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A Review of Radioimmunoassay for Steroids[1])

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Summary: This review, based on the current literature, considers the practical aspects of steroid radioimmunoassays. The problems associated with the raising of specific antisera and their characterization are discussed. Features of assay design, reliability criteria and practicability of radioimmunoassays for steroids are considered.

Radioimmunoassays für Steroide, eine Übersicht

Zusammenfassung: Die vorliegende Übersicht befaßt sich, basierend auf der einschlägigen jüngeren Literatur, mit der praktischen Durchführung von Radioimmunoassays für Steroide. Die Möglichkeiten, spezifische Antiseren gegen Steroide zu gewinnen und ihre Charakterisierung werden beschrieben. Die verschiedenen Aspekte des Aufbaus eines Steroid-Radioimmunoassays, die Verläßlichkeitskriterien und die Praktikabilität werden diskutiert.

Introduction

The prime requirement on which the principle of radioimmunoassay is based is the antiserum. Steroids are not intrinsically antigenic because of their low molecular weights, but they can be so rendered by coupling to carrier proteins. Substances with molecular weights lower than 1000 are non-immunogenic per se (1), and for antibody formation it has been recommended that substances with molecular weights lower than 3000 should be conjugated (2).

Hapten	+	Carrier	= Antigen
Steroid	+	Macromolecule	= Steroid-Conjugate
↓			↓
not immunogenic			immunogenic

Attempts have been made to couple steroids to carrier proteins since the early 1930's (3), but stable conjugates capable of eliciting antibody formation in animals were only synthesized some twenty years later (4, 5, 6, 7, 8).

Failure of some earlier attempts may have been due to the biologically unstable ester bond that was used to link the steroid to the protein (9). However, the antisera developed were used primarily for the study of the biological effects of steroids (10, 11). It remains an irony of the history of endocrinology that radioimmunoassays for protein hormones were in use for almost 10 years (12) before the same principle was combined with the steroid antisera (then also available for the previous decade) to develop the first radioimmunoassay for a steroid (13, 14). From these beginnings the number of assays available has increased rapidly, until today it is possible to measure almost every steroid of biological or pharmacological significance (tab. 1).

The greatest advantage of radioimmunoassay in the measurement of steroids is the high sensitivity which can be achieved, namely of the order of 10^{-12} g (1 pg), compared to the microgram quantities detected by previous methods (tab. 2).

The essential requirements for a steroid radioimmunoassay are

(i) the antiserum,
(ii) the pure radiolabelled steroid,
(iii) some preliminary preparation of the biological specimens in which the steroid is to be measured.

[1]) This review is based on a lecture held at an International Atomic Energy Agency Training Course on radioimmunoassay techniques (Lima/Peru, October 1974).
[2]) Supported by the Deutsche Forschungsgemeinschaft.

Tab. 1. Steroids measured by radioimmunoassay

Estrogens	(63, 65, 74)
estrone	(69, 73)
estradiol	(62, 68, 69, 71, 72, 73, 75, 76, 77, 78)
estriol	(64, 73)
estrone-sulfate	(66)
2-hydroxyestrone	(67)
15 α-hydroxyestriol	(70)
Corticosteroids	
cortisol	(81, 82, 83)
corticosterone	(79)
deoxycorticosterone	(80)
aldosterone	(84–94)
Androgens	
testosterone	(95, 97, 98, 100–103, 105–107, 114)
dihydrotestosterone	(114)
testosterone-glucuronoside	(99, 109)
dehydroepiandrosterone	(112, 117)
dehydroepiandrosterone-sulfate	(104, 112)
androstenedione	(107, 110)
androstene-3β, 17β-diol	(113)
androsterone	(108, 111)
Progestins	
progesterone	(118, 119, 122, 123, 124, 125)
17-hydroxyprogesterone	(120, 121)
pregnenolone	(115, 116, 117)
17-hydroxypregnenolone	(113, 117)
Synthetic Steroids	
medroxyprogesterone	(126)
dexamethasone	(127)
prednisone	(128)
cortisone	(129)
hydroxycortisone	(130)

Tab. 2. Sensitivity of methods for estimation of steroids

Method	Sensitivity (ng)
Bioassay	100–10000
UV-absorption and spectrophotometry	100–2000
Fluorescence methods	5–50
Double isotope dilution	0.5–10
Gas liquid chromatography	
– flame ionization	5–20
– electron capture	0.05–1.0
Radioligand assays	
– plasma proteins	1–10
– receptor proteins	0.01–0.1
– antisera	0.005–0.1

