Recent developments in steroid radioimmunoassays

S. L. Jeffcoate

*Department of Chemical Pathology, St. Thomas's Hospital, London SE1, and
National Institute for Biological Standards and Control, Hampstead, London NW3, U.K.*

The rapid progress of steroid radioimmunoassays since their introduction in 1969 has resulted in their widespread application to mammalian physiology and experimental and clinical medicine. The technical aspects of this progress up to the middle of 1974 have been covered in a number of books and reviews (see, for example, Abraham, 1974, 1975; James & Jeffcoate, 1974; Cameron, Hillier & Griffiths, 1975; Gupta, 1975). This review will be a somewhat personal assessment of current developments (up to mid-1976) and their possible place in the future; it will be neither exhaustive nor, I hope, exhausting.

The technique of steroid radioimmunoassay (RIA) has, like the ages of Man, seven stages and these will be considered in turn: antisera, tracers, sample preparation, reagent dispensing, separation of free and bound radioactivity, counting and data processing, quality control and standardization.

Steroid antisera

In the past 2 years, there have been no significant developments in the production, assessment or treatment of antisera for steroid RIA (apart from separation methods, discussed later). It was established by mid-1974 that the nature of the immunogen, i.e. the steroid hapten, the chemistry, site and number of linkages, and the nature of the protein carrier, governed the properties of the resulting antisera. The site of linkage to the steroid nucleus was found to be particularly important and chemists were induced to try all the possible sites. For testosterone, for instance, virtually every carbon atom has been tried in an attempt to limit the cross-reaction with 5 α -dihydrotestosterone (DHT). Even the most specific antisera for testosterone still show, however, a 15% cross-reaction with DHT which is little improvement on the antisera raised against the easily prepared 3-linked conjugates.

Affinity chromatography has been used in attempts to improve the specificity and affinity of steroid antisera. In my view, these attempts are doomed to failure, primarily because the cross-reaction from closely-related steroids is not the result of a separate population of antibodies which can be removed but is an inherent property of the binding site of the antibody. As regards the selecting out of the high affinity antibodies, this is made difficult by the fact that it is these antibodies that bind most avidly to the solid-phase ligand and are the most difficult to elute without damage.

Steroid tracers

Tritium-labelled steroids are most widely used as tracer ligands in the assay and also for recovery checks. Indeed, other tracers cannot be used for the latter purpose since they will not behave identically to the unlabelled steroid in partition and chromatographic systems. With the move toward less preparation of the sample before assay this use of [^3H]steroids will become less necessary. The advantages and disadvantages of [^3H]steroids have been discussed elsewhere (Jeffcoate, Edwards, Gilby & White, 1975) and are summarized in Table 1. The need to ensure radiochemical purity is most important; it should not be taken for granted that manufacturers have provided the correct steroid in the stated amount and with the desired degree of purity. In one study, wide ranges of purity were found (Manlimos & Abraham, 1975) and attributed to the shipment of the steroids from the manufacturer in solution at ambient temperatures. Stability was improved by shipping in the dry state after addition of carrier cholesterol (1 $\mu\text{g}/1 \mu\text{Ci}$) which should not cross-react with the antiserum. Purity should be checked frequently, e.g. every 3 months.

The need to check the radiochemical purity of [^3H]steroids to some extent negates the main

Table 1. A comparison of the advantages and disadvantages of ^3H and ^{125}I -labelled tracers

^3H	^{125}I
<i>Advantages</i>	
1. Available commercially	1. Easier counting
2. Chemically defined	2. Higher specific activity
3. Long half-life	3. Higher affinity
<i>Disadvantages</i>	
1. Expense	1. Preparation
2. Lower specific activity	2. Higher affinity
3. Isotope effects	3. Cost of iodine

disadvantages of ^{125}I -labelled tracers, the short half-life and the need for frequent preparation. The use of ^{125}I -labelled tracers has been reviewed elsewhere (Jeffcoate, 1975) and in my view their advantages outweigh their disadvantages. The routine plasma testosterone assay at St. Thomas's Hospital has utilized a testosterone-histamine- ^{125}I tracer without trouble for the past 3 years and the results are comparable to those obtained using [^3H]labels when collaborative assays are carried out between laboratories (M. J. Wheeler, personal communication). Another gamma emitter, ^{75}Se with a half-life of 120 days, has been used in a competitive protein-binding assay for cortisol (Chambers, Glover & Tudor, 1975), but this isotope has not yet been used successfully in an RIA. At present the specific activity of ^{75}Se is low compared with that of ^{125}I .

