. The titer is expressed as the final dilution which gave 50% of specific binding of tracer in the absence of unlabelled standard.

Standard calibration curves are constructed and the cross reactivity calculated as the relative percentage mass of substance to drug which gives the equivalent 50% depression of the BO value for the drug.

At the beginning of the immunoassays, the aim was to obtain the most specific assay. It was particularly interesting to study the hormonal patterns in endocrinology. The objective was to determine the physiological levels in the most precise way. In the field of the residues detection, the problem is relatively different because the immunological measurements or assays are used as screening methods to identify the positive samples before confirming them with a physico-chemical technique.

Unfortunately, during the past few years, we have been confronted with an increase and a diversification of the molecules used. That is why we try to produce antibodies which allow the detection of all the molecules of a drug family instead of trying to find an immunoassay for each substance. The exact identification is done during the confirmation by mass spectrometry.

An example of this approach is the assay of the β_2 agonists. The molecule of this group contains a tert-butyl or isopropyl group at the right side of the structure.

Our anti-salbutanol antibody has a larger specificity and higher cross-reactivity for several β_2 agonists (table 2) because we have coupled the carrier of this group on the aromatic ring. This test is very interesting for the screening of β_2 agonists.

Substances	% Cross-reaction
Clenbuterol	100.0
Salbutamol	87.5
Brombuterol	92
Mabuterol	35
Cimaterol	11.7
Cimbuterol	10.7
Terbutaline	31.8
Mapenterol	27
Fenoterol	< 0.01

Table 2: Percentage of relative cross-reactivity of the anti-salbutamol

4. ASSAY DEVELOPMENT

Each immunoassay is based on the detection and measurement of the primary antibody-antigen reaction. A wide variety of immunoassays are now used in the field (4).

ELISA (Enzyme Linked Immuno Sorbent Assay) is one of the most common assay systems (5). The binding elements (antibody) are attached to a solid support. An unknown quantity of analyte is added to a constant quantity of labelled antigen. Both antigens are in competition with the antibody for the available binding sites. After washing away excess of non-bound antigens, a chromogen substrate is added, which leads to the appearance of colour; the colour intensity is inversely proportional to the quantity of analyte.

Various supports like multiwell plates, plastic tubes, cuvettes, dipsticks have been used. Since a few years, dipsticks are used to detect pregnancy in cattle by dosing the progesterone in milk. Concentrations of progesterone in order of ppb are measured.

For your information, this table shows the different ELISA kits produced in our laboratory (table 3). There are also others companies which produce kits in the same field.

For the β_2 agonists, we have developped two kits: one specific for clenbuterol and one that allows the detection of all the β_2 agonists. This test was prepared by using a salbutamol antibody and a clenbuterol conjugate. Apart from fenoterol and ractopamine, most of the β_2 agonists are detected.

Concerning the androgenes, we have a test for methyltestosterone and one for nortestosterone. The methyltestosterone antibody has been coupled on position 3. The nortestosterone kit allows to measure 19 nortestosterone and its main metabolite, the α nortestosterone.

For trenbolone, we have two specific kits: one for α and one for β. The use of trenbolone is allowed for the fattening of cattle in some countries (USA). That is why a specific method is

required in order to control the Maximum Residues Limits (MRL's). Thus, we have prepared the antibodies by coupling the carrier on position 3.
For oestrogenes, we developed a kit for zeranol and one for ethynyloestradiol.
Finally, for corticosteroids, we have a more specific system for dexamethasone and another kit that allows to detect all the corticosteroids unfortunately with cross reactions with endogenous compounds.

