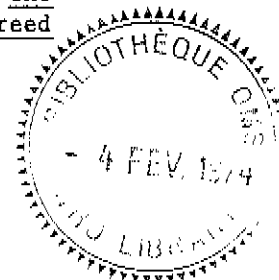




DRAFT RECOMMENDATIONS ON PRINCIPLES FOR THE ASSESSMENT OF  
RADIOIMMUNOASSAY SYSTEMS (AND OTHER LIGAND ASSAY SYSTEMS) FOR HUMAN HORMONES

Note: This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein and for preparation of material to be considered by the Expert Committee on Biological Standardization. The text, therefore in its present form does not necessarily represent an agreed formulation of the Expert Committee. Any communication regarding these proposals should be addressed to the World Health Organization, Geneva, Switzerland, attention: Biological Standardization Unit.



General considerations

Until formal General Requirements for Immunoassay Reagents and requirements for material used for particular immunoassays can be formulated, draft recommendations have been formulated for the assessment of radioimmunoassay systems and of the quality of reagents used with them for the immunoassay of human hormones. In many instances, however, in the recommendations which follow, only suggestions can be made on particular aspects of the preparation of the reagents. For making an adequate assessment of each immunoassay system, adequate data should be requested and taken into consideration on the extent to which the recommendations have been followed.

It is important that the nature and form of each particular hormone as it is present in the samples of biological fluids and which it is of clinical importance to measure should be known, before the competence of any immunoassay system can be judged. In general the properties of the reagents used for immunoassay of a particular hormone should be related to each other, for that immunoassay system as a whole. The inter-related quality of reagents should be that which provides optimal performance of the whole assay system in accuracy of quantitation, specificity and sensitivity.

The specificity of the whole immunoassay system should be determined for the particular form of the hormone and type of test material it is intended to assay. For each immunoassay system, therefore, depending on the hormone, there is a need for certain characterized reference materials which would be used to control the specificity of the system by testing for the absence of cross-reaction with related hormones which may be present in test samples. For peptide hormones these may consist of pure samples of the intact hormone and of sub-units, as well as perhaps similar pure preparations of related hormones. For steroid hormones, pure samples of various other known related steroids would be used. In assessing the specificity of each immunoassay procedure, it is advisable also to test a number of samples from persons in physiological and various pathological conditions in which different levels and forms of the hormone occur, as well as samples of known hormone content.

A similar procedure should be used to determine the level of sensitivity of the immunoassay system. The achievable level of sensitivity determined for each particular hormone should be stated.

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Recent interest in immunoassay of hormones has developed to such an extent that in each of several countries there are many laboratories carrying out such assays for clinical and research purposes. It is of public health importance therefore for each country to ensure the highest possible quality and consistency of assay performance by their laboratories. Assessment of immunoassay performance requires both the initial evaluation of the accuracy, specificity and sensitivity of the immunoassay system but also the continued monitoring of assay performance in each laboratory making the immunoassays. The former consideration would determine the circumstances for which particular immunoassay systems would be suitable and the conditions for which they would be relevant in clinical medicine. Control of laboratory performance on the other hand would be the responsibility of the laboratory services.

Laboratory performance should be based on the following considerations:

- (a) Development of a national system for immunoassay services involving the relevant laboratories in the country. Such a national system should include at least one major laboratory with specialized competence in immunoassays of particular hormones as well as regional or local laboratories which would work in liaison with the central laboratory. Such a national system would also organize continued monitoring of performance. For this, coded test samples typical of materials assayed routinely (e.g. pooled plasma or urine) and if necessary blank samples (e.g. for steroid hormones where the active material in plasma is extracted before assay) and samples with added known amounts of hormone would be immunoassayed by the participating laboratories. Analysis of the results received will enable an assessment to be made of the general pattern of the values obtained and the relative position of each laboratory's results within that pattern. This would identify imprecise or anomalous results and would enable laboratories to examine the possible reasons for this. Problems in the statistical analysis of results which may differ even for the same assay method are also relevant to study on a national scale. Another important consideration for a national service is the designation and, if applicable, registration of laboratories which perform immunoassays and of establishments which supply radioactive and non-radioactive reagents and services.
- (b) Quality of materials and suitable procedures used for immunoassay, accuracy of quantitation, assessment of intra-assay and inter-assay variation for particular immunoassays in the same laboratory, routine inclusion of samples of known hormone content and blanks.
- (c) The use of any available international services or materials. The WHO services at the present time consist of the provision of certain international standards and international reference preparations for bio-assay and one for immunoassay. Individual laboratories may make available working reagents and materials to other laboratories which are unable to prepare or obtain their own; information on the availability of these materials throughout the world has not been compiled.