Non-isotopic tracers for RIA are being developed: such markers include:

- (a) viruses, e.g. bacteriophage;
- (b) enzymes, e.g. alkaline phosphatase;
- (c) fluorescent;
- (d) spin-labelling;
- (e) immunological FETs

and some of the potential advantages are:

- (a) a long shelf life;
- (b) cheapness of materials and equipment;
- (c) no separation step;
- (d) automation;
- (e) simultaneous assays;
- (f) no isotope health hazard and disposal problem.

The prospect of cheap automation is the main attraction of these methods, although at present the price of lower sensitivity has to be paid. Future developments in this area should be expected.

Sample preparation

The extent to which a biological sample has to be purified before steroid RIA is proportional to the amount of error (losses, blanks, transcriptional) introduced and inversely proportional to the number of samples that can be processed in unit time. For both these reasons, there is a gradual move to less and less pre-assay preparation. This approach obviously carries its own potential danger, that of loss of specificity because of the presence in the sample of cross-reacting steroids or other interfering, 'non-specific' substances.

The ultimate goal, that of no sample treatment at all, is only possible for steroids present in biological fluids in very high concentration ($\mu\text{mol/l}$), e.g. cholesterol and cholesterol esters, dehydro-epiandrosterone (DHA) sulphate or steroid glucuronides (Kellie, 1975).

For steroids like cortisol, which are present in moderate concentration (nmol/l), non-extraction methods are also possible but in this case only after cortisol-binding globulin has been inhibited.

This can be done by (a) heating to 60°C for 1 h, (b) treatment with ANS (8-anilino-naphthalene sulphonic acid) as used in thyroid hormone assays or (c) precipitation of plasma proteins with ethanol. These methods could also be applied to sex hormone-binding globulin (SHBG).

For most steroids a solvent extraction is required. Although there has been little recent development in this area, four points are worth making. First, the purity of the solvent should be initially high and frequently checked: a few ml evaporated and run as a sample in the RIA will soon reveal if pseudo-steroid 'blank' material is being added. Secondly, the choice of solvent can add specificity to the assay. This can be illustrated with respect to progesterone: it is unnecessary to extract with a polar solvent like ether when a much 'cleaner' extract can be obtained with a less polar solvent, such as hexane, with a minimum reduction in recovery. The logical extension of this view is that each steroid has its own extraction recipe tailored to its polarity so as to produce the cleanest extract with maximal recovery.

This conclusion does however militate against the third consideration, that of assay standardization (see also the final section). The more solvents in use in a laboratory and within a group of laboratories, in which attempts are being made to achieve comparability of assay results, the more difficult it is to control the quality of the reagents. It may thus be best to select one (e.g. ether) which will extract all the steroids of interest, and to specify its purity and mode of use in as much detail as possible.

Finally, there have been developments in speeding up the rate-limiting step in steroid RIA, the separation and drying of the organic phase. A neat method of separation is to freeze the aqueous subnatant either in solid CO₂/acetone or with a cryoprobe (use of a deep-freeze is not recommended if ether is the solvent) and then to decant the organic supernatant without fear of contamination with the water phase. Evaporation machines are becoming commercially available. These will hold up to 100 tubes and will shake or warm them in a flow of nitrogen or air.

