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Hormone Assays in Biological Fluids

Edited by

Michael J. Wheeler

J. S. Morley Hutchinson

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Preface

Initially, hormones were measured by biological methods, either internally or externally. Internally, within the patient, for example, estrogens could be monitored by examination of vaginal cytology and androgens by the onset of hirsutism. External bioassays involve(d) either whole laboratory animals or specific tissues or cells in incubation or culture to provide suitable endpoints for response. In general, bioassays, although often exquisitely specific are, with notable exceptions, either expensive or time-consuming or not sensitive enough to measure hormones in body fluids.

Hormones, particularly steroids and their metabolites, have been measured chemically, particularly in urine, for the past 60 years. Such methods have proved to be valuable diagnostic tools and methods are described in this book using high-performance liquid chromatography and gas chromatography–mass spectrometry, procedures that give high specificity and sensitivity. It was, however, only with the development of immunoassays by Berson and Yalow, and by Ekins, that methods sensitive enough to measure levels of hormone circulating in blood were possible. Further sensitivity was achieved with the introduction of monoclonal antibodies and the development of immunometric assays. Early immunoassay methods used radioactive labels that were unsuited to automation. Automation was achieved when enzyme, fluorescent, and chemiluminescent labels were introduced and today the majority of hormone assays are available on automated immunoassay analyzers. Although convenient, these assays are often expensive and can suffer from nonspecific effects.

The high precision and sensitivity of today's assays enable hormones to be measured at very low concentrations in a variety of such biological fluids as serum, cerebrospinal fluid, and saliva. Not only have studies revealed seasonal variations more clearly but also, by measuring frequent samples throughout the day, the episodic secretion of a number of peptides. The more sensitive assays have also allowed the measurement of hormone levels in children and in hormone-deficient states. Therefore, not only have the assays extended our knowledge of the biochemistry and physiology of hormones, but they have also opened up new knowledge that aids in the diagnosis and treatment of disease.

In the present reviews of methods of hormone assay, a wider view of a hormone than envisaged by Bayliss's and Starling's definition has been taken, with the word hormone being applied to any specific chemical messenger or its metabolites in blood and other body fluids. We have used SI units throughout the book for consistency. Where no international standard is available we have used the most common unitage quoted in the literature.

It has not been possible to cover all the hormones, but many of the techniques described in *Hormone Assays in Biological Fluids* are transferable to those hormones

that are absent. Where reference ranges are quoted, these are only given for guidance. It is always recommended that the assayist should establish the reference range for the method used and the population being investigated. The first chapters give an overview of the methods commonly used in the measurement of hormones. These are followed by chapters on specific hormone groups. The emphasis has been on noncommercial assays so that readers can set up their own methods. In some cases commercial assays are described for comparative purposes. As already mentioned, most assays are available as commercial kits and many are automated. However, some of the advantages of in-house assays are that they are often less expensive, may be tailored to give a working range suitable for the specimens to be measured, and can be modified to reduce interference effects and provide more precise, more sensitive, and more accurate results. We believe there is still an important place for developing one's own assays.

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1

Immunoassay Techniques

Michael J. Wheeler

Summary

No other development has had such a major impact on the measurement of hormones as immunoassay. Reagents and assay kits can now be bought commercially but not for the more esoteric or new hormones. This chapter explains the basics of the immunoassay reaction and gives simple methods for immunoassays and immunometric assays and for the production of reagents for both antigenic and hapten hormones. Alternative methods are given for the preparation of labeled hormones as well as several possible separation procedures. The methods described here have been previously used in a wide range of assays and have stood the test of time. They will allow the production of usable immunoassays in a relatively short period of time.

Key Words: Immunoassay; antibodies; tracers; separation techniques; peptides; protein hormones; steroids.

1. Introduction

The first immunoassays were described by Yalow and Berson *(1)* and Ekins *(2)* for the measurement of insulin and thyroxine, respectively. As they say, the rest is history. There are now hundreds of immunoassays for scores of analytes including hormones, tumor markers, bone markers, drugs, antibodies, and cardiac markers covering the fields of endocrinology, oncology, haematology, toxicol-

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Table 1
Basic Requirements of an Immunoassay

An antibody to the analyte to be measured
A labeled form of the analyte (competitive immunoassay)
Or a labeled second antibody to the analyte (reagent excess immunoassay)
A separation system to separate antibody bound tracer from unbound tracer

ogy, serology, infectious diseases, and so on. Developments in antibodies, labels, and automation have resulted in highly specific and sensitive assays. This chapter provides an overview of immunoassay technology and some general methods for the production of antibodies, labeled analytes, and solid-phase separation, but assays specific to different analytes will be found in the assay-specific chapters.

The basic requirements of an immunoassay are given in **Table 1**. Both polyclonal and monoclonal antibodies are employed in current immunoassays as will be explained in **Subheading 1.1.** Labels attached to analytes and antibodies may be radioactive, usually iodine-125 (radioimmunoassay and immunoradiometric assays), enzymes such as alkaline phosphatase and horseradish peroxidase, (enzyme immunoassay or immunometric assay, or enzyme-linked immunosorbent assay [ELISA]), chemiluminescent (e.g., acridinium ester), fluorescent (e.g., fluorscein), or an earth chelate such as europium or terbium. All the nonradioactive labels are used in different commercial-automated immunoassays. Manual assays using radioactive or nonradioactive labels are available for a great number of different analytes and the only expensive equipment required is for measuring the end-point. Because γ-counters and colorimeteric plate readers are generally available in-house, assays have tended to use either radioactive or enzyme labels. Both types available are fairly easy to use and each has its own advantages and disadvantages. The main advantage of a radioactive label is that the assay is relatively free from interference and counting can be delayed for several days if necessary. Enzyme labels remove the hazard of

radiation and radioactive contamination but can be affected by inferring substances that can inhibit enzyme activity or affect the colorimetric reading. Manual assays are useful where a study is for a limited amount of time or when the number of samples is small, whereas automated assays are more convenient for the routine diagnostic laboratories that carry out large numbers of determinations of a defined repertoire of analytes from week to week. Raising antibodies is fairly simple but lengthy, whereas producing labels, especially the majority of nonradioactive labels, can be rather complex so it is much easier to set up an assay if the antibody and label or tracer are available commercially.

The basic immunoassay reaction is as follows:

$$\text{Antibody (Ab)} + \text{Antigen (Ag)} \rightleftharpoons \text{Antibody.Antigen (Ab.Ag)}$$

An antibody specific to the endogenous antigen (analyte) in the sample binds to the antigen after a period of incubation. In order to quantify this reaction a labeled form of the antigen is added that competes for a limited number of antibody-binding sites with the endogenous antigen.

$$\text{Ab} + \text{Ag} + \text{Ag}^* \rightleftharpoons \text{Ab.Ag} + \text{Ab.Ag}^*$$

In this system, the amount of labeled antigen bound to antibody is inversely proportional to the amount of endogenous antigen in the sample. In order to measure the amount of labeled antigen that is bound, the bound moiety must be separated from the unbound reagents. Greater assay precision is achieved with the most specific separation system.

Hormone immunometric assays use labeled antibodies in a "sandwich" assay. The antibodies are either both monoclonal or one is monoclonal and the other polyclonal.

$$\begin{aligned} \text{Ab}_1 + \text{Ag} &\longrightarrow \text{Ab}_1\text{.Ag} \\ \text{Ab}_1\text{.Ag} + \text{Ab}_2{}^* &\longrightarrow \text{Ab}_1\text{.Ag.Ab}_2{}^* \end{aligned}$$

The systems that have been developed for general diagnostics use a capture antibody that is usually bound to a solid phase such as

the reaction tube or microtiter well surface or to a particle, such as a plastic bead or cellulose. Sandwich assays use excess reagents because the assay depends on occupancy of binding sites rather than competition for the binding sites. This assay design can produce fast, sensitive, and highly specific assays.

1.1. Antibodies

Immunoassay techniques have been made possible by the unique characteristics of the mammalian immune system. These are:

1. A foreign substance (antigen) entering the body stimulates the immune system to produce antibodies against that substance.
2. Antibodies recognize specific and sometimes unique characteristics of the antigen.
3. Once antibodies have been produced there is an enhanced production of antibody following a second challenge with the antigen.

Antibodies used in immunoassays fall into two groups, polyclonal and monoclonal antibodies.

Polyclonal antibodies may be produced in mammals such as rabbits or sheep. When a foreign substance enters the body, it stimulates the immune system to produce antibodies to the substance making it attract macrophages that will attack the foreign substance. Using this natural reaction, an analyte in as pure a form as possible is injected into the animal stimulating the production of antibodies. In the case of many proteins (hormones, tumor markers), this may involve the injection of the protein without modification because the protein of one species is usually different enough from the same substance in the host to cause an immune reaction. Some substances such as drugs and steroids are too small (and are called haptens) to produce an antigenic reaction, and natural steroids are the same as those circulating in the target animal. These latter substances can be made antigenic by attaching them to a protein such as albumin or keyhole limpet haemocyanin. The conjugated molecule is large and antigenic but as a hybrid substance is now seen as a foreign substance by the host animal.

Monoclonal antibodies (MAbs) use the same characteristics of the immune system but provide unique specificity by recognizing only one epitope on an antigen. The method for the production of MAbs was developed by Kohler and Milstein in 1975 ***(3)*** and led to the development of very specific and sensitive sandwich or two-site immunoassays. The first step is to produce a polyclonal antiserum in a mouse or rat. The lymphocytes are harvested and fused with a myeloma cell line. The cells are then cultured in a medium containing hypoxanthine, aminopterin, and thymidine (HAT medium). Aminopterin blocks the main biochemical pathway for DNA synthesis, whereas hypoxanthine and thymidine provide substrate for an alternative salvage pathway. The myeloma cells lack the salvage pathway and die and the lymphocytes are short-lived and also die after about 7 d. The remaining cells are the hybrid cells where the alternative biochemical pathway for DNA and RNA synthesis has been inherited from the original lymphocytes and immortality has been inherited from the myeloma cells. The surviving cells are diluted, plated out, and grown on in microtiter plates. The medium is tested for antibody, and where present, the individual clones can then be plated into microtiter plates and grown further. These cell lines will be from a single original hybrid cell and will produce one type of antibody recognizing a single epitope on the antigen. These wells are further screened to assess antibody quality and the chosen wells can be investigated further or cryopreserved for later investigation. Full details of monoclonal preparation and cryopreservation may be found in **ref**. ***4***.

2. Materials

2.1. Preparation of Steroid–Albumin Conjugate

1. Dioxane (VWR International Ltd, Lutterworth, UK).
2. Histamine (Sigma-Aldrich Company Ltd, Dorset, UK).
3. 1 *M* Hydrochloric acid.
4. Isobutylchloroformate (VWR).
5. 1 *M* Sodium hydroxide.

6. Steroid-3-conjugate, e.g., testosterone, cortisol and other steroids with a 3 carboxyl group (Steraloids Inc, Newport, RI).
7. Tri-*n*-butylamine (VWR).

2.2. Polyclonal Antibody Production

1. Freund's adjuvant (Gibco Life Technologies, Paisley, UK).
2. Saline: 9 g sodium chloride in 1 L distilled water.
3. Steroid conjugate, hapten conjugate or any antigen (Steraloids).

2.3. Production of Labeled Antigen or Antibody (Tracers)

2.3.1. Iodination Using Chloramine T Oxidation

1. Chloramine T (Sigma-Aldrich).
2. 0.1 *M* Hydrochloric acid.
3. Peptide/protein.
4. 0.5 *M* Phosphate buffer, pH 7.4: dissolve 57 g disodium hydrogen orthophosphate and 13.4 g potassium dihydrogen orthophosphate into 800 mL distilled water and check pH. Adjust pH if necessary with 0.1 *M* NaOH or 0.1 *M* HCl and make up to 1 L.
5. 0.05 *M* Phosphate buffer, pH 7.4: make up 100 mL 0.5 *M* phosphate buffer to 1 L with distilled water.
6. 0.1 *M* Sodium hydroxide.
7. Sodium iodide.
8. Sodium [125] iodine (Amersham International plc, Amersham, Bucks, UK).
9. Sodium metabisulphite.

