

Chapter 9

Comparison of the Use of β -Scintillation Counting and γ -Counting for the Radioimmunoassay of Steroids

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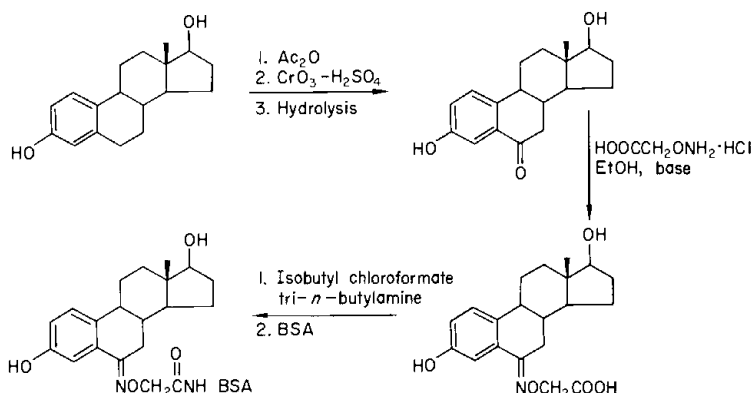
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INTRODUCTION

The relatively insensitive colorimetric and fluorometric assays for steroid hormones have given way during the last 10-15 years to assays based on the principle of saturation analysis. Initially, steroid binding proteins - uterine cytosol for 17β -oestradiol; pregnant guinea pig serum for progesterone and dog plasma for cortisol - were extensively used, together with tritiated steroids as the labelled components. The non-specificity of these proteins, together with their limited sensitivity potential, soon allowed antisera raised to steroid conjugates to gain in importance and practicability. Tritiated steroids, however, still continued to be used in the radioimmunoassay. Even with the increased specificity often achieved with these antisera, preliminary extraction of the steroid from the biological fluid was a necessary prerequisite for almost all assays, together with recovery experiments, and in some cases, chromatography. The combination of this purification step together with β -scintillation counting has rendered steroid immunoassays both complex and time consuming and therefore expensive. The rapidly increasing use of the radioimmunoassay technique especially in clinical research and the routine laboratory has resulted in the need for simple, cheap and reliable methods. Not surprisingly, therefore, attention has turned to steroid assays that are both direct (without extraction) and incorporate a γ -labelled tracer. The advantages of γ -counting over β -liquid scintillation counting are substantial in terms of cost saving for both materials and technician time. Considerable technical problems are associated with the introduction of a γ -emitting radioisotope into the steroid nucleus and thus tritiated steroids will still continue to be extensively used. This paper discusses the reasons for recent attempts to develop simplified steroid radioimmunoassays by comparing and contrasting the isotopes commonly used in terms of their practical advantages, availability and the technical problems associated with their incorporation into clinically validated assay procedures.

ANTIBODIES TO STEROIDS

A basic component of any saturation analysis technique is the specific receptor, and antisera have advantages over other binding proteins. Unfortunately, however, steroids are non-immunogenic and it is necessary to attach to the steroid a macromolecule in order to elicit an antibody response. In addition, steroids do not in general contain functional groups that can be linked directly to the macromolecule (for example, bovine serum albumin). The usual approach is to attach a side chain containing a terminal carboxylic acid residue to the steroid and then link the acid group to an amino group of the carrier protein. This conjugation can be effected by either the mixed anhydride or carbodiimide method. Scheme 1 illustrates a typical procedure and shows the synthesis of the ESA conjugate of 17β -oestradiol-6-(O-carboxymethyl)-oxime. The steroid is functionalized at the 6-position, derivatized and then conjugated to the carrier protein. The site of attachment



Scheme 1 Preparation of the BSA conjugate of 17 β -oestradiol-6-(α -carboxymethyl)-oxime.

of the carrier protein to the steroid is most important as it determines the ultimate specificity of the antibodies produced². Further discussion on this aspect of assay development is, however, beyond the scope of this paper.

TRITIUM-LABELLED STEROIDS

The main physical features of tritium, iodine-125 and selenium-75 are summarized in Table 1. Many tritium labelled steroids are readily available commercially and they have therefore found widespread use in radioimmunoassays. In addition, assay kits for many of the steroids of clinical interest are available and these, in nearly all cases, also incorporate tritiated tracers. Some of the advantages and disadvantages of tritium-labelled steroids are shown in Table 2.