Chromatographic separation of steroids from biological fluids has been a traditional approach of steroid assayists but with the gradual improvement of reagents and the increasing pressure of sample numbers, it is becoming less necessary and less popular. It still has two important places. First, non-chromatographic methods need to be validated against those in which chromatography has been used. The second application is in obtaining multiple steroid analyses of a single sample. This may be of value when several related steroids are of pathophysiological interest in a specific situation. Although other systems have been used, Abraham has developed this approach using extracts of 1 ml plasma on celite columns, e.g. for 4 androgens (Abraham, Manlimos, Solis & Wickman, 1975) or for 4 progestins (Abraham, Manlimos, Solis, Garza & Maronlis, 1975). Celite column chromatography was also used by Parker, Ellegood & Mahesh (1975) for assay of 7 steroids of interest in reproductive endocrinology after separation (in < 2 h) from a single 1–2 ml sample of plasma.

Reagent dispensing

The move toward increased mechanization of steroid RIA is occurring within the context of increasing automation of RIA and clinical chemistry in general. There has been a rash of dilutors, dispensers and pipetting stations on the market in addition to the relatively simple hand-held repeating pipettes. All these have their place, as do the completely automated machines under development which take in a sample at one end and, after a suitable delay, push out an answer at the other end.

Separation of free from bound radioactivity

After the incubation of the reagents for an appropriate time the next important phase in steroid RIA is the separation of free from bound radioactivity. Many methods have been used (Ratcliffe, 1974) and perhaps the aims of these methods should be stated before discussing how developments might advance steroid RIA. These are: no mis-classification error (i.e. all the bound and none of the free is in the bound fraction); cheapness and simplicity; potential for automation; ability to be standardized within and between laboratories.

Adsorption of the free fraction onto activated charcoal, uncoated or coated with dextran, albumin or gelatin is the most widely used method in steroid RIA. It has the advantage of cheapness and simplicity and can be fairly well standardized: one large batch of charcoal can be purchased to serve a number of laboratories for many years. Its disadvantages are (i) the dependence on time between addition and centrifugation; there is an increase in the radioactivity adsorbed to the charcoal caused either by dissociation of the bound complex and adsorption of the freed tracer or, more likely, adsorption of the bound complex itself, and (ii) the difficulty of using charcoal in automatic equipment.

Polyethylene glycol (PEG) is easier to handle in these respects (Schiller & Brammall, 1974, 1976) and recently has been widely used. One disadvantage of PEG is the need for higher centrifugation speeds (at least 1500 g) compared with dextran-coated charcoal (500 g). This may limit its universal applicability.

The double-antibody methods widely used in peptide assays can also be applied to steroids but they seem to be unnecessarily expensive and time-consuming when other techniques are available.

Although the first steroid RIA published was a solid-phase (coated-tube) method, these techniques have been slow to gain ground. They appear to have many advantages particularly in terms of cost, ease and potential automation. The routine plasma testosterone RIA at St. Thomas's Hospital mentioned above has employed antibody-coated tubes for several years. Temperature control at the coating stage is the only critical factor.

Other solid-phase methods that seem to be increasingly used are those in which antibody is covalently bound to particles which can be easily centrifuged for separation (e.g. Sepharose or glass beads). A novel approach (Nye & Landon, 1976) is the use of antibodies coupled to magnetic particles which can be simply and automatically removed from the incubation medium with a magnet.

In summary, developments in this area of steroid RIA are in the direction of methods that are either cheap, automated or easily standardized and preferably all three.

Counting and data processing

The counting of the radioactivity in either the antibody-bound or free fractions (or both) requires automatic counters. These machines are expensive and becoming more so. Two tendencies are discernible however: first, in the direction of more complicated counters that will do a certain amount of data processing in addition to counting; and secondly in the direction of cheaper, simpler bench-top counters with, for example, a single channel and limited sample capacity. Different users will have different needs and this will govern the choice of counter.

Scintillation-counting of β -emitters such as [^3H]steroids has been advanced by the use of polypropylene inserts which are produced by several manufacturers. The sample plus scintillation fluid is put in the insert which is then placed in a standard counting vial. Three benefits accrue: less scintillant is used; the waste scintillant and the insert can be easily disposed of by incineration thus reducing the health hazard; washing of counting vials is reduced to a minimum.