2.3.2. Iodination Using Lactoperoxidase Oxidation

1. Hydrogen peroxide.
2. Lactoperoxidase (Sigma-Aldrich).
3. Prolactin (National Institute for Biological Standards and Control, Potters Bar, UK).
4. 0.5 *M* Phosphate buffer, pH 7.4 (*see* **Subheading 2.3.1., item 4**).
5. 0.05 *M* Phosphate buffer, pH 7.4 (*see* **Subheading 2.3.1., item 5**).
6. Sodium [125] iodine (Amersham).

2.3.3. Iodination of Steroids and Other Haptens

2.3.3.1. Iodination of Histamine

1. Chloramine T (Sigma-Aldrich).
2. Histamine (Sigma-Aldrich).
3. 0.5 *M* Phosphate buffer, pH 7.4 (*see* **Subheading 2.3.1., item 4**).
4. Sodium [125] iodine (Amersham).
5. Sodium metabisulphite.

2.3.3.2. Formation of a Mixed Anhydride

1. Dioxane (Sigma-Aldrich).
2. Testosterone-3(*O*-carboxymethyl)oxime (Steraloids).
3. Tri-*n*-butylamine (Sigma-Aldrich).
4. Isobutylchloroformate (Sigma-Aldrich).

2.3.3.3. Conjugation

1. 0.01 *M* Hydrochloric acid: prepared as 10% dilution from 0.1 *M* solution (Sigma-Aldrich).
2. Mixed anhydride prepared from **Subheading 3.3.3.2**.
3. 0.05 *M* Phosphate buffer, pH 7.4 (*see* **Subheading 2.3.1., item 5**).
4. Sodium hydroxide.
5. 0.01 *M* Sodium hydroxide.
6. Toluene.

2.3.3.4. Separation and Purification of Iodinated Product by TLC

1. Ethanol.
2. Glacial acetic acid.
3. Methanol.
4. Silica gel TLC plates ultraviolet absorbing at 254 (VWR).
5. Toluene.

2.3.3.5. Separation and Purification of Iodinated Product by HPLC

1. Acetonitrile (Sigma-Aldrich).
2. High-pressure liquid chromatography (HPLC) column 0.5 × 10 cm C18-ODS column.

3. Methanol.
4. Sodium acetate.

2.4. Separation Methods

2.4.1. Coating of Microtiter Plate Wells and the Inside of Polystyrene Tubes for Solid Phase Assays

1. Carbonate buffer, pH 10.0: 13 m*M* sodium bicarbonate, 6.4 m*M* sodium carbonate.
2. Phosphate-buffered saline (PBS) 0.05 *M*, pH 7.2 with 0.1% bovine serum albumin (BSA).

2.4.2. Separation of Antibody-Bound and Unbound Antigen by a Second Antibody Method

1. Donkey anti-rabbit immunoglobulins serum (Guildhay Ltd, Guildford, UK).
2. 0.05 *M* PBS, pH 7.2 with 0.1% BSA.
3. Polyethylene 6000 (Sigma-Aldrich).
4. Rabbit serum (Guildhay).

2.4.3. Antigen Separation of Tritiated Tracers Using Dextran-Coated Charcoal

2.4.3.1. Preparation of Dextran-Coated Charcoal

1. Charcoal.
2. Dextran.
3. Dextran-coated charcoal: 0.05 g Dextran + 0.5 g charcoal in 100 mL 0.05 *M* phosphate buffer, pH 7.4.

2.4.3.2. Separation With Dextran-Coated Charcoal

1. Dextran-coated charcoal: 0.05 g Dextran + 0.5 g charcoal in 100 mL 0.05 *M* phosphate buffer, pH 7.4.
2. Scintillation fluid: Optiphase Hisafe 3 (EG and G Ltd, Milton Keynes, UK).
3. Scintillation vials (LIP Ltd, Shipley, UK).

3. Methods

3.1. Preparation of Steroid-Albumin Conjugate

1. Place a stoppered conical glass test tube in water at 10°C.
2. Add 5–10 mg steroid 3-carboxymethyloxime, 15 µL tri(m)butylamine and 300 µL dioxane.
3. Shake gently to dissolve steroid.
4. Add 5 µL isobutylchloroformate.
5. React for 20 min, shaking periodically.
6. To a small (25-mL) conical flask, add 20 mg human albumin in 2 mL distilled water:dioxane (1:1).
7. Place the flask on ice and cool to 8°C.
8. Add the reactants in the conical test tube to the conical flask and stir for 4 h (*see* **Note 1**). (After 1 h add 25 µL 1 *M* sodium hydroxide.)
9. At the end of 4 h dialyse overnight against distilled water.
10. Precipitate the protein conjugate by slowly adding very small drops of 1 *M* HCl (*see* **Note 2**).
11. Stand overnight at 4°C.
12. Centrifuge, pour off supernatant, and freeze dry.
13. Confirmation of conjugate can be carried out spectrophotometrically.

3.2. Polyclonal Antibody Production

There are a number of different methods that have been used to produce polyclonal antibodies. Subcutaneous, intradermal, intralymphatic, intrasplenic, intramuscular injections, and injections into the footpad *(5)* have been used. In the United Kingdom, regulating bodies tend to give permission for subcutaneous injection only unless there are very good reasons for trying a different route. The following procedures have been used in this laboratory to prepare a number of steroid and peptide antibodies, but modifications (given in the notes) may be required to meet local licensing requirements for animal work.

1. Dissolve 500 µg to 1 mg steroid conjugate (100 µg peptide) in 1 mL saline (*see* **Note 3**).
2. Add 1 mL Freund's complete adjuvant (*see* **Note 4**).
3. Mix on a vortex mixer or by some other means to produce a stable emulsion.

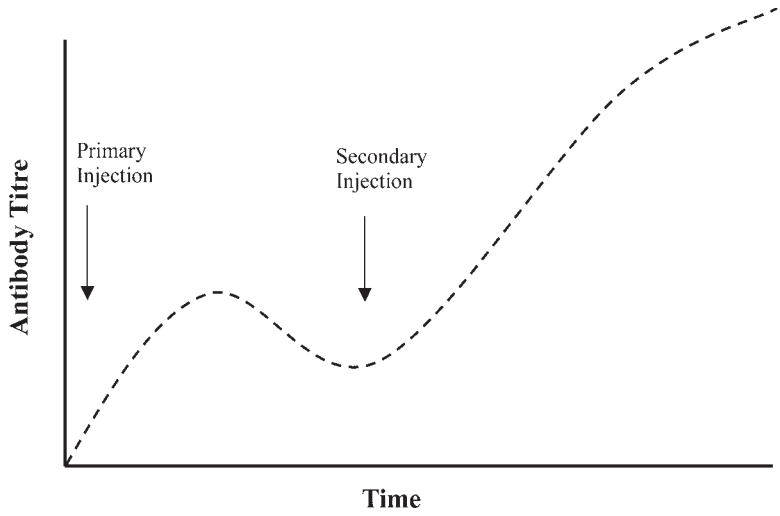

Fig. 1. A diagram showing the trend in antibody titre for a hormone anti-serum raised in a rabbit.

4. Inject subcutaneously along the back of the rabbit into about 12 sites (*see* **Note 5**).
5. Repeat this procedure after 4 wk substituting Freund's incomplete adjuvant for the Freund's complete adjuvant.
6. Take a small amount of blood from an ear vein after a further 4 wk for testing (*see* **Note 6**).

A diagrammatic graph is shown in **Fig. 1** of typical antibody production measured as titer. Antibody has to be tested for titer (the number of antigen binding sites available), binding affinity (how tightly antigen is bound), and specificity. As this chapter is restricted to reagent and assay development, the reader is referred to two general immunoassay books that fully describe the assessment of immunoassay performance *(6,7)*.

Polyclonal antibodies can be produced in amounts that are far in excess of the needs of the individual researcher. For example, 1 mL of a modest antibody that can be used at a dilution of 1/10,000 will, using 100 L per tube, be sufficient for 100,000 tubes and would last almost 4 yr if one were to carry out an assay of 100 tubes every working day of the year. It is relatively easy to collect up to 10 mL of blood by ear

bleed from a rabbit or about 60 mL by exsanguination. Nevertheless, this production is an insufficient amount for commercial requirements. Polyclonal antibodies can also suffer from lack of specificity because antibodies to all the epitopes on an antigen will be present. Some of these epitopes will be common to other similar antigens and will result in cross-reaction with these other antigens. An example is polyclonal antibodies to luteinizing hormone that crossreact with chorionic gonadotrophin, follicle-stimulating hormone, and thyroid-stimulating hormone owing to the presence of a common α subunit. The degree of crossreaction and the binding affinity is unpredictable because this will vary between the animals used to raise the antibody as well as between different bleeds from the same animal.

3.3. Production of Labeled Antigen or Antibody (Tracers)

The ideal tracer will be a homogeneous preparation that has the same affinity between antibody and antigen as the unlabeled substance. Competitive immunoassays are generally used for small molecules such as drugs and steroids. In such assays, a labeled form of the substance is prepared. Although steroids can be labeled directly with radioactive iodine, the product has poor affinity for the antibody and is unstable. Steroids and other small molecules are usually labeled by first producing a reactive bridge to which the label (iodinated amine, enzyme, and others) can be attached. The most common intermediate products are carboxymethyloximes and hemisuccinates. The former can be attached through a carboxyl group, whereas the latter is usually linked through a hydroxyl group. Enzymes and fluorescent and chemiluminescent substances can then be attached through this bridging structure. In the case of iodinated tracers, histamine or tyramine is iodinated first and then attached to the bridge structure. The chemical bridge increases the distance between the substance and the attached label, thus reducing inhibition of antibody binding.

Peptides and proteins can be labeled by direct methods because of the availability of reactive tyrosine groups in most of them. The most common methods are: (1) Chloramine T (*N*-chloro-*p*-toluenesulphonamide), (2) Lactoperoxidase, (3) Iodogen, and (4)

the Bolton-Hunter reagent (5-[*p*-hydroxyphenyl]propionic acid *N*-hydoxysuccinimide ester).

3.3.1. Iodination Using Chloramine T Oxidation

The iodination reaction may be carried out in a small (LP3) polystyrene tube. After each solution is added, the reactants should be mixed by gently flicking the end of the tube.

1. Add 10 µL iodine-125 (37MBq), 10 µL 0.5 M phosphate buffer, pH 7.2 (*see* **Notes 7** and **8**), 2 µg peptide in 10 µL 0.05 *M* phosphate buffer, pH 7.2 and 10 µL chloramine T in 0.05 *M* phosphate buffer (1 mg/mL).
2. Mix for 10 s.
3. Then add 10 µL sodium metabisulphite in 0.05 *M* phosphate buffer (4 mg/mL) and 200 µL potassium iodide in 0.05 *M* phosphate buffer (10%).

The iodinated peptide can then be separated from free iodide by running down a column of Sephadex. Where there is only one form of peptide a 1 × 10-cm column of Sephadex 50 or Sephadex 100 may be used. Where the peptide exists as, or breaks down during the labeling process to, other isoforms, longer columns are required using Sephadex 200. **Figure 2A** shows the elution profile of radioactivity from 0.5 × 10-cm column of Sephadex G100 after chloramine iodination of LH.

3.3.2. Iodination Using Lactoperoxidase Oxidation

Chloramine T is a powerful oxidizing agent and may cause degradation of the protein molecule. Therefore, a more gentle procedure may be required. Hydrogen peroxidase may be used as oxidizing agent for the iodination of prolactin. Again, the reaction may be carried out in a small (LP3) polystyrene tube, mixing after the addition of each solution.