A variety of kits is now available for certain immunoassays of human hormones. Reagents are also made available in bulk form. If kits are made from such bulk materials it will be advisable for the performance of the kits to be checked against

assay performance by the use of the bulk materials. In addition, since kits are often made in a series of consecutive batches consistency of performance both within and between batches should receive particular attention.

Since radioimmunoassay involves the handling of biological fluids which may be a potential source of viral infection, and of radioactive material, personnel performing these assays should have appropriate training and the immunoassays should be carried out in suitable specified places in each laboratory. Consideration should also be paid to the proper disposal of containers and waste material and to the treatment of apparatus.

Attention should also be drawn to certain recommendations of the Twenty-first Expert Committee on Biological Standardization in connection with expressing the results of immunoassays (Wld Hlth Org. techn. Rep. Ser., 1969, No. 413, p.9). When the results of immunoassays are given, there should be added the qualification "by immunoassay"; in addition if the results are given in units, it is essential that the reference material on which the unit was based should also be stated. In all cases, if any preparation has been used to calibrate the reference material in the assay system, this should be identified.

In the case of certain hormones (e.g. human pituitary hormones) source materials for the preparation of immunoassay reagents are scarce and difficult to obtain. The development of centralized national services for collecting and processing such material (e.g. human pituitaries) is therefore of considerable importance for national immunoassay services.

#### 1. Antigen for labelling

The antigen should be homogeneous, and should preferably be homologous with the hormone to be measured, unless an antigen from another species, or synthetic material has been shown to be suitable.

Wherever possible the identity of the hormone should be shown by classical biological activity in vivo by comparison with a characterized preparation of the hormone; this may be dispensed with when the identity of the hormone is not in doubt, e.g. steroids. In some cases the starting material for preparing the antigen is from natural sources and it would be preferable to use that with the highest specific biological activity obtainable. After purification the antigen should consist of intact hormone molecules unless it has been shown that a particular fragment serves the purpose as well and can be more conveniently prepared. In other cases synthetic materials are used, e.g. tetracosactide ( $\beta$  1-24 corticotrophin), synthetic gastrin, angiotensins I and II. Purity of the antigen for labelling would be related to freedom from known likely contaminants, such as precursor forms of hormones (e.g. proinsulin) and degradation products (e.g. C peptide of insulin, deamidated insulin) and structurally related hormones and subunits (e.g. glycoprotein hormones) as determined by tests including the following:

- (i) tests of homogeneity (a) of molecular size as shown by gel filtration;
- (b) of surface charge by isoelectric focussing and acrylamide gel electrophoresis; (c) of N terminal amino acid by dansylation or Edman degradation; and
- (d) of C terminal amino acid by analysis with carboxypeptidases;

- (ii) analysis of amino acid and carbohydrate composition after hydrolysis;
- (iii) reaction with specific antisera which distinguish between the intact hormone and likely contaminants, such as subunits in preparations of glycoprotein hormones.

In some cases the antigen for labelling may retain these properties indefinitely. It would be preferable however for an expiry date to be stated.

The container should be marked with:

the name of the hormone followed by the words 'antigen for labelling' or 'labelled antigen' whichever is applicable;

batch number;

conditions for storage;

whether it does or does not require further purification before use;

nature and concentration of any added substances including any bacteriostat that may have been added;

expiry date;

if applicable, identity and amount of radioactivity (isotope) present at the earliest date the material may be used after isotope labelling.