Since the tritium atom and hydrogen atom are similar in size, one of the more important advantages of this isotope in the radioimmunoassay is that no discrimination is observed in binding to the antibody between radioactive and unlabelled forms of the steroid being measured. (Jeffcoat *et al.*³, have, however, reported an isotope effect with tritium in an oestradiol system but found no difference in binding in a testosterone assay.) The long half-life of tritium compared with the commonly used γ -emitting isotopes is not the real advantage it seems, since intermittent purification of the tritiated steroids is recommended.

The major disadvantage of employing tritiated steroids is that they are uneconomic especially when a large throughput of samples is being considered, for example in the routine clinical laboratory. The cost of sample preparation in terms of technician time, scintillation fluid and vials is considerable. Additional disadvantages are associated with the well known problems of tritium counting. Long counting times are necessary (5-10 min) and therefore automatic counters are required and chemiluminescence and quenching may cause difficulties. Finally, there is the problem of waste disposal of the counting mixture. These disadvantages considerably outweigh the advantages of ready availability and similarity between the tritiated and unlabelled material.

γ -LABELLED STEROIDS

The routine laboratory is designed to function economically. This economy is readily realized for protein radioimmunoassays since they all utilize γ -counting. The ideal situation in the laboratory would be to standardize all radioimmunological procedures not only in terms of the methodology used, but also in the counting procedures employed.

Table 1. Physical characteristics of radioactive isotopes commonly used in radioimmunoassay.

Isotope	Half-life	Emission	Energy of emission/MeV	Specific activity Ci μgatom^{-1}	
				Theoretical	Practical
^3H	12.35 years	β	0.018 (max.)	29	29 ^a
^{75}Se	121 days	γ -and X-Ray	0.14 0.27 0.28	1.07×10^3	20 - 40
^{125}I	60 days	γ -and X-Ray	0.035 0.027	2.16×10^3	theoretical

^aNormally 4 ^3H atoms are incorporated per mole, specific activity $> 100 \text{ Ci } \mu\text{mol}^{-1}$

Table 2. Tritium-labelled steroids in RIA

Advantages

Commercially available and cheap

Long half-life, but intermittent purification necessary

Similar in size to ^1H

Disadvantages

Problems of β -counting

Uneconomical in practice, cost of scintillator vials, sample preparation time, etc.

Waste disposal problem.

Table 3. Iodine-labelled steroids in RIA

Advantages

γ -emitting isotope (no scintillation fluid, etc.)

High specific activity (theoretical)

Short counting time (manual counting possible)

Solid or liquid phase counting

Disadvantages

Short half-life, regular preparation needed

Products not readily available commercially

Large atom - antibody may discriminate between radioactive and non-radioactive antigens

Preparation can present handling problems/safety hazard

Secondary containment should be plastic

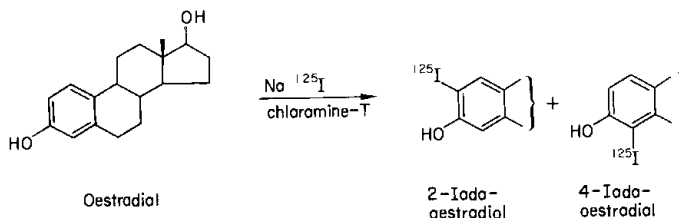
A γ -emitting isotope of carbon, oxygen or one of the smaller elements would be the ideal tracer for steroid radioimmunoassay systems but, unfortunately, none is available. The two isotopes which have been used are ^{125}I and to a lesser extent ^{75}Se . With these isotopes either the liquid or more conveniently, the solid phase resulting from the separation procedure employed, can be counted directly and the problems associated with β -counting are avoided.

IODINE-125 LABELLED STEROIDS

Steroids can be labelled with ^{125}I to high specific activity. Atom for atom, ^{125}I gives approximately one hundred times the count rate provided by ^3H and short counting times can be achieved thus making manual counting a reality (Table 3). For example, the NE-1600 manual counter which can count 16 samples simultaneously, is ideal for this purpose.