1. Add 10 L 0.5 *M* phosphate buffer, pH 7.2, 5 µL iodine-125 sodium (18.5 MBq), 2 µg prolactin in 10 µL 0.05 *M* phosphate buffer, pH 7.2, 10 µL lactoperoxidase (1 mg/mL in 0.05 *M* PBS, pH 7.2) and 10 µL

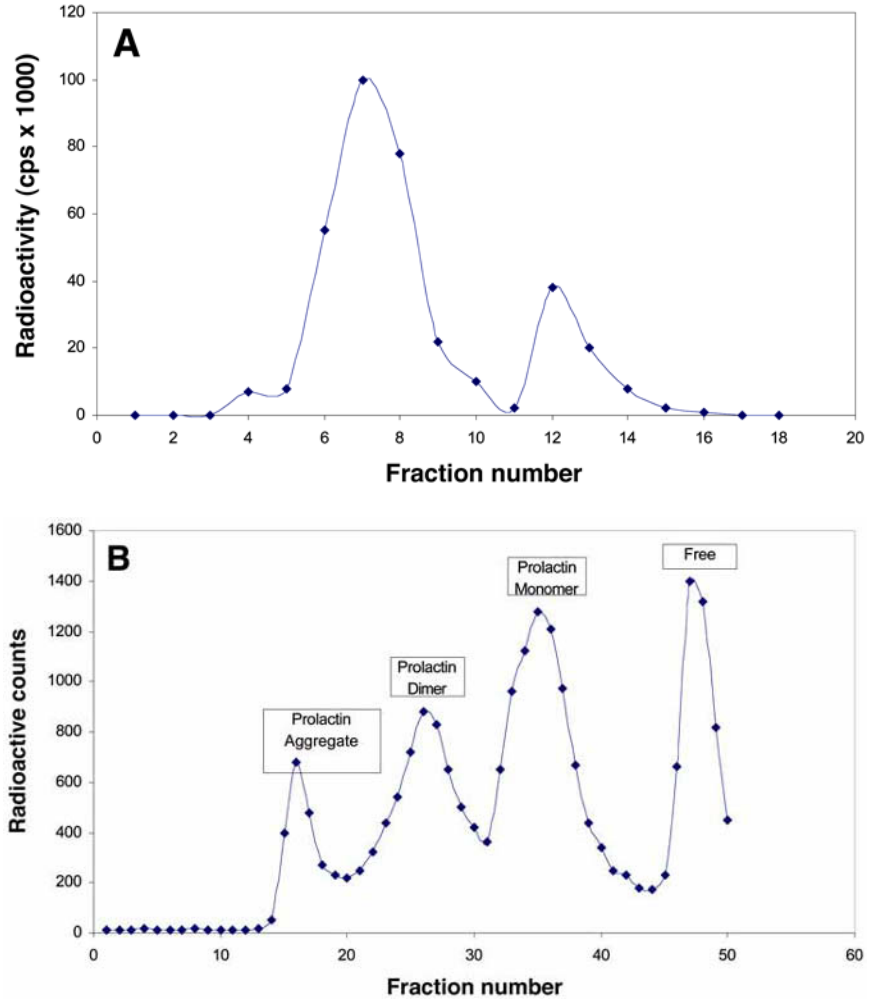

Fig. 2. **(A)** Radioactivity profile from a 0.5 cm × 10 cm Sephadex G100 column following iodation of LH. **(B)** Profile from a G200 column of radioiodine after the iodination of prolactin.

hydrogen peroxide solution (1:12,000 dilution of 30% solution in distilled water) (*see* **Notes 7** and **8**).

2. Cap the reaction tube, mix gently, and incubate for 5 min at room temperature.
3. Add 10 µL hydrogen peroxide solution and incubate for a further 5 min.

4. Separate the iodinated products (monomer, dimer, aggregate, and iodinate enzyme on a 1.5 × 500-cm Sephadex G200 column. A separation profile is shown in **Fig. 2B**.

A number of different procedures have been published. Coupling the lactoperoxidase to cellulose particles allows the iodinated enzyme to be removed by centrifugation.

Iodogen, available commercially, is also a gentle oxidizer, but we have found that it is not very efficient for amounts of less than 10 g protein. Some proteins are deficient in tyrosine and histidine, which are iodinated in the above reactions, and so cannot be iodinated by the methods described previously. Bolton and Hunter developed a method using 3-(*p*-hydroxyphenyl) propionic acid *N*-hydroxysuccinimide ester that is iodinated with chloramine T. The ester is added to a solution containing the polypeptide and combines with lysine residues or the N-terminal.

3.3.3. Iodination of Steroids and Other Haptens

The addition of a radioactive iodine atom to a hapten is more complex. Although there have been a number of published methods for the direct incorporation of an iodine molecule into the structure of a hapten, such as a steroid, these have not been very successful. This is because the iodine molecule is one-third of the size of the whole hapten and causes disruption of its structure. This makes the molecule unstable as well as sometimes not being recognized by the antibody. Therefore, the procedure with haptens is to label histamine or tyrosine and then attach it to the hapten via a chemical bridge. The following method describes the iodination procedure we have used for many years for the iodination of testosterone, cortisol, and other steroids having a 3 carboxyl group and is based on the method of Nars and Hunter ***(8)***.

3.3.3.1. Iodination of Histamine

The reaction should be carried out in a glass-stoppered conical pyrex tube behind appropriate shielding. After the addition of each

solution, the reactants should be mixed by gently flicking the bottom of the tube.

1. Add 10 µL histamine (2.2 g) in 0.5 *M* PBS, pH 7.2, 10 µL sodium iodide-125 (37 MBq) and 10 µL chloramine T (5 mg/mL water).
2. Incubate for 30 s at room temperature.
3. Add 10 µL sodium metabisulphite (30 mg/mL).

The iodinated peptide can then be separated from free iodide by running down a column of Sephadex. Where there is only one form of peptide a 1 × 10-cm column of Sephadex 50 or Sephadex 100 may be used. Where the peptide exists as, or breaks down during the labeling process to other isoforms, longer columns are required using Sephadex 200. **Figure 2A** shows the elution profile of radioactivity from a 0.5 × 10-cm column of Sephadex G100 after chloramines iodination of LH.

3.3.3.2. Formation of a Mixed Anhydride

The reaction should be carried out in a glass-stoppered conical pyrex tube behind appropriate shielding. After the addition of each solution, the reactants should be mixed by gently flicking the bottom of the tube.

1. Add 100 µL testosterone-3(*O*-carboxymethyl) oxime, 10 : tri-*n*-butylamine in dioxane (1:5) and 10 µL isobutylchloroformate in dioxane (1:10).
2. Incubate at 10°C for 20 min frequent mixing.
3. Add 3.45 mL dioxane and place the tube in ice.

3.3.3.3. Conjugation

1. Add 50 µL of the mixed anhydride solution to the iodination mixture followed by 10 µL 0.1 *M* NaOH.
2. Incubate in ice for 1 h with occasional mixing.
3. After 1 h, add 10 µL 0.1 *M* NaOH.
4. Incubate in ice for a further hour with occasional mixing.
5. Add 1 mL 0.1 *M* HCl followed by 1.0 mL toluene.
6. Mix for 1 min on vortex mixer.

7. Remove and discard the solvent layer.
8. Add 1.0 mL 0.1 *M* NaOH and 1.0 mL 0.5 *M* sodium phosphate buffer, pH 7.2.
9. Add 0.5 mL toluene (*see* **Note 9**) and carefully mix on a vortex mixer for 1 min to extract the iodinated steroid conjugate.
10. Remove the toluene layer into a clean conical glass tube.
11. Repeat last step and combine the toluene extracts.

3.3.3.4. Separation and Purification of Iodinated Product by TLC

1. Reduce the volume of the extract to approx 250 µL under nitrogen or air.
2. Add a solvent mixture of toluene:methanol:glacial acetic acid (75:24:1) to a TLC chromatography tank.
3. Draw an origin line on a 5 × 20-cm silica gel plate.
4. Carefully streak the reduced extract along the origin, using about 20 µL at a time, and dry with an air or nitrogen stream.
5. After the TLC tank has equilibrated for about 1 h add the TLC plate.
6. When the solvent front has reached to about 1 cm from the top of the plate, remove the plate and air dry.
7. Place the dry TLC plate on top of an X-ray film for about 30 min (*see* **Note 10**).
8. Develop X-ray film and locate radioactive band on TLC plate.
9. Scrape off the silica gel carefully onto a large no. 1 Whatman filter paper that has been folded down the middle.
10. Fold the filter paper and tip the silica gel into a stoppered conical tube.
11. Add 5 mL ethanol and mix for 1 min.
12. Place the tube in a lead container and allow to settle in a suitable refrigerator overnight.
13. The next day the radioactivity in the solution can be assessed by counting a small aliquot in a γ-counter in order to determine the specific activity and the concentration required for an assay.

Most steroids made in this way are stable for several months. Small amounts may be taken, dried down, and buffer added to give a working solution for radioimmunoassay (RIA).

3.3.3.5. Separation and Purification of Iodinated Product by HPLC

1. Take the toluene extract to dryness under nitrogen.
2. Add 100 µL column solvent mixture (*see* **Note 11**).

3. Run on a lead-shielded 0.5 × 10 cm C18-ODS reverse phase column using the same solvent mixture as the mobile phase.
4. Collect 1 mL fractions.
5. Count the fractions in a γ-counter and, if necessary, pool the fractions in the first radioactive peak that indicates the iodinated hapten.
6. Take about 10 µL and count the radioactivity to determine the specific activity and the amount to be used in an assay.

Enzyme, luminescent- and chemiluminescent-labeled hormones may be prepared in a similar way to iodinated tracers with only slight modifications in some instances ***(9)***. These methods were developed in the early days of immunoassay and their simplicity and robustness have withstood the test of time. Little detail of the methods used is given in current texts and one has to go back to early publications to search these out ***(10,11)***.

3.4. Separation Methods

Once the antigen and antibody have reacted, the mixture contains both bound and unbound antigen and antibody. In order to determine the amount of antigen bound to the antibody it is necessary to separate the bound and unbound moieties ***(12)***. Nonspecific methods, such as adding ethanol or ammonium sulphate solution, have been used but these methods give rise to high nonspecific binding that reduces the sensitivity of the assay. More specific methods are preferred for routine methods. Antibodies can be attached to solid surfaces, such as a coating on the bottom of the polystyrene reaction tubes or microtiter plates, plastic beads, or on cellulose particles. Commercial methods frequently used magnetized particles. The advantage of the latter is that these are held in suspension during the reaction providing shorter incubation times and can be quickly pulled out of the mixture by applying a magnetic force either at the bottom or sides of the reaction tube. The solution can then be aspirated and the particles thoroughly washed. During the wash step the magnet is moved away from the tube during addition of the wash solution and reapplied so that the solution can be aspirated. Several wash steps may be used to reduce the nonspecific binding to a minimum. A method for coating the inside of a polystyrene reaction tube, microtiter well, plastic particles, or beads is described.

3.4.1. Coating of Microtiter Plate Wells and the Inside of Polystyrene Tubes for Solid-Phase Assays

1. Add antibody diluted in carbonate buffer pH 10.0 (*see* **Note 12**) to the microtiter plate wells and incubate at 4°C overnight.
2. Aspirate the liquid, add 0.1% BSA 0.05 *M* PBS pH 7.2 and then leave at room temperature for 1 h.
3. Aspirate to dryness before washing with 0.05 *M* phosphate buffer pH 7.2
4. Aspirate to dryness.
5. Place the microtiter plate in a plastic bag, preferably with a small amount of silica gel, and store in a refrigerator for no more than 24 h.

3.4.2. Separation of Antibody-Bound and Unbound Antigen by a Second Antibody Method

Another simple separation method is the double antibody method. After the initial reaction, a solution is added containing a second antibody to the first antibody. The solution also contains nonspecific serum of the animal where the first antibody was raised. For example, if the method uses a rabbit anti-hormone antibody, the separation solution contains an antiserum to rabbit immunoglobulins plus rabbit serum. The anti-rabbit immunoglobulin antibody will bind to the rabbit anti-hormone antibody and to rabbit immunoglobulins in the rabbit serum. As the second antibody can bind more than one first antibody molecule a protein matrix forms that precipitates out. This usually takes about 12 h but the precipitation can be speeded up by adding the second antibody and rabbit serum in an 8% solution of polyethylene glycol 6000. Precipitation is almost instantaneous but is probably best left for about 20 min. The precipitate is centrifuged down. In the case of iodine tracers the supernatant is aspirated and the amount of radioactivity in the precipitate is determined in a gamma scintillation counter. For enzyme tracers the precipitate can be resuspended in a substrate solution and the color determined.