#### 1.1 Unlabelled antigen

If the antigen is supplied for radioactive labelling by the user, it should be issued in a form suitable to do so (amount, concentration, diluent buffer and carrier, container) accompanied by appropriate instructions including those for reconstitution and storage conditions thereafter where necessary.

It would be desirable if users would examine such material as they label for themselves in the light of the recommendations in the following section 1.2.

#### 1.2 Labelled antigen

When the antigen is issued already radioactively labelled the following guidelines for manufacturing control should apply:

- (a) radiochemical purity of the isotope used for labelling should preferably comply with criteria for such isotope preparations used for administration to man. Similar criteria should be formulated for other isotopes used for labelling, such as tritium;

(b) after hydrolysis of an antigen labelled with  $^{125}\text{I}$  or  $^{131}\text{I}$  the majority (say 70%) of the radioactive iodine should be, for example, as moniodinated tyrosine; only a small amount (say 5%) should be as diiodinated tyrosine;

(c) information should be provided on:

(i) the approximate amount of the labelled hormone; and

(ii) the approximate proportion of the radioactivity present in damaged hormone or fragments, aggregated hormone and fragments, small molecular constituents such as iodide.

These data should be provided for the labelled antigen at the earliest time at which it may be used after labelling and as expected (on the basis of experimental data) at the end of the expiry period;

(d) the performance of the labelled antigen should be consistent within the period it is recommended for use; and when tested by the assay procedure with serum samples containing known amounts of the hormone, the results at the beginning and at the end of the expiry period should be within acceptable limits;

(e) ideally, labelled antigen should be purified (or re-purified) immediately prior to use in an assay. This is particularly applicable to labelled steroids. Instructions should be included for a suitable purification procedure (e.g. TLC or paper-chromatography, gel filtration) and also other recommendations, e.g. composition and size of column, buffer for elution or chromatography, and any bacteriostat which is compatible with the product.

## 2. Reference materials (to be included in each assay)

The reference material used for the assay should consist of the homologous hormone so that the hormone in the test sample and the reference material are as nearly identical as possible (i.e. human and preferably from the same source, plasma or urine) so as to obtain the greatest similarity in molecular constitution and configuration. This may be easier to achieve with some hormones, e.g. steroids and small peptide hormones, than with others. If the hormone has been synthesized, synthetic material may be suitable (e.g. angiotensins).

The identity of the hormone must be demonstrated as in the case of antigen for labelling.

The degree of purity of the reference material should be such that any impurities present do not interfere with the assay. This would depend on the type of hormone to be assayed and the type of sample to be tested, plasma or urine; in the cases of growth hormone and LH it has been possible to achieve this more easily than in the case of FSH. The reference material should also be calibrated with any reference material

issued nationally or internationally, or, where appropriate, with the pure chemical substance, e.g. steroids and prostaglandins, in the same immunoassay system, under the same conditions and with the same reagents. Where the biological activity of the material has also been estimated by bioassay the potency should be expressed in units of biological activity, e.g. international units. Since different assay methods, e.g. classical in vivo methods and in vitro methods may give different estimates of biological activity, the method of bioassay and the standard used should be stated.

If a series of batches of reference material of the same hormone are made, it is recommended that the manufacturer set aside a batch of material for use as a house standard. If national or international reference material is available, this house standard should be calibrated, and if possible the biological activity measured as recommended above. Whether or not calibrating material is available, successive batches could be compared with the house standard by immunoassay using the same immunoassay system, and preferably the same reagents. The effect of this would be to ensure consistency of behaviour of these batches of reference material, provided they are used in the same system with the same reagents.

The stability of the reference material under the recommended storage conditions should be determined by immunoassay using the same assay system, conditions of assay, with the same antiserum and other reagents, with the exception of the labelled antigen which should be of approximately the same quality. The test for stability should be an accelerated degradation test of suitable design.