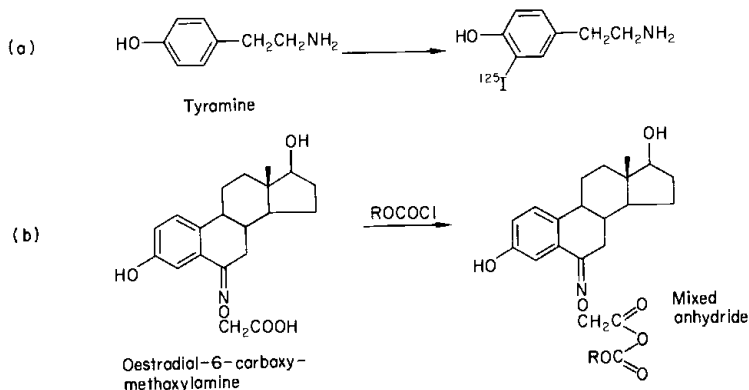
PREPARATION OF ^{125}I -LABELLED STEROIDS

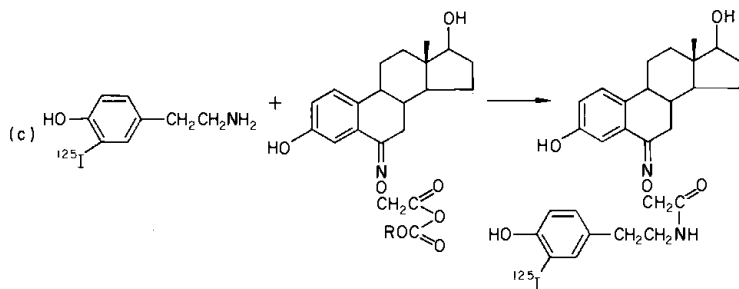
Unfortunately ^{125}I has a relatively short half-life (60 days) and this makes regular preparation of the tracers essential. Iodinated products are not commercially available (except in radioimmunoassay kits) and in-house or custom preparation is required. The Greenwood, Hunter and Glover method, or modification thereof, is generally used to introduce the iodine atom. The method involves the oxidation of Na^{125}I to iodine with chloramine-T. For iodination purposes steroids can be divided into two groups: phenolic (oestrogens) and non-phenolic (progesterone, cortisol, etc.). For phenolic steroids direct iodination is possible and results in the incorporation of the iodine atom into the A-ring (see Scheme 2) producing a mixture of 2- and 4-iodo derivatives



Scheme 2 Direct iodination of oestrogenic steroids (17 α -oestradiol).

together with a small amount of the diiodo compound. These derivatives have only recently been successfully used as the labelled components in radioimmunoassays. The example described in the literature cites directly iodinated oestradiol. The usual strategy for phenolic steroids is to iodinate another reactive compound such as tyrosine methyl ester (TME), tyramine or histamine and then link the amino group of the iodinated material to the carboxylic acid group previously introduced into the steroid. The procedure is analogous to the preparation of protein conjugates for antiserum production and Scheme 3 shows the various reactions involved in the indirect

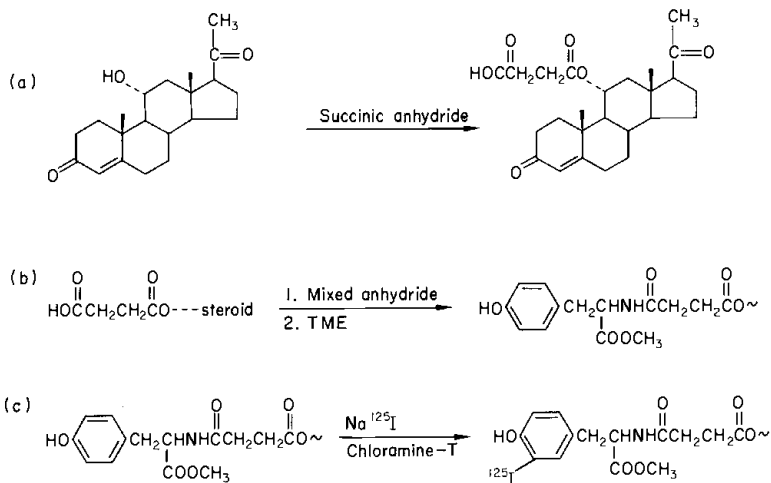




Scheme 3 (a) Tyramine. (b) Oestradiol-6-carboxy-methoxylamine. (c) Indirect iodination of oestrogenic steroids (17β -oestradiol).