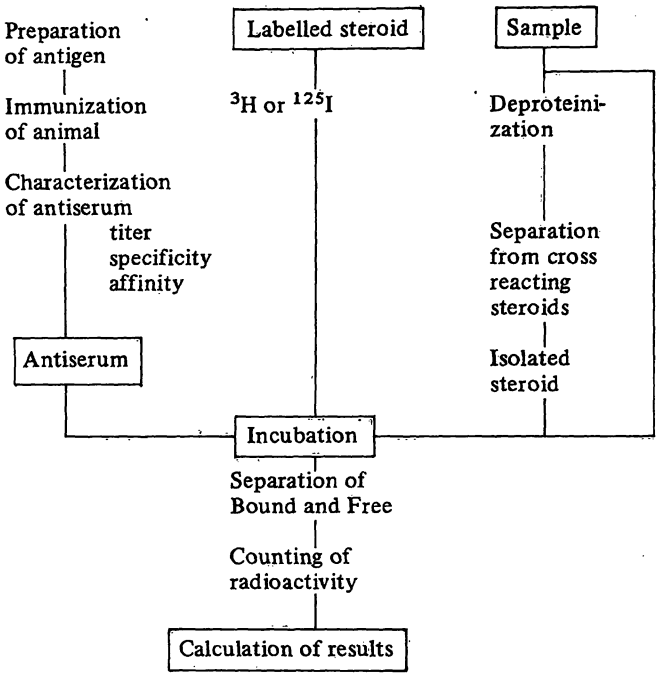

Fig. 1. Basic steps in steroid radioimmunoassay

The interrelation of these three factors is outlined in figure 1. Each of these requirements will be discussed in more detail, in order to provide an introduction to and a review of the field of steroid radioimmunoassay.

Antiserum

Preparation of Antigen

Coupling Reactions

Steroids contain two types of oxygen functions (oxo and hydroxyl groups) which can be used to link the steroid moiety to a protein by stable peptide bond formation. An intermediate derivative is first formed at this reactive group; acyl chloride and hemisuccinate at the hydroxyl group and oxime at the oxo group (4, 5, 15); thiol derivatives have also been used recently (16). Coupling of this steroid derivative to the protein is achieved either by a mixed anhydride reaction (4) or by the carbodiimide method (17). The latter method has been criticized because of the degree of cross-linking produced within and between the protein molecules (18) and the mixed anhydride reaction seems to be the preferred method. The coupling of dehydroepiandrosterone to bovine serum albumin via oxime formation and using the mixed anhydride reaction is shown in figure 2. Sites on each of the four rings (A–D) of the cyclopentanoperhydrophenanthrene skeleton can be used for coupling purposes. Figure 3 shows examples of steroid conjugates, with coupling sites circled.

Carrier Proteins

Bovine serum albumin is the most commonly used of the carrier proteins. It is probably used because it is readily available at a low cost. Since the antibody response generally increases with the molecular weight of the protein (1, 19), larger molecules such as thyroglobulin, hemocyanin and polylysyl lysine have been tried as carrier proteins. In our experience, antisera from animals immunized with testosterone-3-hemo-

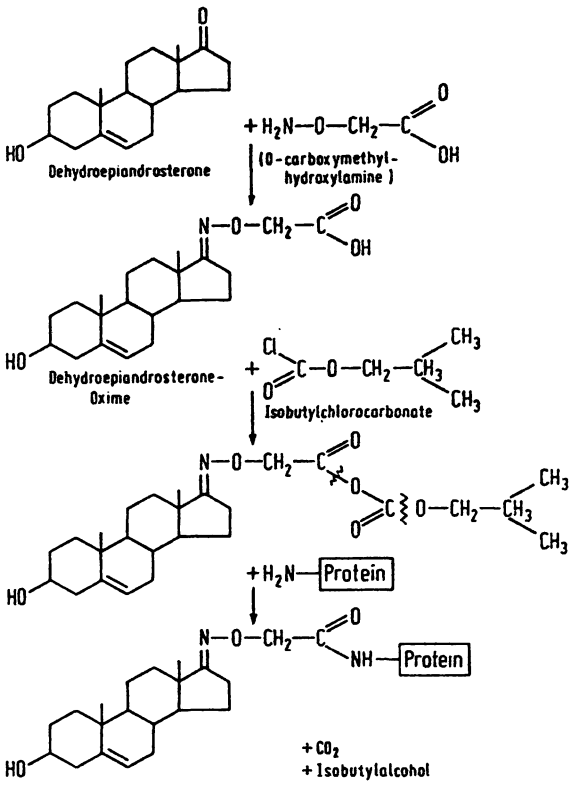

Fig. 2. Conjugation of dehydroepiandrosterone with a carrier protein

cyanin were not superior to those immunized with testosterone-3-bovine serum albumin.

In the final conjugate the molar ratio of steroid molecules coupled per protein molecule is usually 8:1–30:1. No convincing evidence has yet been produced which

Tab. 3. Immunization schedules for antiserum production (Data from 41 randomly selected publications 1969–1974)

ANIMAL SPECIES USED		
Rabbit	72%	(67, 69, 70, 71, 72, 73, 75, 78, 81, 84, 86, 91, 95, 96, 97, 101–106, 108, 119, 121, 122, 128, 130)
Sheep	18%	(115, 116, 118, 98, 86, 83, 62)
Goat	8%	(124, 125, 126)
Guinea pig	2%	(77)
ROUTES OF IMMUNIZATION with booster		
subcutaneous	46%	(62, 67, 69, 96, 98, 101, 102, 108, 116, 118, 121, 124–126)
subcutaneous & intradermal	6%	(105, 37)
intramuscular	17%	(70, 81, 86, 91, 95, 130)
intradermal	6%	(73, 104)
footpads & subcutaneous	8%	(71, 106, 122)
intramuscular & subcutaneous	3%	(84)
without booster		
intradermal	14%	(72, 75, 78, 97, 103)