The containers of reference material should be marked with:

the name of the hormone, followed by the words "reference material for immunoassay";

the concentration or content of hormone;

batch number;

conditions for storage;

nature and amount of any added substances;

expiry date;

instructions for reconstitution, if applicable, for the diluent to be used (e.g. buffer, protein content, hormone free serum), and for conditions for storage.

### 3. Antisera

(i) In the case of large molecule hormones used as immunogen for preparing antisera the antigen should preferably be the purest preparation available using for example material conforming to the specifications for antigen for labelling (section 1 above). When this is not feasible, antigen of less purity may have to be used. In order to preserve the specificity of the assay system the permissible degree of impurity would have to be related to the purity of the labelled antigen and of the reference material used in the particular assay system.

- (ii) Small molecule hormones (such as steroids, short peptide hormones and  $T_3$  &  $T_4$ ) may have to be conjugated to another (usually larger) molecule in order to make it suitably immunogenic. In such instances the site of chemical conjugation should be remote from those parts of the molecule which confer the characteristic activity of the particular hormone, so that the antibodies produced are more likely to be specific. The large molecule used for such conjugation should be one that is not a component used subsequently in the assay, e.g. bovine serum albumin in buffer diluting fluid or in the test samples.
- (iii) In the case of certain groups of hormones in which portions of their primary aminoacid sequences are similar the antiserum prepared should contain antibodies to the molecular configuration which is specific for the hormone and not to the common configuration. Examples are glycoprotein hormones (TSH, FSH, LH, HCG), gastro-intestinal hormones (secretin, gastrin, glucagon, cholecystokinin/pancreozymin) and the group human placental lactogen, growth hormone and prolactin. In the case of glycoprotein hormones, this may be achieved by using the specific  $\beta$  subunit, and for groups of steroid hormones  $C_{18}$ ,  $C_{19}$  and  $C_{21}$  steroids. When, however, an antiserum is found to be non-specific the unwanted antibodies in some instances may be removed by various procedures, e.g. affinity chromatography or by absorption with an excess of cross-reacting antigens.
- (iv) The sensitivity of the assay system should be sufficient to permit direct estimation in test samples of physiological concentrations of hormone without the necessity for extraction and concentration. In general antisera containing antibodies with high affinity (high association constant,  $K$  value  $>10^9$  litre per mol) are more suitable. In the case of steroid hormones and prostaglandins, however, extraction of test samples is unavoidable.
- The antiserum should also be tested and shown not to react with known likely contaminants or other substances (e.g. serumproteins) that may occur in test samples and which may interfere in the assay.
- (v) Rabbits and guinea pigs have generally been used to prepare antisera. The choice of the species may be influenced by the amount of immunogen available, and the procedure used to separate bound from free hormone in the assay system.
- (vi) Information should be provided on the properties of the serum including its specificity and affinity and the final dilution at which a stated proportion of a stated amount of labelled antigen in a stated volume would be bound.

#### 4. Other binding substances

In the case of ligand assays other than immunoassays, other means of binding are used instead of antisera. These include plasma hormone binding proteins (e.g. for cortisol, testosterone, progesterone) or preparations of cell membranes from tissues of target organs carrying hormone binding sites (e.g. kidney receptor sites for parathyroid hormone, ovarian or testicular receptor sites for LH and HCG). Such carrier preparations, to be effectively used, require that the antigen for labelling in the assay system retains its specific biological activity.

5. Test samples

Detailed instructions should be supplied on the preparation of the patient including avoidance of drugs incompatible with the assay system, the taking of the test samples, any treatment of the test samples (e.g. with a suitable anticoagulant, enzyme inhibitor or bacteriostat) and conditions of storage to prevent loss of the hormone prior to the assay.

If test samples have to be transported suitable conditions (for example storage in dry ice) should be used for preserving the sample.

6. Treatment of assay data

There may be a variety of methods of statistical analysis of immunoassay results. This could pose a problem if different methods of treatment of data from the same method of assay give divergent results. Such problems are best handled by immunoassay services on a national basis, rather than by individual laboratories. International exchange of information on statistical methods used nationally will be useful to all countries.