KIT	CONJUGATED	ANTIBODY	CROSS-REACTION	SENSITIVITY
β2 agonist	Clenbuterol-N=N-HRP Diazotation	Salbutamol-HMS-BSA Carbodiimide	Clenbuterol 100% Salbutamol 87.5%	15 pg
Clenbuterol	Clenbuterol-N=N-HRP Diazotation	Clenbuterol-N=N-BSA Diazotation	Clenbuterol 100% Salbutamol 5.5%	40 pg
Methyltestosterone	Methyltestosterone-3-CMO-HRP Carbodiimide	Methyltestosterone-3-CMO-BSA Carbodiimide	Methyltestosterone 100% Methylboldenone 100% Methandriol 47% Testosterone 32.7% Stanozolol 18.4%	17 pg
19-Nortestosterone	Nortestosterone-17-HMS-HRP Carbodiimide	Nortestosterone-17-HMS-BSA Carbodiimide	19-Nortestosterone 100% 19-Norepitestosterone 55% Norethandrolone 57% Norgestrel 32%	5.5 pg
Zeranol	Zeranol 6'-HMS-HRP Carbodiimide	Zeranol 6'-HMS-KLH Carbodiimide	Zeranol 100% Zearalenone 19.6%	1.5 pg
Ethynyloestradiol	Ethynyloestradiol-6-CMO-HRP Anhydride mixte	Ethynyloestradiol-6-CMO-BSA Anhydride mixte	Ethynyloestradiol 100% 17 β oestradiol 0.3%	2 pg
Dexamethasone	Dexamethasone-21-HMS-HRP Carbodiimide	Dexamethasone-21-HMS-BSA Carbodiimide	Dexamethasone 100% Cortisol 5.6%	32 pg
Corticosteroids	Prednisolone-21-HMS-HRP Carbodiimide	Dexamethasone-21-HMS-BSA Carbodiimide	Dexamethasone 100% Prednisolone 186% Betamethasone 50% Flumethasone 30% Cortisol 16%	16 pg

Table 3: Main ELISA kits produced in our laboratory (molecules dosed, conjugates and antibodies used, cross-reactions and sensitivity of the assays)

IMMUNOAFFINITY CHROMATOGRAPHY

The extract purification techniques are of a great importance in the analysis of traces (6). These techniques must have a high degree of purification and a high recovery of all compounds of interest without separation. One of the most successfull techniques for purification developped in the past years is immunoaffinity chromatography. The coupling of immuno and physico-chemical methods improves both the sensitivity and performance of analytical method. It is of an increasing interest for laboratories dealing with complex samples (7).
Antibodies specific to a drug or a family of compounds are bound to a solid support which is then placed in a small chromatography column. An aqueous extract of the sample is passed through the column and drug residues are retained by interaction with the antibody while all other components of the extract are washed through. An elution solution is then used to dissociate the drug from the antibody. The drug is present in the eluate, free from interferences and can be quantified by mass spectrometry.
The basic components required for IAC are the following:
1) a suitable antibody
2) coupling the antibody onto an inert support
3) loading the sample on the column, if possible in an aqueous solution
4) washing step to remove the interferences
5) elution step with dissociation of the antibody-antigen complex in order to allow the identification of the substance
6) re-equilibration and re-use of the column

1. Antibody

The problem of the production of the antibody is identical to the ones described previously. If we try to quantify a special molecule or family of compounds, everything will be based on the specifity of the antibody (as seen previously). In order to increase the number of molecules to be purified several gels prepared from different antibodies are mixed in the same column. In this case, it is called multi-immunoaffinity chromatography (MIAC).

2. Coupling of the antibodies to the matrix

As soon as the choice of the antiserum is made, it is good to isolate the Ig G fraction before the coupling.
The two procedures the most frequently used are precipitation with ammonium sulfate or affinity chromatography with protein A-sepharose. The protein concentration of Ig G is estimated by the Lowry technique.

Most of the antisera used in our laboratory for the immunological assays and IAC have been prepared by hyperimmunization of rabbits. The concentration in Ig G after purification is about 15 mg/ml of serum.
The quantity of Ig G that can be coupled varies with the capacity of the column required. Usually, for an antiserum with a good affinity, 0,5 mg/ml will allow to obtain a capacity of 50 ng for the gel. However, in some cases, it is possible to couple 3 mg Ig G or more, in order to increase the capacity.
Cyanogen bromide (CNBr) activated soft agarose gels have been used for many years. Their main disadvantage is the limited stability of the protein binding. During the last few years, a variety of activated matrices have been available: tresyl-activated sepharose (Pharmacia), toyopearl tresyl actived, fractogel-actived (Merck). Coupling procedures are clearly described by the respective manufacturers.
After the preparation of the gel, there are several parameters to be controlled. Among these ones, the capacity is a parameter very often asked by the users. If labelled material is available , the capacity is quickly and easily estimated by applying a solution containing, for example 1 ng analyte per ml and a small amount of labelled analyte. The « break through » point can be determined by monitoring the radioactivity of the eluate.
The capacity required varies with the sensibility of the detection method used and with the volume of the starting sample.

3. Loading the column with the sample

The extraction of the samples is often carried out by using methanol. Such extracts require dilution with water to reduce methanol concentration at maximum 5 %. The flow of the column must be regular in order to allow a correct binding. A to important flow could be responsible for a decrease of the yields.