When a second antibody method is used the amounts of second antibody and animal serum have to be optimised to give maximum formation of the protein matrix. Poor matrix and thus poor precipitation occurs

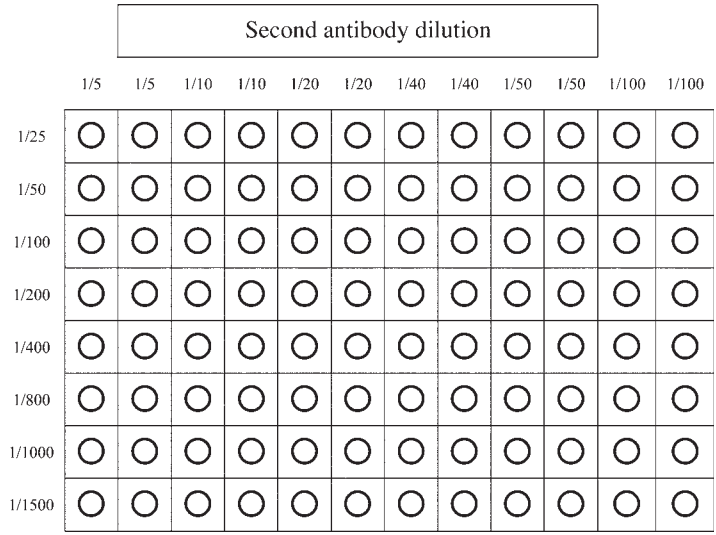

Fig. 3. Arrangement of tubes and dispensing for optimizing the second antibody and nonimmune animal serum.

if either the antigen or the second antibody is in excess. Optimum conditions may be determined in the following way and is described for a simple RIA using a rabbit polyclonal antiserum to a hormone.

1. Prepare solutions of anti-rabbit immunoglobulin antiserum in 8% polyethylene glycol (PEG) solution: typically 1/10, 1/25, 1/50, 1/100.
2. Prepare solutions of non-immune rabbit serum in 8% PEG solution: typically 1/100, 1/250, 1/500, 1/1000.
3. Label 32 reaction tubes and set up as in **Fig. 3**.
4. Add 100 µL assay buffer (0.1% BSA in PBS 0.05 *M*, pH 7.2), 100 µL tracer solution to give 10,000 cpm and 100 µL anti-hormone antibody to all tubes.
5. Add 100 µL tracer solution to two tubes, cap, and put to one side (total count tubes).
6. Mix and incubate overnight at 4ºC.
7. Add 250 µL each rabbit serum dilution to two columns of tubes as shown in **Fig. 3**.
8. Add 250 µL each second antibody solution across each row as shown

in **Fig. 3**.

9. Mix the tubes and incubate for 20 min.
10. Centrifuge for 30 min at 1500*g*.
11. Decant or aspirate the supernatant.
12. Count the radioactivity in the precipitates and calculate the percentage binding of tracer.
13. Determine which tubes contain the maximum amount of radioactivity and note the dilutions of second antibody and rabbit serum used. If this was rabbit serum at a dilution of 1/500 and second antibody at 1/25, then for the routine assay 500 µL PEG solution, containing rabbit serum at 1/1000 and second antibody at 1/50, would be added to each tube at the separation stage.

Once the optimum conditions have been found further experiments can then be carried out on first antibody and tracer concentrations and first incubation times in order to optimize the assay for sensitivity and precision. Therefore, in the development of any immunoassay it is important to have optimized the separation step first.

3.4.3. Antigen Separation of Tritiated Tracers Using Dextran-Coated Charcoal

Some steroid assays use tritiated tracers. These may be bought commercially and obviate the need to prepare iodinated tracers that require special facilities. Tritiated tracers may also be available for other haptens.

3.4.3.1. Preparation of Dextran-Coated Charcoal

This may be carried out in a large glass or plastic beaker.

1. Wash the charcoal to remove fines by adding distilled water (about five times the volume of charcoal) to several grams of charcoal.
2. Allow to settle for 60 min.
3. Aspirate most of the water from the top of the charcoal.
4. Repeat the **step 1** at least five more times.
5. On the final occasion, aspirate the water from the top of the charcoal as completely as possible and dry the charcoal in an oven at 100ºC.

6. To 100 mL assay buffer (*see* **Note 13**) add 0.5 g charcoal and 0.05 g dextran.
7. Store the reagent at 4ºC and always add to the assay at 4ºC while keeping the charcoal in suspension by gentle mixing on a magnetic stirrer.

3.4.3.2. Separation with Dextran-Coated Charcoal

1. 500 µL dextran-coated charcoal is added to each tube.
2. The reaction tubes are incubated at 4ºC for 15 min (*see* **Note 14**).
3. The supernatant is poured into a scintillation vial and scintillation fluid added.
4. The radioactivity in each tube is counted in a beta scintillation counter.

This is a very brief account of the methods used in immunoassay development, but it provides sufficient information for setting up a basic immunoassay. However, further development work is required to achieve an assay that fulfills the need for a sensitive and precise method. Development includes investigating different volumes of reagent in the assay system, the concentration of reagent constituents, the need for a wash step, the temperature for the reaction, and the length of incubation times. There have been several books written on immunoassay techniques over the years that will give many references although the older books give more detail of the methods ***(6,7,10,11)***.

4. Notes

1. The method adopted to keep the reactants at the right temperature and to mix continuously was to place the flask at 45°C in a beaker of iced water. A small flea could be placed in the liquid and the whole apparatus placed on a magnetic mixer. Extra ice was added to the iced water at intervals to keep the reactants cool.
2. This step has to be carried out with great patience and care as the end-point, when the conjugate precipitates, is very sudden. If too much acid is added the precipitate goes back into solution. The pH is just under 7.0. The precipitate is usually fairly fluffy in appearance, but if a fine precipitate forms it is better to continue, but retain the supernatant after centrifugation for further manipulation.

3. Smaller volumes can be taken. We use a total volume of about 500 µL when we carry out intradermal injections. In this case the back of the animal has to be shaved first.
4. In the United Kingdom, the use of incomplete adjuvant is preferred by the regulatory body.
5. For intra-dermal injection we inject at about 30 sites along the back.
6. Some researchers advocate taking some blood after the first 4 wk to check for antibody production before the second injection. This is to save injecting precious material into an animal that is not producing antibodies. We have found this unnecessary.
7. Sodium iodide I^{125} is provided in alkaline solution.
8. The higher molarity buffer neutralizes this solution before analyte is added. The 30% stock solution should be stored at 4°C for no longer than 1 yr.
9. Toluene may be used for the less polar steroids such as testosterone, but ethyl acetate is used for polar steroids such as cortisol.
10. To position the TLC plate after the X-ray plate has been developed, punch two holes with a pin through the paper envelope of the X-ray holder one at each of the two bottom corners, and one hole at the top center of the plate. This allows a small amount of light through that produces three black spots when the plate is developed.
11. For testosterone we use isocratic separation using acetonitrile:water 1:1; for progesterone acetonitrile:sodium acetate 1:4.
12. The antibody dilution used in this laboratory has been between 1/2000 and 1/10,000 found by experimentation.
13. The concentrations and solutions described here have been used in many assays by many centers, but variations occur. Some researchers have described assays where water is used in place of buffer and others have not used dextran.
14. These are typical conditions to reduce stripping of the antigen from the antibody. This occurs if the reactants are kept too long in the presence of the charcoal. Optimum conditions may be found by incubating the charcoal suspension in a blank tube (tracer only) and a zero tube (tracer plus antibody solution) for increasing periods of time. The counts in the blank will increase as stripping occurs.

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2

High-Performance Liquid Chromatography for Hormone Assay

John W. Honour

Summary

High-performance liquid chromatography (HPLC) is a refinement of traditional column chromatographic techniques. The speed of analysis and the resolution are increased with new column-packing materials and eluant pumped through the column at high pressure. The potential for achieving measurements of hormones in small volumes of plasma or urine is limited, both in normal and pathological situations. Using HPLC with ultraviolet absorption, the detection limit is only nanogram amounts of hormones per milliliter of blood serum. The applications of the technique to specific hormones from recent and older literature will be used throughout this chapter to illustrate aspects of the technology.

Key Words: HPLC; UV absorption; reproductive steroids; andrenal steroids; thyroxine; insulin; angiotensins; melatonin; neuropeptides; endorphins; renin.

1. Introduction

High-performance liquid chromatography (HPLC) is often used for purification of samples prior to an indirect detection technique such as immunoassay (IA). Metabolic studies are also performed using HPLC to resolve the metabolic products. Some HPLC meth-

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ods come close to standards for reference technology. For peptide hormones the major uses of HPLC have been for purification, qualitative analysis, and structural determination. Clinical studies are sometimes dependent on indirect measurements when, for example, monitoring hormone treatment. Therefore, in insulin-treated diabetics, management of patients has been improved by monitoring glycated haemoglobins in blood samples, separated by HPLC with reverse phase (RP) and cation exchange. The quantitative analysis of hemoglobin A1C is used as a marker of insulin action and glucose control in those patients ***(1)***. With the introduction of new technologies for producing hormones, the testing of pharmaceutical preparations for purity is an area where HPLC has been particularly useful.

HPLC analysis of hormones in biological fluids has to compete in the laboratory with IA that is capable of measuring hormone concentrations rapidly in numerous small samples (10–50 μL) of body fluid equivalent to as little as 10 pg/mL. There are diurnal variations in the concentrations of hormones in blood as well as pulsatile secretory patterns. Several samples may need to be taken to interpret results with time. Measurement of a hormone concentration in plasma provides insight to the tissue exposure of the hormone. Plasma is therefore a more popular medium for analysis than urine. The latter still provides an important source of information because the steroid content of a 24-h urine collection is an integer of the daily production.

HPLC is an important chromatographic technique for hormones for several reasons.

1. High temperatures are not required.
2. Material can be recovered from the column eluates for further analytical procedures.
3. Resolution is superior to TLC and paper chromatography.
4. HPLC offers the potential and versatility for separation of different forms of hormones such as free and conjugated steroids, pro-hormones, hormones, and peptide fragments.
5. The range of detectors. UV absorption is the most useful of current detectors, other hormones can be detected with a refractive index or an electrochemical detector.

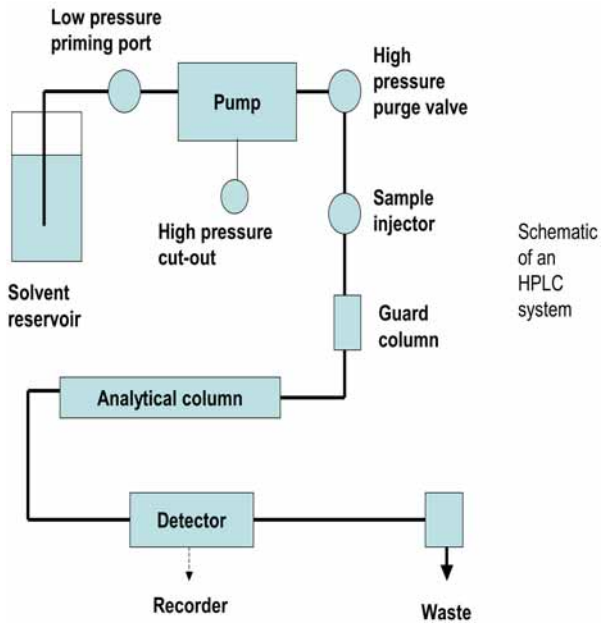

Fig. 1. Schematic of an HPLC system for hormone analysis.

2. Materials

Figure 1 is a block diagram of a typical HPLC system. The mobile phase is contained in the solvent and is delivered to the column through the action of a pump that controls eluant flow at up to 6000 psi. Gradient elution of the column may require more than one reservoir, pump, and eluting reagents. The flow of the separate pumps is regulated to generate a varying mixture of the eluting reagents with ability to have gradients of different shapes as part of the computer-controlled package. There are two main types of pump, the syringe and reciprocating pump. The most popular applications use constant flow of column eluant. The advantage of syringe pumps is that the flow is pulse free, this is particularly important with electrochemical detectors. A pressure display is important to monitor the outlet pressure. Changes in pressure indicate blockages or declining column conditions. The column is placed between the column injector and the detector. A system may also include provision for pre- or post-

column reactions (derivatization). The detector then couples to a recorder or back to the computer operating/data management.