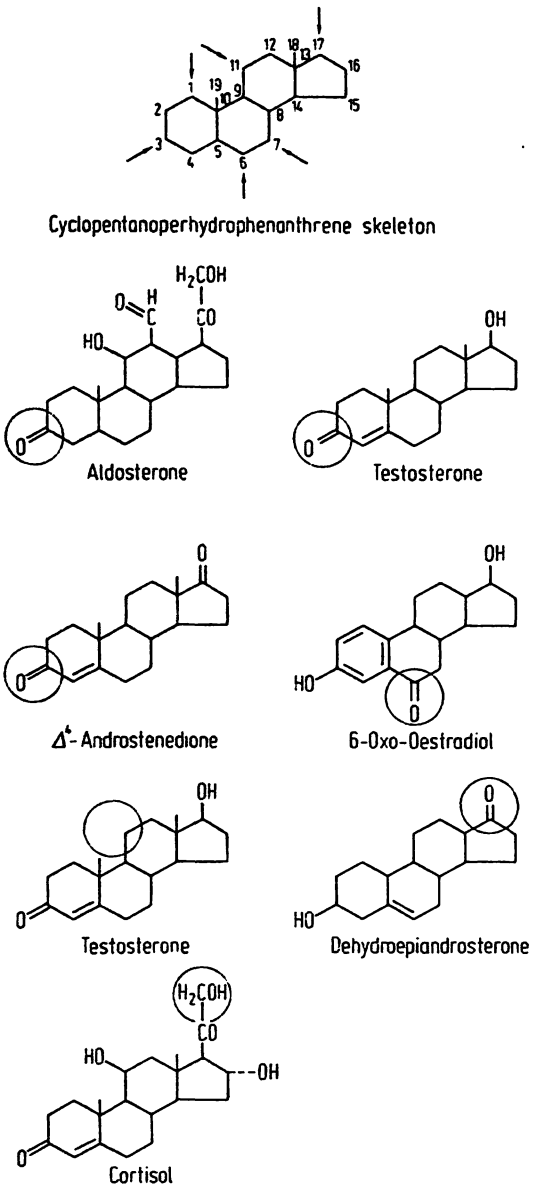

Fig. 3. Possible sites for conjugation with proteins on the steroid skeleton (indicated by arrow), and concrete examples (with coupling sites circled)

demonstrates that the actual steroid/protein ratio influences the quality of the antiserum produced, within these limits.

Immunization of Animals

All knowledge of the choice of animals, the route of administration of immunogen and the use of adjuvants apppears to be rather empirical. Although laboratory aspects of radioimmunoassay have been much improved, little effort has been made to rationalize immunization procedures. It is not possible to ensure reproducibility in the production of antisera when the immunization schedules are purely arbitrary.

Choice of Animal

Although theoretically any species of the animal kingdom could be used for immunization purposes, the rabbit is the one most commonly chosen (tab. 3). The

rabbit probably holds this favored position because it has large ears with readily accessible vessels for drawing blood and a blood volume large enough for antiserum production for radioimmunoassay purposes, and it is good natured. It is also easily kept under laboratory conditions. Other animals, such as sheep, goats and guinea pigs are also used, but they have the disadvantage of either requiring special housing facilities or of being too small to accumulate large volumes of antisera. Some animals, rats and pigs for example, are very susceptible to "adjuvant sickness" and are therefore not suitable for antiserum production.

Routes for the Administration of Immunogen

All investigators unanimously use *Freund*'s adjuvant as the emulsifying agent in which the immunogen is introduced into the animal (20, 21). There is, however, a large variation in the route of administration (22). All possible routes of immunization, such as subcutaneous, intradermal, intramuscular, intraperitoneal, thymus transplanatation, into the lymph nodes or footpads, have been used, either with or without booster injections (tab. 3). Only immunization by the intradermal route over multiple sites on the back of the animal has been successful without booster injections, and this schedule has the further advantage that very small doses of the immunogen are required (50–100 µg per animal) (23, 24). High titers are achieved within 6 weeks of immunization. The development of titers produced by our single, multiple-site, intradermal immunization schedule is shown in figure 4. The distribution of the titers of antisera produced in 128 rabbits is shown in figure 5.

Characterization of Antisera

Titer

The titer is defined as the antiserum dilution at which 50% of a given amount of labelled steroid is bound (25) (figure 6). The assay conditions used to assess the titer of an antiserum must be held constant in order to compare several antisera from different animals, or to monitor changing antiserum levels in one animal. The conditions used are therefore similar to those of the final assay system.

When establishing the assay conditions, the titer is used as the working dilution of the antiserum and can be adjusted later for optimization of the assay. If antisera from different animals or from several bleedings of one animal are available, the antiserum with the highest titer is usually selected for the assay. In published assay methods, the antiserum titers range from 1/5,000 to 1/100,000. Few assays have been reported using titers exceeding 1/100,000 or below 1/1,000.