iodination of 17β -oestradiol⁶. Tyramine is iodinated by the chloramine-T method and the product reacted with the mixed anhydride derivative of the steroid to give the labelled tyramine conjugate. Not surprisingly, due to the complex nature of the method, multiple products are obtained and the yield of the required labelled material is frequently very low.

A more satisfactory alternative can be employed for non-phenolic steroids since the reactive group may first be attached to the steroid, and the TME conjugate then iodinated⁷. Scheme 4 illustrates the approach for progesterone. 11α -hydroxyprogesterone



Scheme 4 Indirect iodination of non-phenolic steroids using progesterone as the example.

is converted into the hemisuccinate, which is conjugated to TME via the mixed anhydride. The TME derivative is then iodinated by the usual chloramine-T method. The yield from this iodination reaction is usually high. Even so, purification of the labelled products is essential - both unwanted radioactive products and cold material have to be removed by thin layer or column chromatography. The preparation of iodinated steroids in the laboratory can present handling problems and there is also a safety hazard to personnel to be considered.

The tritium analogues of some steroids, particularly the synthetic steroids, are not readily available and the iodinated versions are often more accessible. ^{125}I -labelling may also be a more useful alternative in drug radioimmunoassays⁸.

ANTIBODY DISCRIMINATION

As Table 3 shows, the major disadvantage of ^{125}I over tritium is the size of the atom. For proteins this disadvantage is less important than for steroids and in any case, the specific activity attainable for proteins with either ^3H or ^{14}C is insufficient to allow practicable counting times. The iodine atom when directly introduced into the steroid nucleus frequently results in products which are completely non-immunoreactive. This non-immunoreactivity means that the antibody is often sufficiently specific to be able to distinguish completely between the naturally-occurring steroid and the steroid possessing a bulky iodine atom. As noted above, non-phenolic steroids cannot be directly iodinated and attachment of a reactive group is necessary. The result is a steroid possessing an even bigger iodinated side chain which readily allows the antiserum to discriminate against the tracer. To overcome this problem, a suitable combination of antibody and tracer, which can be used to quantitate the naturally-occurring steroid, must be sought.

HOMOLOGOUS AND HETEROLOGOUS SYSTEMS

In an homologous system both the protein (the immunogen) and the reactive group (the tracer) are attached through the same bridging group and the same site to the steroid nucleus. As might be expected, the resultant antibody has a much higher affinity for the tracer than for the naturally-occurring steroid. The tracer binds so tightly to the antibody that it cannot easily be displaced by the native material. The result is a very insensitive assay. The antibody may be capable of recognizing slight changes in the labelled group. The standard curves obtained with iodinated TME, tyramine and histamine derivatives of progesterone-11 α -hemisuccinate are shown in Fig. 1. With an antiserum raised to a progesterone-11 α -hemisuccinate-protein

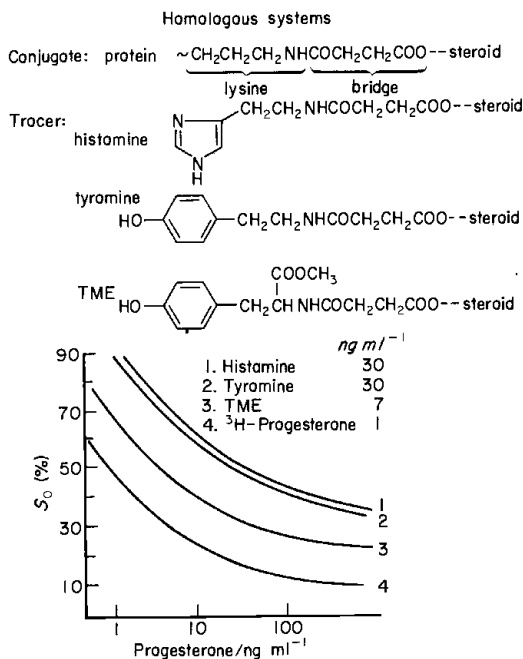


Fig. 1 Homologous steroid RIA effects of variation of the reactive group. For further description, see text.