2.1. Columns

Separation in HPLC is effected as components move in the liquid mobile phase through interactions with the packing of the column. Typically columns are 10–25 cm in length and less than 5 mm in diameter. Porous, silica-based packing materials, of 3–40 μ*M* in particle size, of rigid material can withstand high pressure. Sinter disks (frits) are used to cap the ends of the column and act as filters. The packings can be coated with a stationary phase or be chemically bonded. The separation of hormones with HPLC can be effected by absorption, partition, ion-exchange, RP, and RP ion-pair chromatography.

High-performance silica and alumina columns give excellent separation of steroids, but are less popular these days. RP phases with C_{18}, C_8, C_2, and phenyl substitution have been used. The chromatography depends largely on partition so that separation will vary with the carbon chain length and the nature of the mobile phase. The size and consistency of the porous packing will also affect analytical performance. RP columns that have 60,000 or 80,000 theoretical plates per meter offer excellent resolution and sharp peaks permit detection to around 1 ng of many hormones (*see* **Table 1**) ***(2–14)***.

Cyano and amino phases have been used to effect separation of corticosteroids ***(15)*** and oestrogens ***(16)***. HPLC of polar oestrogens has been achieved on ion-exchange columns ***(17)***. Size exclusion analysis has been used for the separation of insulin and its degradation products ***(11)***. Micro bore columns of less than 2 mm id may permit increased sensitivity by narrowing the elution peak unless the volume of sample and the total mass of material in the extract cause a loss of peak shape and resolution. Antibodies to thyroxine (T4) were purified and subjected to proteolytic degradation. The resulting peptides were purified using microbore columns ***(18)***.

Naturally occurring mixtures of steroid hormones have a wide range of polarities, but steroids of similar polarity, that may be

Table 1
Reverse-Phase HPLC for Hormones

Hormone	Source	Reference
Gonadotrophin surge-attenuating factor	Ovarian follicular fluid	*2*
Corticotrophin-releasing hormone and POMC peptides	Human skin	*3*
POMC and ACTH peptides	Skin	*4*
α-melanocyte stimulating hormone	Phaeochromocytoma tissue	*5*
Recombinant IGF-1		*6*
Calcitonin	Incubation with enzymes and gut mucosa	*7*
Anabolic steroids	Urine	*8*
Proinsulins	Rat pancreas	*9*
Insulin and related peptides		*10*
Insulin	Pharmaceutical preparation	*11*
25-hydroxy-vitamin D	Serum	*12*
25-hydroxy-vitamin D	Serum	*13*
Arginine vasopression	Pharmaceutical preparation	*14*

derived from different metabolic pathways, tend to elute in clusters. Careful selection of the stationary phase from the range of commercially available products can enable a system to be devised with high selectivity *(19,20)*. Silica packings, bonded to octadecyl or diol groups, are the most popular for general use. Supports differ in particle size, porosity, and levels of residual-accessible silanol groups. Synthetic polymers may be more inert than silica. The physical characteristics of packings have been studied with various solvent gradients. The resolution and elution order of steroids has been reported in a number of publications. There seems to be no easy means, however, to identify the most suitable packing for a particular separa-

tion. Selective differences cannot be firmly attributed to alkyl chain length or to shape of the packing.

Supports can have variable and often incomplete coverage of residual silanol groups, which affects separation, peak shape, and recovery. Some packings with uncapped silanol groups are chemically reactive with hormones owing to intramolecular hydrogen bonding. This leaves the phase acidic and may explain the instability of certain hormones. Aldosterone and 18-hydroxylated steroids are susceptible to a number of reactions on certain columns that can influence the quality of the HPLC result. Acid residues, such as residues found on uncapped HPLC supports, can lead to ring closure of such steroids with a bridge of C-18 to C-20 or C-21. In the presence of methanol this may lead to formation of methyl ethyl ketals. Other products, dimers, and isomers are possible leading to a number of peaks in the HPLC analysis of a single compound. These products can have retention times spread throughout a solvent gradient elution of a RP column. This may be disastrous in interpretation of a metabolic study unless products are characterised by other means. Such supports are not recommended for aldosterone and related steroids, e.g., 18-hydroxycorticosterone ***(21)***. The extent to which a packing is not covered can be determined by a methyl red absorption test ***(22)***.

Porous graphitic carbon is a new micro-crystalline packing material for RP chromatography that is chemically stable to strong alkali and acid. As it contains no unreacted silanol groups commonly found on silica based RP materials it is considered to be a pure RP adsorbent and may overcome some of the difficulties of silica-based columns described previously. Corticotrophin-releasing factor (CRF) and gonadotrophin-releasing hormone (GnRH) were among the hormones purified with columns of high carbon content ***(23)***.

Very nonpolar material will accumulate on an RP column and decrease separation but this can be reduced by the use of a guard column (30–70 mm in length) containing a larger (30-µm particles) pellicular equivalent of the analytical column. In an assay for simultaneous assay of cortisol, prednisone, and prednisolone a C8 guard column was used before the C18 analytical column ***(24)***. Guard columns are cheap and can be dry-packed. The first few millimeters of packing from the analytical column can also be replaced at intervals.

2.2. Mobile Phases

Useful separations of hormones can be achieved using chromatography on silica with isocratic (fixed) mixtures of two (binary) solvents *(25–27)*. RP columns eluted with polar binary solvent mixtures, usually methanol or acetonitrile with water, are now used widely for separations of hormones. The polarity of the solvent is an important factor in the separation of molecules using HPLC. The elution ability of a solvent is reversed with RP-HPLC, the more polar the phase, the longer the retention time. Other factors need to be considered. The lower the viscosity of a solvent the lower the pressure needed to operate the column. Solvents and any impurities they contain may affect the detection system (particularly the UV absorption). Solvents such as ethers form unstable peroxides when exposed to oxygen and may need stabilisers to combat this. Methyl-*t*-butyl ether is a useful substitute for diethyl ether. Tetrahydrofuran is a good solvent for higher molecular weight analytes. The eluting solvents should be degassed to avoid bubbles in the low pressure of the detector and filtered before use. Mobile phases need careful preparation. Aqueous components need to be adjusted for pH.

The separation of a range of related hormones is best achieved with gradient elution. Additional pH, ion-pair, and modifier effects can be incorporated. Retention times are reproducible between runs provided that the column is equilibrated to the starting solvent mixture. Methanol/water gradients effect the separation of the major adrenal steroids. Dioxane is a better choice for the separation of polar adrenal steroids and acetonitrile is preferred for resolving testicular steroids. Peak shape and resolution can be improved by maintaining the column at a fixed temperature above ambient, e.g., at 45–60°C when analyzing steroids, but this could lead to degradation of peptide hormones. At higher temperatures, the eluent viscosity is reduced *(28)*. If working at ambient temperature it is advisable to have a room with well-controlled temperature to achieve reproducible retention times.

The use of three and four solvents in a mobile phase system has overcome the difficulties in choosing the appropriate column pack-

ing for a particular steroid separation. Systematic, statistical procedures for solvent optimisation have been developed *(29–32)*. Derks and Drayer *(33)* reported the separation of very polar 6α and 6β hydroxylated metabolites of cortisol by isocratic elution from a silica column with water:chloroform:methanol. A computerized system for optimization of conditions for RP-HPLC with respect to temperature and gradient steepness has been tested for a number of compounds, including corticosteroids and androgens *(34)*.

Phosphate can be incorporated into the mobile phase *(35)* and, with this system, the buffer anion and pH exert significant effects on the separation *(36)*. Salts are used in the eluting solvent in the analysis of oestrogen conjugates, but this may in the long-term corrode the steel of the columns and tubing. Oestrogens can be effectively separated when silver nitrate is included in the mobile phase to give 2 g of silver nitrate with 60 mL methanol, 40 mL water at 0.55 mL/min *(37)*. To prevent metallic silver building up on the column a water:methanol (50:50 v/v) mobile phase is used each evening to flush excess silver nitrate from the system. Even so, a small build-up can occur that requires a rinse with dilute nitric acid or replacement of the tubing when back-pressure rises.

Steroid conjugates can be separated using ion-pair chromatography. Andreolini et al. *(38)* show excellent separations of oestrogens on RP-18 packings by eluting with a gradient of acetonitrile/methanol and phosphate buffer containing cetyltrimethylammonium bromide. Cyclodextrins have been used for micellar chromatography of steroids with variable success *(39–41)*. Trifluoroacetic acid has been used in eluting solvents for insulin *(42)*, steroids *(43)*, CRF and GnRH *(23)*, and angiotensin II *(44)*.

Gradient elution is usually necessary to elute a series of hormones after extraction from biological fluids. Gradient elution reduces analysis times and depending upon the gradient shape can optimize separation and improve peak symmetry. Nonlinear, stepped, and linear gradients of solvent proportions have been largely dictated by the available facilities for programming the pumps. Flow and temperature programming can also be used. Simultaneous variation in temperature and gradient steepness for RP-HPLC of steroids may

be useful for improved resolution of corticosteroids, but not substituted testosterone compounds *(34)*.

2.3. Sample Injection

The sample injection system is usually a loop on a valve system controlled by opening and closing valves to direct eluant flow in several directions. The sample loop itself can be between 1 and 100 µL. A syringe with the sample, or an automated sample injection system, inserts the sample through a septum. Dead volume is kept to a minimum in the system by using narrow-bore stainless steel tubes with low dead-volume connections.

After extraction the hormones are usually dissolved in the mobile phase. The addition of a suitable macromolecular matrix, e.g., polyethylene glycol to the extracting solvent prior to evaporation can improve the recovery because the hormones may dissolve poorly in the mobile phase alone. Injectors that encompass rubber septa should be avoided. At the high instrument sensitivities, often used for the analysis of hormones spurious and irreproducible peaks, may occur in the chromatogram. These may reflect the action of injected solvents on the septum. Injection valves are, therefore, preferred.

2.4. Detection

The simplest detectors for hormones are single wavelength spectrophotometric detectors. Variable wavelength detectors are now common and very sensitive. Detection cells should have small dead volumes. Diode array detectors provide a repeated UV spectrum of the column eluate and, thus, for a component eluting in a single peak from the HPLC there is precise identification.

Peptide hormones are easily detected by UV absorption of peptide bonds at 210 nm or tryptophan residues at 280 nm. Static/dynamic light scattering has been used to detect insulin *(11)*. Steroid hormones with the α, β unsaturated ketone in the A-ring absorbs UV light with maximum around 240 nm and extinction coefficients 12,000–20,000. Isolated carbonyl groups absorb with a

maximum at 280 nm (275–285 nm) and molar extinction coefficients of 17–155. The natural oestrogens have peak absorption at 280 nm because of the aromatic A-ring and can be detected themselves with sensitivity limits of 100–10 pg/mL. Although steroids can absorb UV below 200 nm, in practice it is difficult to attain a clear signal from noise without a reduction in sensitivity particularly when solvent gradients are used to elute the steroids. With some gradient elution systems it is necessary to correct for baseline variation by comparison of the response of the eluate from the analytical column with the flow of solvent alone through a reference cell. Interference in UV detection of a synthetic progestogen (Medrogestone) by endogenous steroids in HPLC analysis was achieved by reaction with oxalyl chloride and UV detection at 242 nm *(45)*.

Nanogram quantities of steroid hormones can be detected by the use of fluorescence *(46)*, refractive index *(47)*, and electrochemical detectors *(48,49)*. Melatonin has been detected by fluorescence *(50)* and electrochemical detection *(51,52)*. Chemiluminescence has been used for proinsulin and C-peptide *(53)*.

In some cases, it has been necessary to react the hormones in the eluate from the column with reagents to form UV-absorbing derivatives. Post-column derivatization methods are restricted to very fast reactions limiting the scope of application *(46)*. Using HPLC with post-column derivatization melatonin was shown to be unstable in commercial formulations when exposed to light *(54)*.

Most of the urinary steroid metabolites are inert in HPLC detectors. Reactive groups have been utilized in order to make derivatives for spectrophotometric detection. HPLC has, thus, been used to separate individual oxo-steroids after conversion to phenylhydrazone derivatives. 3α-Hydroxysteroids are detected using a dehydrogenase reactor *(55)*. Melatonin in pineal gland extracts were determined by HPLC with pre-column derivatization *(56)*.