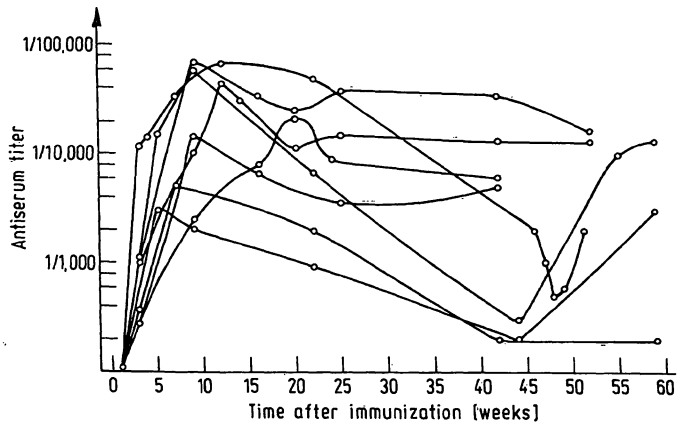

Fig. 4. Development of antiserum titers in 8 male rabbits immunized against testosterone

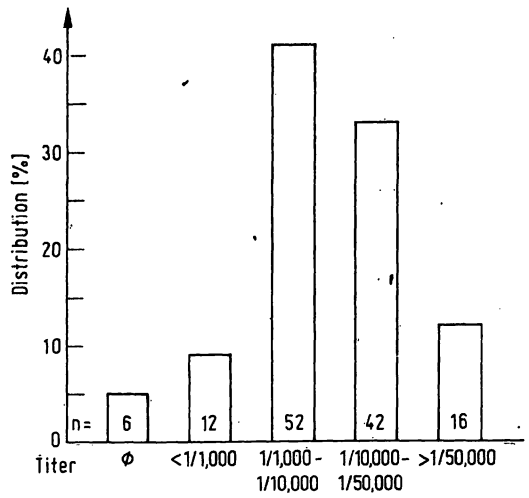

Fig. 5. Distribution (%) of antiserum titers produced in 128 rabbits after one-time, multiple site, intradermal immunization with various steroid conjugates

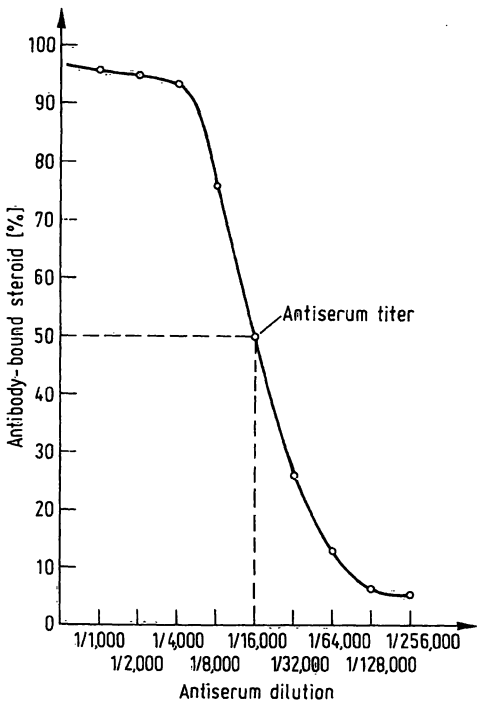

Fig. 6. Determination of antiserum titer

Note that the titer of the antiserum is not necessarily proportional to the antibody concentration (it is, however, proportional to the product of the antibody concentration and the affinity constant (K_a) (26)), nor does the titer give any indication of the specificity of the antiserum.

Affinity

The binding affinity of the antibody towards the steroid hapten governs the sensitivity and detection limit of the assay (2, 25). The affinity constant can be calculated from either a *Scatchard* plot (27) or from *Michaelis-Menten* curves (28). In the second approach the saturation data used in the calculation gives an average affinity constant for the total heterogeneous population of antibody binding sites, while *Scatchard* plots give an indication of the number of antibody populations present. The K_a constant is defined in the *Michaelis-Menten* method as the reciprocal of the free (unbound) steroid molar concentration at half saturation of the antibody binding sites (figures 7 and 8).

Binding affinities calculated for populations of steroid antibodies are of the order of 10^9 l/mol and are thus about 100-fold higher than the affinities of the specific binding proteins, such as transcortin and testosterone-binding globulin, but are in the same range as the binding affinities of the cellular steroid receptors.