conjugate, the histamine and tyramine labels give almost identical standard curves. These are less sensitive than curves obtained with the TME derivative. The side chain ($-\text{COOCH}_2$) of the TME derivative may lower the binding to the antibody and thereby produce a more sensitive standard curve. The side chain of the other two tracers has an identical configuration ($-\text{CH}_2\text{CH}_2-$) to the lysine part of the conjugate. All three labelled derivatives give standard curves which are less sensitive than that from the corresponding tritiated assay. This result emphasizes that the antibody is able to discriminate between the iodinated and native forms of the steroid molecule.

Further improvements in assay sensitivity can be made by more drastic changes to the steroid derivative. This can be effected by altering the bridge between the steroid and the reactive group (heterologous bridge, homologous site system) or the site of attachment to the steroid of the reactive group (heterologous site system). It may be necessary to try many different combinations of antiserum and tracer in order to attain the required level of sensitivity. Our work in this direction will be more fully reported elsewhere.

SELENIUM-75 LABELLED STEROIDS

The only other γ -emitting isotope to have found use in steroid radioimmunoassays, albeit to a limited extent, is selenium-75¹⁰. The additional advantages over iodine-125 (see Table 4) are the long half-life and the slightly smaller size of the selenium atom. Introduction of the selenium atom is more difficult to accomplish than iodine-125. The only reported example is the cortisol analogue shown in Fig. 2 with the selenium atom attached directly to the steroid nucleus.

Table 4. Selenium-labelled steroids in RIA

Advantages

γ -emitting isotope

Short counting time

Longer half-life than ^{125}I

Secondary containment less demanding than ^{125}I

Slightly smaller atom than ^{125}I

Disadvantages

Not in general use

Different window settings to ^{125}I required for maximum efficiency

More difficult to introduce ^{75}Se than ^{125}I

Costly to prepare

Specific activity lower than the theoretical value

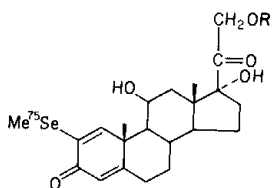


Fig. 2 Cortisol analogue labelled with selenium-75⁷.

Outweighing the advantages is the fact that ^{75}Se is not readily available and it is costly to prepare compared with ^{125}I . Different 'window' settings are required for maximum counting efficiency, but satisfactory counting can be achieved using the ^{125}I settings. Unlike ^{125}I the specific activity so far attainable is considerably lower than the theoretical maximum and this limits its use to assays for substances present in serum in high concentrations, e.g. cortisol.

CONCLUSION

From the foregoing, it is clear that a number of problems need to be overcome in the development of iodinated steroid assays. The advantages to the user, once a satisfactory system has been validated, are, however, considerable. We have discussed the improvements inherent in γ -counting compared with β -counting in the routine laboratory in terms of simplicity and therefore cost effectiveness, but have not so far described how the problems of extraction and recovery correction can be overcome. Essentially this may be achieved by a direct assay on the serum sample if the antiserum is sufficiently specific to obviate extraction and the combination of antiserum and γ -labelled steroid tracer is such that the sensitivity attained allows very small serum samples to be used - thus minimizing the 'serum effect'.

Work in our laboratory has been moderately successful in developing such steroid radioimmunoassays, which can be used on biological fluids to provide simple, rapid inexpensive assays of steroid hormones of clinical importance. The clinical validity of these novel assays has been demonstrated.

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DISCUSSION

P. ROSS: Could you please explain why the iodinated haptens give high backgrounds?

M.R. REDSHAW: The insolubility of the iodinated hapten in the assay buffer probably accounts for the high backgrounds. Unstable tracers would also give high background levels and the values would be expected to change with the age of the tracer. However, the iodinated haptens we have prepared have been stable and we have attributed any high levels to insolubility.