Several laboratories have demonstrated the variety and complexity of intermediates and products formed when radioactive steroids are incubated with steroid metabolizing tissues. Flow-through radioactivity detectors have been useful for examining the products of reac-

tions with labeled substrates *(57,58)*. T metabolism in human hair follicles *(59)*, the action of Formestane (a steroidal aromatase inhibitor) in breast cancer patients *(60)*, and dehydroepiandrosterone sulphate (DHAS) transport across the blood–brain barrier *(61)* are recent examples. A number of metabolites of dexamethasone formed in vitro in human liver were found using this approach and later identified by LC coupled with mass spectrometry (MS) *(62)*. The short resolution time of the sample components in the radioactive counting chamber limits sensitivity. Current detection limits for tritium are 10,000 dpm with flow cells incorporating scintillant (around 1% efficiency) to 1000 dpm (50% efficiency) when the column effluent is mixed with liquid scintillant before passing through a flow cell.

The coupling of HPLC with MS is more widely used for hormone assay than gas chromatography (GC)-MS. The coupling of LC with MS necessitates the removal of a solvent mobile phase that can flow at rates up to 2 mL/min. Interfaces that rely on heat for the production of a fine spray of solvent (Thermospray) are now relatively common. The spray is directly heated as the sample passes through a fine capillary leading from the HPLC to the MS. The fine droplets continue to vaporize as they pass into the source. A portion of this vapor and the ions produced in the ion source pass into the mass spectrometer through a sampling cone and the remainder is pumped away by the mechanical vacuum pumps. Direct ion evaporation involves evaporation into the gas phase of ions present in solution. This process is improved if there is a volatile buffer like ammonium acetate in the LC mobile phase. A secondary type of ionization uses a filament to initiate chemical ionization. Thermospray can be used in negative or positive ion modes.

In electrospray, an electric field is used to cause ions to evaporate from solution. Compressed air is used to force the sample from the HPLC through a fine capillary to a jet into the MS. This system operates at ambient temperatures and ionization is gentler than with thermospray. The combination of micro-bore column HPLC with thermospray or electrospray MS seem to be the most promising systems. Spray interfaces are used mainly with quadrupole MS. A disadvantage of mild ionization is in the lack of fragmentation that

reduces specificity of the technique as a detection system. Increased structural information can be obtained if molecular ions (or fragment ions) are induced to fragment further by collision induced dissociation in a collision cell placed between two MS systems (tandem MS). LC-MS is now used for a range of hormones, including steroids *(63–69)*, neuropeptides *(70)*, β-endorphins *(71)*, enkephalins *(72)*, melatonin *(73)*, angiotensin I and II *(74)*, arginine vasopressin *(75)*, plasma renin activity *(76)*, and thyroxine *(77)*.

2.5. Identification

A homogeneous peak at an elution time that coincides with that of the reference compound under similar conditions are indications of the identity of material in a chromatogram. 3 Keto-4-ene steroids can be distinguished from other possible compounds eluted from the column by monitoring the UV absorption at further wavelengths using a photodiode array detector *(78)*. Photodiode array has also been used for vitamin D metabolites *(79)*. A mass spectrum is a fingerprint of any compound.

A separate analysis with a different column (preferably of opposite polarity) or a different gradient elution system adds confidence to the analytical specificity. Should the identification in each system coincide with the same standards it is highly probable that each chromatogram reflects the same steroid content. These criteria have not been rigidly applied in the published work relating to hormones. The combination of LC column retention time with a mass spectrum or UV absorption spectra comparable to those properties of reference compounds are taken as high standards in identification.

3. Quantitative Methods

The chromatographic peak height or area is measured manually or with the aid of an integrator and, ideally, the response of the analyte is compared to the response of an appropriate internal standard. The ratios of response for the analyte to the signal from the internal standard are plotted for the concentration range of interest. The concentration of an unknown amount of hormone in the sample is

determined from a calibration curve. There are a vast number of synthetic steroids and hormones available that can be used as internal standards. Because a number of steroid-based drugs are widely used in hospital patients, there is potential for interference in steroid assays. The use of two very different internal standards (e.g., 19-nortestosterone and 6α-methyl prednisolone) ***(80)*** prevents erroneous results in the case of medication by either one of the steroids selected provided that they behave in a similar way to the analyte during the analytical procedure.

When internal standards are not used, the extraction and injection must be carefully controlled before peak response can be reliably derived from a calibration curve on injected standards. A deferred standard technique can be adopted in which a known amount of the analyte is injected in pure form some time after, but during the chromatographic run of the unknown sample.

One major drawback to HPLC lies with the inability of UV detectors to provide suitable sensitivity and selectivity for analysis of many of the hormones in plasma or tissue extracts. Coupling with IA provides the requisite sensitivity to many hormone detection systems. The eluates from an HPLC analysis can be collected, dried down, and reconstituted in buffer for IA. This has been used for steroids ***(81–83)***, CGRP ***(84)***, thyroxine ***(85)***, angiotensins ***(86)***, and gastric inhibitory peptide ***(87)***.

3.1. Preparation of Samples

In general the analysis of hormones in biological materials requires the following:

1. Treatment of the specimen—extraction, hydrolysis of conjugates.
2. Pre-treatment of samples to produce derivatives that will enhance separation or increase sensitivity of detection.
3. HPLC analysis and detection of the individual components including post-column derivative formation to enhance detection.

3.2. Extraction

Internal standards of tritium-labeled hormone can be added to a sample or extract in order to check recovery. The sample should be

left for several hours, e.g., overnight at 4°C, in order to attain equilibrium of labeled with endogenous hormone. Steroids have been traditionally extracted from aqueous solutions and tissues by use of organic solvents, e.g., dichloromethane, ethyl acetate. A high ratio of solvent to aqueous phase is required to avoid emulsions. Serum is usually mixed for several minutes by vortexing or rotation. The layers are separated by centrifugation and the aqueous layer is usually removed by suction. Freezing the aqueous phase (in a bath of dry ice and methanol) provides a useful method of retaining water in the tube while decanting an organic layer. To avoid large "solvent front" effects in the chromatogram the extracts are washed with 0.1 *M* base or with hexane to remove saponifiable lipids or nonpolar materials. The organic phase is partly dried by addition of anhydrous sodium sulphate. A completely dry extract is produced by evaporation under nitrogen or by rotary evaporation under vacuum.

Solid-phase extraction using small cartridges containing silica modified by a functional group covalently bonded to the surface, e.g., octadecylsilyl groups (Sep Pak C18, Waters Associates; Bond Elut C_{18}, Analytichem) are ideal for extraction and recovery of hormones including metabolites and conjugates that are very water soluble ***(88)***. Some highly polar metabolites of cortisol (notably 1β and 6α hydroxylated metabolites of cortisol that are important excretory products of the hormone in urine of newborn infants) ***(33)*** and aldosterone (found particularly in renal tissue) ***(89)*** are highly polar and are poorly recovered from water with organic solvents. SPE has been incorporated into a quantitative assay for insulin in blood ***(42)*** and plasma renin activity ***(76)***.

The particle size of the solid phase and character of the solvent requires that the sample be forced through or sucked through the columns. The following basic steps are utilized:

1. Column preparation.
2. Sample application and clean-up.
3. Analyte elution.

Methanol is passed through the solid phase to wet the surface of the packing material. A flush of water or buffer is then used to dis-

place the methanol. The volumes of washes used in these steps are not critical—typically being 2–5 mL. The sample is then passed through the column and the compound(s) of interest are retained. One or more washes with water or solvents can be used to selectively remove salts and other undesirable compounds. The compound(s) of interest are then eluted with a relatively small amount of solvent (such as methanol, acetone, or ethyl acetate) (2–5 mL) so that solvent can be subsequently evaporated rapidly to leave a dry extract. Certain undesirable compounds that are absorbed on the column may be selectively removed by washing with a specific solvent or buffer prior to final elution. Compounds of interest are eluted with a solvent selected on the basis of polarity and or acidity. These columns have also markedly improved the purity of mixtures injected onto HPLC columns, essentially acting as pre-columns.

The techniques based on extraction of solutes with solid matrix cartridges have been incorporated into automated analysis. The initial extraction is still a manual or semi-automated process. However, an automated sample—pretreatment procedure ***(90)*** was reported for the analysis of a synthetic steroid, Triamcinolone, in urine. An automated technique based on dialysis and SPE trace enrichment (ASTED) ***(91)*** has been applied to the analysis of cortisol and corticosteroids in serum ***(92)***.

Lipidex 1000 is inert with low polarity and with the absence of irreversible absorption. The gel acts as a solvent with high capacity for lipids. Inorganic and polar organic materials are readily removed with water. This extraction procedure is superior to the use of silica, which binds steroids strongly and causes decomposition of some steroids ***(93)***.

A powerful clean-up of samples can be achieved with immunoaffinity chromatography before LC-MS analysis. Antibodies to the analyte are supported on column packing support, such as Sepharose, and enriche the analyte of interest. Sepharose cannot withstand the high pressures of an HPLC system and is therefore an independent enrichment of sample. Rigid polymers such as polyhydroxyethylmethacrylate are now available that can be incorporated in a column on-line with the HPLC ***(94)***.

3.3. Pre-Column Derivatization

Steroid hydrazones formed by reaction of ketosteroids with 2,4-dinitrophenyl-hydrazine have strong UV absorption (maximum 260 nm and extinction around 10,000) as well as visible absorbance (maximum 350 nm, extinction 10,000) giving detection limits for DHAS of 80 ng/mL *(95)*.

Kawasaki et al. *(96)* described the measurement of 17-oxosteroid conjugates in urine and serum by HPLC of dansyl hydrazine derivatives coupled with a fluorescence detector. Acidic metabolites of cortisol in urine (cortoic acids, formed by oxidation of the primary C-21 hydroxyl group) have been detected as the pyrenylmethyl-21-oic esters *(97)*. Identification was confirmed by UV, fluoresence spectroscopy, and supported during evaluation by photodiode array and MS. Oestrogen and corticosterone in plasma were derivatized with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole before RP-HPLC and fluoresence detection *(98,99)*.

3.4. Applications of HPLC for Hormone Assays

Many applications have already been cited in references utilized previously to illustrate methodological points. Some additional recent references in these areas have included:

1. Hormone assays in biological fluids and tissues—such measurements may be needed in clinical studies *(100–102)*, outcome of experimental studies in vitro *(103–110)*, and detection of hormone abuse (e.g., doping control in sports) *(112–115)*.
2. Checks on other analytical technologies, e.g., comparison with results obtained by radioimmunoassay to reveal the extent and nature of crossreaction in the IA *(84,116–119)*.
3. Purity and stability checks on pharmaceutical products and reference laboratory materials *(120–124)*.
4. As one of a series of analytical maneuvers to achieve purification of a hormone.

It should now be clear that HPLC has an important analytical role in hormone assays as a stand-alone system for hormone analysis and as a purification technique within a series of steps to achieve high-quality specific assays.

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3

Gas Chromatography-Mass Spectrometry

John W. Honour

Summary

Gas chromatography-mass spectrometry (GC-MS) has been used in hormone assays particularly for steroids in biological fluids. The combination of GC with MS exploits the high-resolving power of gas chromatography to separate closely related molecules, and the ability of the MS to provide precise data for identification and quantification of the separated substances. GC-MS is a very powerful technique for analysis with specificity of hormones in biological fluids. The general principles of GC-MS are described in this chapter along with some examples that illustrate specific applications of hormone analysis.

Key Words: Gas chromatography; mass spectrometry; hydroxyl groups; oxo groups; carboxyl groups; steroids; thyroid hormones; melatonin; diabetes.

1. Introduction

Gas chromatography (GC), or strictly speaking gas-liquid chromatography, is largely performed today with capillary columns and in this chapter the packed column technology will not be considered in detail. As with other forms of chromatography, GC depends on rate of movement of a compound (in the gas phase) propelled by the

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mobile phase (carrier gas) past the stationary phase. In packed columns a long glass or sometimes metal tube (typical dimensions 3 m long and 3 mm wide) is filled with a fine powder (diatomaceous earth as a support phase) coated with a stationary phase. A carrier gas flow of about 60 mL/min is required. A capillary column can be up to 30 m in length with internal diameter up to 0.25 mm of glass or now usually fused silica. The capillary column itself is the support for the stationary phase, the film thickness of which can vary from 0.1 to 0.25 μ*M* in thickness. Gas flow can be 1–2 mL/min. A packed column can achieve separations that are 1000 times better than a thin-layer plate. A capillary column is 100 times better than a packed column.