The sensitivity (s) of an assay is usually taken to be one tenth of the reciprocal of the binding affinity of the average antibody population present:

$$s = \frac{0.1}{K_a} \qquad (29)$$

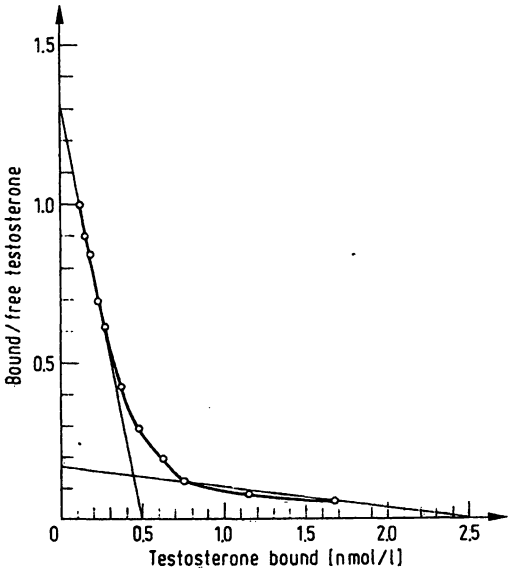

Fig. 7. *Scatchard* plot for calculation of antiserum binding affinity, as exemplified by a testosterone antiserum, diluted 1/25,000.
x-intercept = q; y-intercept = K · q

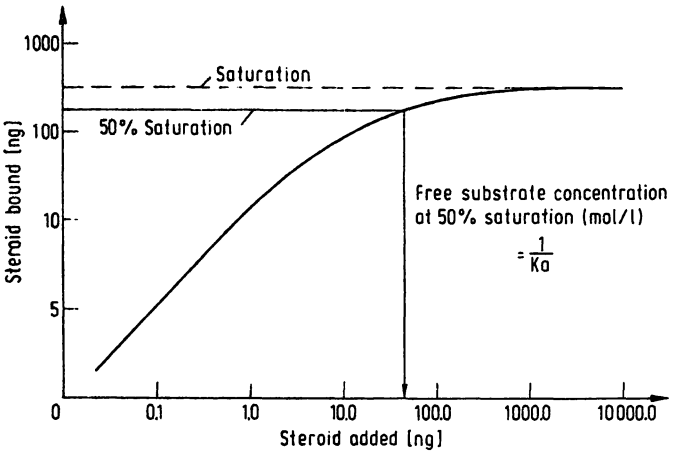

Fig. 8. *Michaelis-Menten* plot for calculation of antiserum binding affinity

Specificity

Specificity depends on the degree of cross reaction of the antiserum with other steroids, and is the most important quality of the antiserum. This cross reaction directly determines the overall specificity of a radioimmunoassay in which the antiserum is used, and indicates the extent to which the samples must be purified before being assayed. Since the chromatographic separation of one steroid from a sample is the rate-limiting factor in the radioimmunoassay, the specificity of the antiserum also dictates the practicability and final cost of the assay.

The specificity of the antiserum is established by displacement studies. The cross reaction is calculated from the ratio of the mass of immunogenic steroid (x) required to displace 50% of the radiolabelled immunogenic steroid to the mass of the cross reacting steroid required to displace the same fraction of the labelled steroid (y):

$$\text{Cross Reaction} = \frac{x}{y} \cdot 100\%.$$

The factors governing specificity of an antiserum are not fully understood. While one cannot determine which part of the conjugate will act as the immunodeterminant site, it was soon recognized that resulting antisera were "far-sighted" (30, 31, 32, 33). That is, steroid antisera are specific for the part of the steroid moiety furthest from the site of conjugation to the carrier. Thus if the steroid is coupled through ring D, configurations on ring A are recognized; while antisera against steroids coupled through position 3 (ring A) show the opposite properties and ring D is recognized. Since steroids are characterized by a specific pattern of reactive oxygen functions, located mainly on the A and D rings of the skeleton, antisera of enhanced specificity have been produced against steroids coupled via the B and C rings. This leaves both the immunodeterminant

Tab. 4. Specificity of antisera raised against different estrogen conjugates, expressed as percent cross reaction (E_1 = estrone, E_2 = estradiol, BSA = bovine serum albumin).

	Conjugate: E_1-17-BSA	Conjugate: E_2-6-BSA
Estrone	100	0.5
Estradiol	80	100
Estriol	66	1

sites available for antibody formation. The best example of this type of coupling is 6-oxo-oestradiol-bovine serum albumin (16, 34, 35, 36, 37) (tab. 4). This does not apply to all steroids. The specificity of the antisera raised against testosterone conjugated through ring B and C is only marginally better than that of antisera raised against testosterone-3-bovine serum albumin (tab. 5). In all these antisera a relatively high cross reactivity to dihydrotestosterone is demonstrated.

It may be possible to elicit a more specific antibody response by interposing a chemical "arm" between the steroid and the protein carrier (38). This is thought to keep the hapten sufficiently distant from the carrier to retain its identity, which may otherwise be masked by steric interactions with the protein molecule. The recent synthesis of oligomeric steroid derivatives may also be an effective means of eliciting specific antibody formation (39).

Affinity chromatography and gel filtration techniques have also been used to increase the specificity of existing antibody preparations, by removing the populations of low affinity antibodies (40, 41, 42).

Labelled Steroids

Either β- or γ-emitting radiolabelled steroids can be used in radioimmunoassays. Tritiated steroids are usually the first choice, as they are readily available commercially. Such labelled steroids have a relatively high specific activity, with the radiolabel incorporated in the skeleton at one, two or four positions eg. [7-^{3}H] dehydroepiandrosterone; [1,2-^{3}H] testosterone; [1, 2, 6, 7-^{3}H] testosterone. These highly labelled steroids are stable for periods of longer than one year, provided they are purified regularly to remove radiation damaged material.