4. Washing conditions

The aim of this step is to eliminate interferences without breaking the antibody-analyte binding. Usually, several volumes of water eventually added with a low percentage of methanol (5 to 10%) are used.

5. Elution from IAC matrix

Dissociation of the antibody-drug couple can be done in various different ways. To facilitate the elution, we must change the conditions within the column. It is usually done by modifying the pH or the ionic strength of the medium or more often by changing the polarity by addition of methanol or ethanol. The elution conditions must be chosen in such a way that the modification of the affinity constant is a reversible process. It is then possible to re-use the column several times. For this reason, elution with water-methanol is very useful but it is very important to

keep a minimum of 10% of water in the elution medium in order to protect the gel that must always remain in a minimum of aqueous phase.

6. Requilibration of the column

After elution, the column must be washed and reequilibrated with buffers containing a preservative (sodium azide) to avoid bacterial contamination. If the conditions of storage (at 4°C - never frozen) are good, the columns can be used several times without loosing their capacity. In our laboratory, some gels for β_2 agonits are regularly used during one year.

7. Applications

Actually, the affinity chromatography is developping in a substantial way.

For your information, I am going to talk about some applications of this procedure, set up in our laboratory.

On a regular basis, we receive faeces from cattle in which we have to detect β_2 agonists and anabolic steroids (8). After the primary extraction, the purification is sequentially sperformed by placing the IAC column for β_2 agonists on the top of the C18 column. After loading the sample, the various extracts are individually processed.

We have also obtained good results in the detection of synthetic corticosteroids (dexamethasone, isoflupredone, prednisolone, ...) by using a mixture of the two gels.Cross-reactions of the antibodies are shown in the table 3.The further detection is done by GCMS.

CORTICOSTEROIDS	Anti-methylprednisolone	Anti-dexamethasone
Dexamethasone	100	100
Prednisolone	50	42
Isoflupredone	74	27
Triamcinolone	30	10
Betamethasone	100	6
Cortisol	30	5
Flumethasone	45	2
Desoxycorticosterone	12	0.5
6 α methylprednisolone	120	0

Table 3: Cross-reactivity (%) of the anti-methylprednisolone and anti-dexamethasone antibodies tested with a dexamethasone conjugate.

CONCLUSIONS

The discovery of the antigen-antibody binding has allowed important developments in the residues determination.
The immunoassays allow to search for traces of substances present at concentrations of about 1 ppb, with a good reliability and at a good price. Furthermore, when the antibody is the mean of purification before making a determination with a physico-chemical method, the limit of detection is significantly better.

REFERENCES

1. M.E. Ploum, W. Hasnoot, R.J.A. Paulussen, G.D. van Bruchem, A.R.M. Hamers, R. Schilt and F.A. Huf, Test strip enzyme immunoassays and the fast screening of norstestosterone and clenbuterol residues in urine samples at the parts per billion level. *J. Chromatogr.* 564 (1991) 413.
2. R.J. Heitzman, Immunoassay techniques for measuring veterinary drug residues in farm animals, meat and meat products. In *Analysis of food contaminants*, ed J. Gilbert, Elsevier Applied Science Publishers, London and New York (1984) 73.
3. E. Märtlbauer, E. Usleber, E. Schneider, and R. Dietrich, Immunochemical detection of antibiotics and sulfonamides. *Analyst* 119 (1994) 2543.
4. J.H. Rittenburg and G. D. Grothaus, Immunoassays: formats and applications. In *Food safety and quality assurance: applications of immunoassays systems* ed. M.R.A. Morgan, C.J. Smith and P.A. Willams, Elsevier Applied Science Publishers, London (1993) 3.
5. G. Degand, P. Schmitz, and G. Maghuin-Rogister, Enzyme immunoassay screening procedure for the synthetic anabolic estrogens and androgens diethylstilbestrol, nortestoosterone, methyltestosterone and trenbolone in bovine urine. *J. Chromatogr.* 489 (1989) 235.
6. N. Haagsma, Sample pretreatment in drug residue analysis. *Euroresidue* (1990) 40.
7. L.A. van Ginkel, Immunoaffinity chromatography, its applicability and limitations in multi-residue analysis of anabolizing and doping agents. *J. Chromatogr.* 564 (1991) 363.
8. M. Dubois, Y. Colemonts, Ph. Delahaut, Development of a screening method for major steroids, corticosteroids and β_2 agonists in faeces. *Euroresidue* III (1996) 392.