Computers are used in RIA for two major purposes: optimal assay design and the calculation and evaluation of results. The theoretical basis and practical application of these have been extensively discussed. In 1974, Rodbard wrote that "the literature on RIA statistics and data processing is large and growing rapidly." This is still true in 1976. The reviews of Rodbard (1974) and Cook (1975) summarize the situation admirably. As regards standard curve-fitting, the logit-log linear transform appears to have gained widest currency and this and other programmes are readily available for use on relatively simple programmable calculators. It is an inefficient use of resources to have an on-line computer system for data calculation.

Quality control and assay standardization

Although the least glamorous aspect of steroid RIA, in my view this is the area in which the most significant developments are taking place in 1976. It has been recognized in public health laboratories (and is even permeating into the thinking in 'pure' research laboratories) that the quality of the

assays performed is at least as important as the number of assays performed. Many of the principles and practices adopted by bioassayists for decades are only reluctantly used by immunoassayists and yet they are essential if the results are to have any meaning.

Quality control is aimed at two interrelated problems, internal and external. Within a laboratory, the assay needs to be as near 'correct' as possible with errors limited and with a mechanism for detecting real or potential breakdown in the assay. This is done by incorporating relevant samples in each assay. Thus the maximal binding (B_0), minimal (non-specific) binding and slope of the standard curve can be assessed. Quality control samples can be included and should preferably cover the range to be expected in the unknown samples which they should resemble as closely as possible. Thus it is better to use the endogenous steroid rather than to 'strip' (e.g. with charcoal) the sample and add known amounts of standard steroid. Cumulative charts showing within-assay and between-assay variance should be kept and make good laboratory wall-paper. Rodbard (1974) and Rodbard, Lenox, Wray & Ramseth (1976) have summarized the use of internal quality control schemes.

External quality control schemes are necessary because (i) many laboratories do not run adequate internal quality control schemes, (ii) results between laboratories need to be comparable so that, in the first place, clinicians and others can use data from physiological and pathological situations without having to readjust to different normal ranges, and secondly, multi-centre research programmes obtain uniform results.

A number of external quality control schemes for steroid RIA are under development. In the United Kingdom, a national scheme has arisen out of the work of the Supra-regional Assay Service (SAS) and all the steroids of major biological interest are covered. In general, about four coded samples are sent out at monthly intervals and the results returned and processed centrally. These schemes give information regarding between and within-laboratory variance and reproducibility. A trouble-shooting element is an essential feature with the organizing quality control laboratory being able to give advice on reagents, assay protocols, etc.

On a world-wide scale, the Human Reproduction Unit of WHO has instituted an external quality control scheme for four steroid hormones in an attempt to increase the quality and comparability of the results obtained by the research laboratories participating in its research programmes in fertility control.

Those who have experienced the results of external quality control schemes for RIA are well aware of the need for assay standardization. International organizations have seen the need for this and laid down guide-lines very clearly (IEAE, 1974; WHO, 1975). The 26th report of the WHO Expert Committee on Biological Standardization is a milestone in this area and covers the need to provide well-characterized, matched reagents for immunoassay and the organization of hormone assay services at district, regional, national and international levels.

The U.K. is ahead of the world here with the SAS scheme and a developing network of regional schemes. The standardization of RIA within the SAS and within the WHO programmes involves a package of matched reagents (well-characterized, in sufficient supply to last for 3–5 years and in ampoules available for distribution) plus detailed assay protocols (buffers, incubation and separation procedures, etc.) and an external quality control scheme. Using such a package, the achievement of the goal of assay standardization and universal comparability of high quality RIA results is brought immeasurably closer.

References

- ABRAHAM, G.E. (1974) Radioimmunoassay of steroids. *Acta endocr., Copenh., Suppl.* **183**, 1–42.
- ABRAHAM, G.E. (1975) The application of steroid radioimmunoassay to gynecologic endocrinology. In *Progress in Gynecology*, Vol. VI, pp. 111–144. Eds M. L. Taymor & T. H. Green. Grune and Stratton, New York.
- ABRAHAM, G.E., MANLIMOS, F.S., SOLIS, M., GARZA, R. & MARGOULIS, G.B. (1975) Combined radioimmunoassay of four steroids in one ml of plasma. I. Progestins. *Clin. Biochem.* **8**, 369–373.
- ABRAHAM, G.E., MANLIMOS, F.S., SOLIS, M. & WICKMAN, A.C. (1975) Combined radioimmunoassay of four steroids in one ml of plasma. II. Androgens. *Clin. Biochem.* **8**, 374–378.
- CAMERON, E.H.D., HILLIER, S.G. & GRIFFITHS, K.