The inclusion of a radioactive atom in the steroid skeleton had been thought to result in a difference in the binding kinetics between the radioactive and non-radioactive steroid (43). However, such an isotope effect has not been demonstrated with ^{3}H- or ^{14}C-labelled steroids (44).

The counting of β-emitting isotopes necessitates the use of scintillation media, and hence β-counting is more expensive and more time-consuming than γ-counting. For this reason, some workers have chosen to use iodinated steroid conjugates. With the exception of the phenolic A ring of the natural estrogens, it is not possible to iodinate the cyclopentanoperhydrophenanthrene nucleus or its substituents directly (45). However, it is possible to introduce the radiolabel on a carrier protein in a similar manner to that of the steroid-protein conjugate (32). This would give the iodinated steroid conjugate approximately the same configuration as the immunogen, but its structure would be very different from the unknown or standard steroid preparation. It is possible to prepare steroid derivatives where only one iodine atom is introduced per conjugate. The steroid is coupled to the methyl ester of tyrosine, which is then used for iodination (46, 47); histamine can be used as an alternative carrier (48). One argument against the use of iodine labels in steroid radioimmunoassays is the size of the iodine atom, which is greater than the A ring configuration of the skeleton. The introduction of such a large atom also affects the kinetics of binding between the labelled antigen and antibody.

Smaller γ-emitting atoms, such as ^{75}selenium, may therefore be of advantage. ^{75}Selenium steroid conjugates of cortisol, testosterone and aldosterone have been

Tab. 5. Specificity of antisera raised against different testosterone conjugates, expressed as percent cross reaction (T = testosterone, 5α-DHT = 5α-dihydrotestosterone, BSA = bovine serum albumin), nr = not recorded.

Steroid	Antiserum against				
	T-3-BSA	T-7-BSA	T-11-BSA	T-17-BSA	5α-DHT-3-BSA
Testosterone	100	100	100	100	100
5α-dihydrotestosterone	60	42	15	nr	100
Androstenedione	0.3	1	2	31	0.5
5β-dihydrotestosterone	8	5	nr	nr	10
Epitestosterone	0.1	1	< 0.9	nr	nr
Dehydroepiandrosterone	< 0.05	< 0.5	< 0.1	0.1	nr
3β, 17β-androstenediol	1.6	7	0.16	0.05	nr
Progesterone	< 0.05	< 0.1	nr	55	< 0.001
Deoxycorticosterone	< 0.001	< 0.5	nr	55	< 0.001
Reference		(16)	(105)	(30)	

prepared, and the use of ^{75}Se-cortisol in a competitive binding assay has been described (49).

Assay Procedure

Deproteinization

Unlike protein hormones, which can be assayed in unprocessed samples, steroid samples must be purified prior to assay. The steroid-binding proteins must be removed, since these compete with the antibody binding sites for the free steroid. Such deproteinization is usually accomplished by solvent partition. The choice of solvent and its degree of purity are important, since a non-specific blank contribution can arise from this step. The following solvents have been applied to the extraction of steroids (tab. 6).

Separation of Cross-Reacting Steroids

With regard to specificity, practicability and cost, the most important question is whether a chromatographic separation is included in the radioimmunoassay, or not. The specificity of the antiserum gives a strong indication of this. Where such a chromatographic step can be avoided, the practicability of the assay is markedly increased and the cost per assay can be correspondingly reduced.

The most commonly used chromatographic systems and an indication of their frequency are given in table 7. Of these systems, partition and adsorption phase paper chromatography have been widely investigated (50), but since such methods are laborious and time-consuming, they tend to have been abandoned in favor of other methods. Column systems have the advantage that they can be re-used after washing with solvent. However, the technician may be overexposed to organic vapors, as many systems using LH-Sephadex columns are benzene-based. Complete separation of steroids with similar polarities is difficult without some chemical transformation (51). Thin-layer chromatography is the most popular technique, since total separations can be obtained by varying the solvent composition with shortened running times. The availability of commercially prepared plates with a variety of support media has led to the standardization of such techniques and reproducibility can be guaranteed.

Tab. 6. Solvents used for extraction of steroids from body fluids

Ether	testosterone, androstenedione, androsterone, dihydrotestosterone, dehydroepiandrosterone, estrone, estradiol, estriol, pregnenolone, progesterone, 17-hydroxyprogesterone
Hexane	progesterone
Hexane/ether	testosterone, androstenedione
Petroleum ether	progesterone
Ethyl acetate	testosterone
Methylene chloride	cortisol, corticosterone, aldosterone, androstenediol, 17-hydroxypregnenolone, dehydroepiandrosterone-sulfate

Tab. 7. Frequency of various chromatographic methods in steroid radioimmunoassays
(Data from 68 randomly selected publications 1969–1974)

Thin-Layer Chromatography		
Silica gel	17%	(66, 71, 75, 81, 82, 97, 99, 104, 108, 112–114, 118)
Alumina	3%	(100, 107)
Column Chromatography		
LH-Sephadex	13%	(63, 68, 69, 73, 76, 77, 90, 101, 110, 121)
Celite	10%	(62, 64, 109, 115, 117, 118, 120)
Alumina	5%	(107, 119, 95)
Paper Chromatography	13%	(80, 84, 87, 90, 104, 113, 114, 127)
No Chromatography	39%	(65, 67, 70, 72, 74, 77, 80, 83, 86, 88, 89, 92, 93, 94, 96, 99, 100, 108, 122, 123, 124, 125, 126, 128, 130)

If chromatographic separation is necessary, it offers the advantage that several steroids can be assayed simultaneously in the same sample. All commonly used chromatography systems can be adapted for this purpose (69, 73, 77, 113, 117).