The two prerequisites for GC are volatility and thermal stability. GC is conducted at elevated temperatures and volatility of the hormone components is enhanced by chemical modification (derivative formation). Most hormones are not themselves sufficiently volatile to vaporize at room temperature. Peptide and protein hormones are not amenable to GC-mass spectrometry (MS) analysis even though there is now potential for analysis by other techniques (fast atom bombardment, thermospray, electrospray, matrix assisted laser desorption/ionization) involving MS (*see* **Table 1** for examples relating to hormone analysis ***[1–13]***). Even when hormones are amenable to GC-MS analysis, the technique often does not compete with other automated techniques in speed, robustness, ease of operation, and maintenance. Biological fluids often need extensive purification before the hormone analysis.

An MS converts molecules into ions that are separated in electric and or magnetic fields. Because the technique involves electrons and ions, that would react with or be scattered by air, an MS operates under vacuum. The ion source is heated to ensure that molecules are in the gas phase. Molecules will fragment in the MS depending on the ionization process and the strengths of chemical bonds within the molecule. The pattern of fragment ions generated within a mass range (mass spectrum) is in essence a fingerprint of the molecule representing the ionized molecule or accountable fragments of the molecule. The spectrum represents the number of frag-

Table 1
Recent Examples of the Analysis of Hormones Using Sophisticated Mass Spectrometric Techniques

Glycoforms of human chorionic gonadotrophin by MALDI-TOF MS	1
Glycoprotein structure by MS	2
Structure of gonadotropin-releasing hormone	3
Posttranslational modifications of luteinizing hormone (lutroropin) receptor	4
Immunoreactive gonadotrophin-releasing hormone by immunoaffinity, capillary electrophoresis, and MS	5
Circulating parathyroid hormone	6
Tyrosine phosphorylated peptides by quadrupole TOF MS	7
Pituitary peptides by electrospray tandem NS	8
Synthetic renin substrate by FAB and hybrid TMS	9
Kidney renin activity	10
Thyrotrophin-releasing hormone like peptides in prostate and other tissues	11
Reduction of disulphide bonds in deamino-arginine vasopressin and arginine vasopressin	12
Arginine vasopressin in lymphocytes	13

ments reaching the detector and the sizes of the fragments are recorded as a mass to charge (m/z) ratio. The spectrum can be compared with those in a library of spectra of known compounds. From the spectrum, one can derive an empirical formula and from the nature of the fragmentation, one can assemble the complete structure from the molecular substructures, akin to a jigsaw puzzle. For quantitative analysis the intensities of the signals associated with ionized molecules and fragments may be correlated with the quantity of the analyte. Depending on the resolution of the MS, the ion can be determined as an integer at low MS resolution or exact mass to a number of decimal places in high MS resolution. Some fragmentation of the ions occurs within the MS either spontaneously or

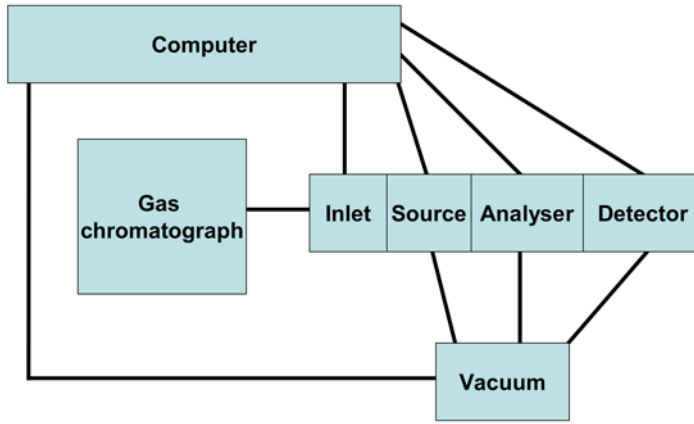

Fig. 1. Components of a GC-MS system.

by induction. The mass spectra obtained from mixtures are usually too complex to be helpful so a mass spectrometer is often coupled as the detector of a separation system such as a GC, and as seen in other examples with high-performance liquid chromatograph (HPLC). This chapter focuses on the general principles in the use of GC-MS for hormone analysis, more detail will be found in selected reviews in the published literature ***(14,15)***.

The detection of isotopes by a mass spectrometer is an important further use of the technique both in an analytical sense and in metabolic and drug studies. In metabolic studies, the use of stable isotopes overcomes the danger of exposure to ionizing radiation. Quantitative analysis based on isotope dilution with stable isotope-labeled internal standards affords highly specific hormone measurements approaching standards of reference methods.

1.1. GC-MS

A GC-MS has a number of segments (*see* **Fig. 1**). These include the following:

1. Sample introduction (injection).
2. Component separation in the GC. The GC operates in the gas phase with trace levels of the analytes (pg to ng). The outlet of the GC is at atmospheric pressure and is led to the high vacuum of the mass spectrometer.

3. Sample transfer from the GC to the MS, this can be a direct link or through a combustion chamber.
4. Ion source—where charged ions are produced. Negative and positively charged ions are formed. The extent of fragmentation is determined by the amount of energy imparted to the molecules.
5. Mass analyzer–where the ions are separated as a function of their mass to charge ratio (m/z).
6. A detector that records the abundance of the ions.
7. A computer, that also plays an important role in data handling, largely controls the operation of a mass spectrometer.

1.1.1. Sample Introduction

The sample is usually injected as a solution into the end of the column itself (on-column injection) or into a heated region at the head of the column. The sample needs to be introduced quickly so only 1–5 μL of solution is injected. For on-column injection the column temperature will be below or close to the boiling point of the solvent in order to discharge the analytes to the stationary phase. In the heated injector zone the sample vaporizes and is flushed out of the injector into the column where it should be deposited as a discrete band. The injection port is usually 50ºC above the final column temperature. The column usually sits with the front end within a glass injector liner of dimensions greater than the end of the column. The sample is injected and vaporizes within the injection port and the components within the gaseous sample mixture condense onto the beginning of the column that is near ambient temperature (cold trapping). Most of the involatile material accumulates on the glass insert, which should be removed and cleaned at frequent intervals. The injection system should introduce a truly representative sample into the column unmodified by thermal degradation or any absorption phenomena. Three principal types of related injector have been used:

1. Split.
2. Splitless.
3. The programmed temperature vaporizer has been added recently.

Split injection was the first method to be successfully applied for capillary GC analysis. The sample after rapid vaporization moves

with high carrier gas flow before being split at the column inlet where the major portion is vented to the atmosphere. In this way the sample size is made compatible with the low capacity of the capillary column. Splitless injection requires similar hardware to that required for split injection. After rapidly evaporating the sample in a heated liner, the vapor is transferred to the column with the split exit closed. After 20–60 s, the split vent is opened to remove remaining vapor from the liner. These techniques have two drawbacks that affect certain sample types. In wider boiling mixtures, there is discrimination against the higher molecular weight components that prevents their quantitative analysis. Thermally unstable compounds are liable to degradation in this injection system. The more recently introduced programmed temperature vaporizer closely resembles the classical split/splitless system but with flexible temperature control to regulate the removal of solvent, volatilisation of the sample, and removal of residue from the injector.

1.1.2. Gas Chromatography

Separation in GC is achieved largely through partition between the mobile phase and the stationary phase. Those components preferentially distributed in the mobile phase move quickly through the column, whereas those preferentially distributed in the stationary phase elute later. Affinity for the stationary phase depends on the physicochemical characteristics of the molecules and the temperature. These characteristics influence for example dipole–dipole energy, hydrogen bonds, polarization, and dispersion forces. GC uses very few stationary phases in hormone assays. The polysiloxanes remain among the major group (SE30–dimethylsiloxane; OV-7 phenylmethydimethylsiloxane; XE-60 cyanoethyl-dimethylsiloxane). The catalogues from suppliers of GC material and columns are very helpful when choosing the column required for a particular separation. A bonded polar phase will meet most requirements for hormone analysis. Cyano-substituted polysiloxanes have selective characteristics useful for improving the separation of compounds with ketones. Chemical bonding of the stationary phase to the column reduces the "column bleed" of the stationary phase and that can influence purity

of the signal at the detector. Columns can be used at temperatures up to 325ºC, even 350ºC for short periods in the analysis. Some chemically bonded columns can be rinsed with solvent to remove accumulated debris and charred materials at the injector end.

The column temperature is selected primarily to maintain the analytes in the vapor form. Changes in temperature markedly influence the vapor pressure of the analyte and its distribution between the mobile and stationary phase and hence the speed of chromatography. Because most applications of GC-MS for hormone assays seek to separate related components, a temperature program over 1 h is the preferred analysis, although gas chromatographs are becoming available that will speed up GC analysis. Isothermal analysis leads to peak broadening with analysis time.

The time of passage of a molecule through the gas chromatograph (retention time) is considered as part of the criteria for identity of the analyte. The nature of the stationary phase, the polarity of the components relative to the stationary phase, the loading of the phase, and column temperature all influence the retention times and separation of components in the mixture. If GC alone is used to separate components with detection limited to nonselective techniques (flame ionization), then confidence in identity can be increased by running on two different stationary phases. Although MS will enable identities of equivalent saturated and unsaturated analytes, retention times may be the only means of identifying isomers that are indistinguishable even by their mass spectral properties.

The carrier gas can affect chromatographic separation and speed of analysis. The gas should be inert, dry, and of high purity. Helium is preferable to nitrogen for GC-MS, although hydrogen can offer some further improvements in speed and resolution. Carrier gas flow needs to be regulated during a temperature-programmed GC run.

Capillary columns can last many months of regular use. The injector liner needs regular cleaning. Chromatography quality may deteriorate with time as evidenced by poor recoveries of some or all components in the chromatograph. Changes in peak shape and loss of resolution of neighboring compounds are further indicators of column deterioration. Some improvements are achieved by removing and repacking the first 5–10 cm of the column.

1.1.3. Sample Transfer to MS From GC

Because the mass spectrometer operates at high vacuum, there are considerable differences in the hardware required in GC-MS to accept gas flow from a packed column compared with a capillary column. A molecular separator is required between a packed column GC and the MS to remove the carrier gas and selectively concentrate the analytes for MS analysis. When a gas flow of 1–2 mL/min is entering the MS system from the capillary GC column the high-capacity vacuum generation systems (turbo-molecular pumps) that are now used for mass spectrometers are capable of maintaining the required vacuum.

In most GC-MS systems, the GC column passes through a vacuum seal of the ion source housing and is positioned to discharge the outlet of the GC into the ion source of the MS.

1.1.4. Ionization of Samples in the Gaseous Phase

Molecules are charged and fragmented when bombarded with electrons (electron impact ionization [EI]) or charged ions (chemical ionization [CI]).

Electron impact achieves ionization of molecules, in the gas phase entering the MS, by displacement of an electron from an analyte molecule:

$$M + e^- \rightarrow M^{+\bullet} + 2e^-$$

$M^{+\bullet}$ is the molecular ion, but this is not always seen in the mass spectrum because it undergoes decomposition to give charged fragment ions. The electrons are produced from a heated filament and are accelerated as they pass through a potential difference of 5–70 V. Negative ions are also produced:

$$M + e^- \rightarrow M^-$$

For chemical ionization, the ion source is maintained with a high partial pressure of a reagent gas such as methane, isobutane, or ammonia that is ionized by electron impact. The ionization of analyte molecules is effected by reaction with reagent gas ions leading to the generation of protonated analyte ions. The reactive species after

ion-molecule reactions in the gas are CH_5^+ from methane and $C_4H_9^+$ from isobutane.

The "soft" ionization produces more reagent gas ions capable of ionizing the analyte molecules than electrons in the EI directly targeting the analyte. When ionizing the analyte, more molecular ions than fragment ions are produced. The outcome is a improved signal for equal amount of analyte compared with electron impact ionization. CI is therefore used more for quantitative analysis and EI for qualitative analysis.