- (Eds) (1975) *Steroid Immunoassay*. (Proc. 5th Tenovus Workshop.) Alpha Omega Alpha Publishing Ltd, Cardiff.
- CHAMBERS, V.E.M., GLOVER, I.S. & TUDOR, R. (1975) (^{75}Se)—radio ligands in steroid immunoassay. In *Steroid Immunoassay*, pp. 177–182. Eds E. H. D. Cameron, S. G. Hillier & K. Griffiths. Alpha Omega Alpha Publishing Ltd.
- COOK, B. (1975) Automation and data processing for radioimmunoassays. In *Steroid Immunoassay*, pp. 293–310. Eds E. H. D. Cameron, S. G. Hillier & K. Griffiths. Alpha Omega Alpha Publishing Ltd, Cardiff.
- GUPTA, D. (Ed.) (1975) *Radioimmunoassay of Steroid Hormones*. Verlag Chemie, Weinheim.
- IAEA (1974) Standardisation of radioimmunoassay procedures. Report of an International Atomic Energy Agency panel. *Int. J. appl. Radiation and Isotopes* **25**, 145–160.
- JAMES, V.H.T. & JEFFCOATE, S.L. (1974) Steroid radioimmunoassays. *Br. med. Bull.* **30**, 50–54.
- JEFFCOATE, S.L. (1975) Use of ^{125}I iodine tracers in steroid radioimmunoassays. In *Radioimmunoassay of Steroid Hormones* pp. 185–195. Ed. D. Gupta. Verlag Chemie, Weinheim.
- JEFFCOATE, S.L., EDWARDS, R., GILBY, E.D. & WHITE, N. (1975) The use of [^3H]-labelled ligands in steroid radioimmunoassays. In *Steroid Immunoassay*, pp. 133–140. Eds E. H. D. Cameron, S. G. Hillier & K. Griffiths. Alpha Omega Alpha Publishing Ltd, Cardiff.
- KELLIE, A.E. (1975) Radioimmunoassay of steroid glucuronides. In *Radioimmunoassay of Steroid Hormones*, pp. 165–183. Ed. D. Gupta. Verlag Chemie, Weinheim.
- MANLIMOS, F.S. & ABRAHAM, G.E. (1975) Chromatographic purification of tritiated steroids prior to use in radioimmunoassay. *Analyt. Letters* **8**, 403–410.
- NYE, L. & LANDON, J. (1976) Automated hormone radioimmunoassays based on the use of antibodies linked to ferro-magnetic particles. *J. Endocr.* (In press).
- PARKER, C.R., ELLEGOOD, J.O. & MAHESH, V.B. (1975) Methods for multiple steroid radioimmunoassay. *J. Steroid. Biochem.* **6**, 1–8.
- RATCLIFFE, J.G. (1974) Separation techniques in saturation analysis. *Br. med. Bull.* **30**, 32–37.
- RODBARD, D. (1974) Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. *Clin. Chem.* **20**, 1255–1270.
- RODBARD, D., LENOX, R.H., WRAY, H.L. & RAMSETH, D. (1976) Statistical characterisation of the random errors in the radioimmunoassay dose-response variables. *Clin. Chem.* **22**, 350–358.
- SCHILLER, H.S. & BRAMMALL, M.A. (1974) A radioimmunoassay of plasma estradiol-17 β with the use of polyethylene glycol to separate free and antibody-bound hormone. *Steroids* **24**, 665–674.
- SCHILLER, H.S. & BRAMMALL, M.A. (1976) Non-chromatographic radioimmunoassay of unconjugated estriol in plasma with polyethylene glycol as precipitant. *Clin. Chem.* **22**, 359–363.
- WHO (1975) Twenty-sixth report of the World Health Organisation Expert Committee on Biological Standardisation (26th ECBS). WHO, Geneva.