Separation of Bound and Free Steroids

The separation of antibody-bound and free steroid is another rate-limiting step in the radioimmunoassay of steroids. It influences the number of tubes which can be handled per time unit, the reliability criteria and the costs of the assay. The various methods in common use are shown in table 8.

These data show that dextran-coated charcoal is widely used (66%) and appears to be applicable to most steroids. This method has the major disadvantage that the reaction is time-dependent (52). More assay tubes can be handled in a given time with separation techniques which are independent of time. In this respect precipitation of the bound fraction with saturated ammonium sulfate solution appears to be the most widely used (20%). The easiest and fastest separation techniques seem to be the solid-phase systems such as antibody-coated tubes or antibodies incorporated into polyacrylamide particles (53, 62, 72, 75, 76). Some researchers have experienced a loss of sensitivity using these techniques, which may have prevented their wider application to date. The solid phase methods have the disadvantage that large amounts of antibody are required for the preparation of

Tab. 8. Methods for separation of bound and free steroid (Data from 70 randomly selected publications 1969–1974)

Adsorption of free fraction		
Dextran-coated charcoal	64%	(64, 66–69, 71, 73–75, 79, 81, 82, 86, 88, 90, 91, 93, 94, 97, 98, 99, 101–103, 105–113, 77, 115–118, 120–124, 129, 130)
Hemoglobin-coated charcoal	2%	(83)
Florisil	2%	(84)
Precipitation of bound fraction		
Ammonium sulfate	16%	(65, 70, 80, 85, 88, 91, 92, 95, 104, 114, 119)
Immunological precipitation of bound fraction		
Double antibody	5%	(96, 100, 126, 127)
Solid phase		
Antibody-coated tubes	3%	(62, 78)
Antibody in polyacrylamide gel particles	6%	(63, 72, 76, 125)
Toluene extraction of free fraction	2%	(87)

the media. The use of a second antibody linked to a solid matrix (eg. DASP), which has recently become available commercially, promises to be of value in the future (54, 55).

The immunosorbents developed for the separation of free and bound protein hormones have also been successfully applied to steroid assays (56, 57). In this method, the first antibody is coupled to dextran particles, so that after equilibration bound and free steroid can be separated by simple centrifugation.

Evaluation of Results

The evaluation of data from radioimmunoassay methods is not a problem specific to steroid assays, and therefore the reader is referred to general reviews of radioimmunoassays for the discussion of this topic (58, 59). As there is no species specificity for steroid hormones, the radioimmunoassay method should be applicable to any biological specimen from any species. However, the specificity of the assay may vary in the different types of specimens, and hence should be checked in each individual case.

Several approaches have been used to evaluate the radioimmunoassay results, and to express the relationship between the mass of steroid and the effect of bound and/or free labelled steroid in the assay. These include the linear logit-log transformation of *Feldman & Rodbard* (27) and the computer models of *Wildman & Powsner* (60), and *Rodbard & Lewald* (61). There is, however, no single way of expressing radioimmunoassay data, since the shape of the standard curve depends on the affinity constant and heterogeneity of the antiserum used. Any computer program used must therefore be capable of forming a "best fit" curve to the data, and not vice versa, to be applicable for all types of antisera.

Factors Affecting Assay Design – Reliability Criteria

The type of steroid-conjugates used for immunization and the hydrophobic character of the steroid haptens account for some of the properties peculiar to steroid radioimmunoassays. These properties include the apparently smaller affinity constants obtained for steroid antisera over protein antisera; the high association and dissociation constants of the steroid-antibody reaction; the spectrum of antibody affinities present and the effect of non-polar substances on the steroid-antibody reaction.

Assay Sensitivity

Two types of sensitivity can be evaluated, that of the standard curve and that of the overall assay. The first depends on the affinity of the antiserum, the mass of antibody and labelled tracer used in the assay, the volume of the incubation media (since the affinity constant is defined in terms of mass/volume) and the error (and hence precision) of the method. The assay sensitivity depends on the sensitivity of the standard curve (indicated by its slope), the blank value of the assay and the recovery of steroid after any purification procedures.

Several factors of assay procedure can be varied to optimize the overall sensitivity.

Assay Blanks

Many of the factors contributing to the method blank are purely chemical and may therefore be avoided.

The affinity constant of the antiserum influences the blank value, with a low affinity causing an increase in the blank contribution. The buffer system used may affect the antigen-antibody reaction, causing an alteration in the shape of the standard curve. Addition of proteins to the incubation media, such as bovine serum albumin, γ-globulin or gelatin to prevent the adsorption of steroids to the tube walls, and increasing the ionic strength of dilute solutions by salt addition can effectively reduce the blank contribution (25, 38).

Reagents may also make a significant contribution to the apparent hormone level measured. This is especially important when chromatographic techniques are included in the method, as the support medium may also contribute significantly to this effect. The reagent blank is not always reduced by using redistilled and high-grade products.

The susceptibility of steroid antisera to the interference of non-polar substances, such as dried residues of organic solvents and lipophilic substances, is due to the influence

of these factors on the hydrophobic steroid-antibody interaction. Coextraction of lipids from plasma may therefore impede the direct assay of crude extracts, even when highly specific antisera are used.

The means of separation of free and bound steroid may also cause a significant blank problem. The use of solid-phase systems has been criticized because of the loss of sensitivity resulting from increased blank contributions.

Incubation Environment

Under normal conditions of assay, the steroid-antibody reaction reaches apparent equilibrium after a relatively short time (1–4 hours at room temperature). The problem of non-equilibrium conditions therefore rarely occurs in steroid radioimmunoassays.

The antigen-antibody complex is stable over a wide pH range, and small changes in the pH of the incubation media have little effect on the formation of the complex. However, the stability is radically affected by the dissociating action of adsorbants such as charcoal. There is a progressive dissociation of the complex with increasing amounts of charcoal and increasing incubation time.

The presence of a heterogeneous population of antibody binding sites can also be demonstrated by the different rates of dissociation of the steroid-antibody complexes present in the incubation medium. The use of charcoal and dextran-coated charcoal, although a very convenient means of separating the free and bound steroid, requires the careful optimization of experimental conditions (in terms of amount of adsorbent added, length of incubation time, etc.) to counterbalance the dissociation effects within the same set of tubes.

Other factors to be considered regarding the acceptability of a steroid radioimmunoassay include accuracy, precision, specificity and finally, practicability.

Accuracy

The accuracy is determined by performing recovery experiments whereby increasing amounts of the steroid are added to plasma aliquots, and the level of steroid present is assayed. A correlation coefficient of 1.00 should be obtained, but deviations may be caused by methodological and personal errors.

Precision

The repeated measurement of a sample in the same assay, and in successive different assays, will give an estimation of the intra- and inter-assay variation. The acceptability of an assay in terms of its precision must be carefully considered, if the assay is to be used to measure steroid levels in the same subjects over a prolonged period of time. The data generally reported in recent publications show a coefficient of variation of about 10% for intra-assay precision, and up to 15% for inter-assay precision.

Specificity

This can be demonstrated in two ways: first, that steroids present in the purified fraction in which the specific steroid is contained do not interfere in the assay either because of low cross reactivity with the antiserum, or because they are present in comparatively small amounts; secondly, that levels of a steroid measured by radioimmunoassay correlate well with the corresponding levels measured by alternative techniques.

Practicability

This is judged according to the degree of skill required in the manipulation of the assay, and also its cost. Hence the inclusion of a chromatographic purification step may be "impractical" in these terms although necessary for increased specificity. Many steroid assays have become more practical as increased use is made of automated techniques. However, the cost of the equipment needed for such steps is the limiting factor in their application to steroid assays.

Conclusions

In the last five years radioimmunoassay has almost completely conquered the field of steroid analysis and has made obsolete almost all previously used techniques for routine analysis. Radioimmunoassay can be applied to most forms of biological material, such as blood, urine and tissue preparations. It is possible to automate at least parts of the assay procedures, and calculation of the results by computer facilitates the handling of large numbers of samples simultaneously. With the inclusion of a chromatographic step several steroids may be measured simultaneously in small sample volumes. The required plasma volumes are also small enough to allow for frequent sampling in the same patient when hormone profiles and rapid fluctuations should be measured. The assays offer satisfactory reliability and the sensitivity is sufficient for many current medical problems. Further improvements can be expected from the development of antisera showing a lesser degree of cross reaction with other steroids.

In the enthusiasm for radioimmunoassay, however, other methods should not be overlooked. The use of mass spectrometry is extremely valuable as a reference method because it offers absolute specificity. Furthermore, radioimmunoassay will always require separation of antibody-bound and free hormone, which will cause problems in the full automation of the assays. This will be necessary in large routine laboratories and for screening programs in preventive medicine. In addition, the counting of radioactivity is expensive and time-consuming. Therefore alternative methods are awaited with great interest. Of the recent developments, enzyme-immunoassays, where the radiolabel is replaced by an enzyme, appear to overcome some of the problems of

radioimmunoassays. Since in these assays the end-point determination will not be the counting of radioactivity but spectrophotometry, enzyme-immunoassays could be introduced into all clinical chemistry laboratories which had been prevented from using radioimmunoassays because of the high costs of counting equipment. Thus, in connection with newly developed antisera showing negligible cross reaction with other steroids, enzyme-immunoassays could offer a suitable alternative to radioimmunoassay in the future.

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