The ions produced by EI, and to some extent CI, are in highly excited states and some energy will be released by the rupture of bonds between atoms giving fragments.

1.1.5. Ion Separation

Mass spectrometers can detect positive or negative ions. The selection of ions is determined by the charge applied to a "repeller" at the back of the ion source that forces ions out into the analyzer.

1.1.5.1. Magnetic Separation

The analyzer is responsible for separating ions according to their mass and charge. A magnetic or electric field is applied for ion separation and in most cases today electric field separation is used. Dedicated mass spectrometers for masses up to 100 are used where gases are being analyzed. High resolution (for greater accuracy in mass assignment) is best achieved with the more expensive magnetic sector instruments that may also have an electric focusing component (double focusing instrument). The quadrupole instruments are popular because of their mass range (up to 4000 Daltons), rapid scanning capabilities, ease of control, size, and cost. Time-of-flight analyzers are not generally used for hormone analysis. Different mass spectrometers are suitable for selected mass ranges. A new technique for steroid analysis involves the insertion of a combustion chamber between the GC and the MS so that the C13/C12 ratio in carbon dioxide from steroids can be analyzed.

The magnetic analyzer is a magnet around a curved tube. The passage through the tube to the detector is a function of momentum and mass to charge ratio, by varying the strength of the magnetic field the ions can be focused to pass through the tube to the detector. An important equation links these functions:

$$m/z = H^2 \cdot r^2 / 2\ V$$

where m is mass, *z* is charge, *H* is magnetic field strength, *r* is the radius of the flight path, and *V* is the accelerating voltage.

By scanning the magnetic field, the range of masses can be brought into focus and the spectrum can be recorded as abundance against mass to charge. By fixing the magnetic field a specific ion fragment of an analyte can be focused (selected ion monitoring SIM) and the intensity of that signal at an appropriate GC retention time would be the basis for quantitative analysis. Further ions of close mass could be focused by varying the energy imparted to the ions from the repellor strength (change the accelerating voltage). The limitation here is that by reducing the energy applied to the charged molecules the lower the energy imparted to the particles and the lower likelihood to succeed in migration through the magnetic sector.

1.1.5.2. Quadrupole Separation

An electric field is created in a quadrupole or ion trap analyzer. In the quadrupole the mass filter is composed of four cylindrical rods (electrodes) at the corners of a square. Opposing pairs of electrodes maintain an electrostatic field (DC) on which is superimposed AC radio-frequency field (RF). The ions from the source enter these fields parallel to the direction of the electrodes and at certain rod voltages ions can undergo an oscillating trajectory of increasing amplitude to reach the detector. Other ions in unstable trajectories are lost between the rods. It is possible to change RF and DC stepwise in order to bring into focus any ion within the mass range (SIM) and this option is widely used in qualitative and quantitative analysis. Time windows covering the retention times of selected hormones can be set and appropriate panels of SIM

will enable different groups of compounds to be detected. This is very effectively used in doping control for anabolic steroid abuse in sport *(16)*.

1.1.5.3. Ion Trap Separation

The ion trap is a three dimensional version of the quadrupole mass filter. It consists of a doughnut-shaped central electrode and two end-cap electrodes. A field is created by applying an RF voltage to the central ring electrode while maintaining the end-cap electrode at ground potential. An electron beam is passed through one of the end caps to ionize the gaseous molecules present in the trap. Increasing the field voltages ejects ions sequentially from the trapping field through perforations in the other end cap before the detector. The ejected ions are detected to provide a mass spectrum. Several ions can be detected in a sequence for SIM.

Tandem MS (MS coupled to MS) can be extended to triple MS. The combinations enable specificity in an analysis particularly an analysis based on single ion monitoring. The ion focused in one mass separation step (first quadrupole) is subject to a further ionization step (in a collision cell between the two separation stages). At a second mass separation either another single ion (daughter ion) is focused to the detector or a spectrum is recorded by scanning that gives partial structural proof to the mother ion.

1.1.6. Ion Detection and Data Handling

The detector of a mass spectrometer is generally of the electron/photo-multiplier type incorporating signal amplification. A mass spectrum of the analyte is obtained by scanning the ion separation device to achieve sequential detection of differing mass. Ions of increasing or decreasing mass impinge on the detector, thus generating a continuous array of signals relating ion abundance to mass to charge ratio. It must be remembered that in GC-MS the sample input will be continuously changing in composition and amounts of analytes and the detection system must be changing focus at a greater rate. A GC peak may typically last a few seconds (2–10 s),

so mass spectra need to be acquired over 0.2–0.5 s to get 10 or more spectra across a GC peak *(15)*. During repetitive scanning the data are stored on computer. A record can be produced called the total ion current chromatogram (TIC) that is a plot of the summed abundances of all the ions in each scan. In GC-MS this TIC will resemble the trace of a GC with flame ionization detection, which is a two-dimensional chromatogram. From the TIC, the point of elution can be determined for each component of a mixture, but there is a third dimension to the data in the form of the mass spectrum. If the molecular weight or unique fragment ions of an analyte are known, then the data can be searched to indicate the point of such elution from the GC and the spectrum plotted from that point in the analysis (selected ion current recording).

2. Sample Preparation for Hormone Assays in Biological Fluids by GC-MS

Some form of sample processing is inevitable before GC-MS analysis. The main reasons for such manipulations are:

1. Removal of proteins, inorganic salts, and other substances that may bind analytes or interfere with the analysis.
2. Concentration of the analytes to levels compatible with the response of the detector.
3. Chemical or enzymic conversion to forms suitable for analysis. Hydrolysis is particularly essential for urine samples in order to release the analytes from glucuronides or sulfates for example.
4. Formation of thermally stable derivatives of hormones for GC analysis

2.1. Initial Sample Treatment

The most simple preparation technique is deproteinization by denaturation and precipitation with acids, such as trifluoroacetic acid. Protein removal has also been done with the addition of some organic solvent, such as acetonitrile or methanol. In general, large ratios of organic solvent volume to aqueous sample volume are needed to complete precipitation. Organic solvent extraction with immiscible solvent, such as chloroform, is effective in many cases for isolation of analytes. Careful choice of the organic solvent or mixture of solvents and the pH

of the aqueous phase are vital. The organic solvent may need to be very pure and may need to be evaporated to increase the concentrations of the analytes. This may need reduced pressure and a moderate temperature. A stream of nitrogen over the sample may be effective. Ultrafiltration is an alternative method of sample purification. Adsorption of the analyte onto an active solid support like charcoal or alumina may be used. More often solid-phase extraction technology is used. Ion exchange, reverse phase, and silica packings are available. Some automation is now possible. Although there are not recent references, some of the approaches to isolation, identification, and quantitation from the 1980s are still worth referring to ***(17,18)***.

Hydrolysis of conjugates and purification of extracts will not be addressed here, specific examples will be found in more detail later. The efficiency of hydrolysis of conjugates remains a critical issue in steroid analysis still being addressed ***(19)***.

2.2. Derivative Formation

Some hormones can be analyzed directly by GC analysis because they are stable at analysis temperatures, but in general hormones need chemical modification (derivatization) to be amenable to GC conditions. A derivatization procedure is nearly always included in the sample preparation of hormones prior to GC separation of components in a mixture of related chemicals isolated from a biological fluid. A derivative of a compound is formed in a microchemical reaction of predictable consequence that retains the structural features of the analyte. The methods for derivative formation are catalogued in a comprehensive review ***(20)***. The rationale for the preparation of derivatives is:

1. Volatility and stability of the analyte to high temperature analysis.
2. Improved chromatographic properties through reduction in adsorption and amplification of structural differences.
3. Assist confirmation of chemical structure of the analyte in terms of numbers of reactive groups, such as hydroxyl and carbonyl functions, that can react separately in separate microchemical reactions.
4. Direct fragmentation of the analyte in the MS in order to assist the assay requirements whether qualitative or quantitative in nature.

2.2.1. Hydroxyl Groups

A number of derivatives of hydroxyl groups are used in hormone analysis. Use of dichloromethylsilane, trimethylchlorosilane, and *bis*-trimethylsilylacetamide (BSA) or *bis*-trimethylfluoroacetamide (BSTFA) will form trimethylsilyl derivatives (TMS) on most hydroxyl groups on hormone molecules. BSA and BSTFA alone will only form TMS derivatives on nonsterically hindered hydroxyl groups, whereas trimethylsilylimidazole (TSIM) will react on sterically hindered hydroxyl groups. Many of the reagents are volatile but TSIM needs to be removed from samples before GC analysis. The best way of removing TSIM reagent is with Lipidex chromatography ***(21)***, but this is time consuming and expensive. TMS derivatives potentiate cleavage of the carbon to oxygen bond so each fragmentation at that point gives rise to an ion of 90 mass units less in the spectrum. The position of the hydroxyl groups is not always apparent from the spectrum although specific fragments of the D-ring and side-chain of steroids are characteristic of certain structures.

The use of tertiary butyldimethylsilyl ethers (TBDMS) for quantitative GC-MS analysis is very common. Loss of C4H9 is the main fragmentation and the resulting abundant ion is usually used in quantitative analysis. Heptafluorobutyrate derivatives are also good for selected ion monitoring (SIM) ***(22,23)***. Vicinal hydroxyl groups can be derivatized to give acetonides and cyclic boronate ester derivatives. These derivatives provide limited structural information in the mass spectra.

Chemical ionization is used for improved sensitivity. The pentafluorobenzoyl derivatives of oestrogens have been shown to be superior to trifluoroacetyl, heptafluorobutyl, penta decafluorooctanoyl, and pentafluorotolyl derivatives ***(24)***.

2.2.2. Oxo Groups

Oxo groups can be derivatized as oximes using methyloxime hydrochloride. A disadvantage in the use of oxime derivatives is the formation of isomeric syn and anti products that are generally separated by the GC process. This can increase the complexity of the

analysis, but in some cases can be diagnostically useful. Using catalytic reactions the oxo group can be enolised to an ene-ol function that will silylate. This enol-TMS derivative technique is used in rapid screening techniques for anabolic steroid residues in urine that indicate drug abuse in sports *(16)*.

Combined derivatives (MO-TMS, pentofluorobenzyloxime-TMS) have been successfully used in qualitative and quantitative work *(25,26)*.

2.2.3. Carboxyl Groups

Glucuronic acid residues and carboxyl groups of amino acids can be protected by esterification. Methyl ester formation is the most frequent derivative used in esterification *(27)*. When steroids are combined with glucuronic acid and converted to methyl ester-methyloxime trimethylsilyl ether derivatives, they have long GC retention times and high molecular weights making analysis difficult with bench top mass spectrometers.

2.3. Quantitative Mass Spectrometry

Internal standards are an important component of quantitative analysis because they can be added at the outset of the analysis and compensate for errors that may occur at any stage in the procedure. The use of the detector response ratio of the analyte to the internal standard plotted against the mass ratios or the concentration of the analyte offers the best way for achieving acceptable accuracy and precision in GC-MS analysis.

An internal standard should ideally meet the following requirements:

1. Resemble the analyte in physico-chemical properties as closely as possible.
2. The internal standard should be stable and not react with the sample or any component under the experimental conditions.
3. The calibration curve should be linear.

Because the mass spectrometer can distinguish molecular weights, it is not essential that the internal standard separates chromato-

graphically from the analyte. In fact co-elution can afford an additional protective carrier effect to the analysis.

Internal standards of hormones can therefore be:

1. Stable isotope-labeled equivalent hormones.
2. Isomeric hormones.
3. Analogues of the hormone.

3. Examples of the GC-MS Analysis of Hormones

3.1. Steroids

Many related steroids in blood or urine can be analyzed in a single GC run over 1 h. Steroids with 18, 19, and 21 carbon atoms are secreted in controlled ways by the adrenal cortex, gonads, and placenta. They circulate in blood, often bound to protein, and are delivered to tissues where they act as transcription factors to direct protein synthesis to regulate tissue and organ processes. Steroids are inactivated in the liver and excreted in bile, feces, and urine. Steroid concentrations can be determined in blood by immunoassay. There are many examples, however, in clinical studies where measurement of steroids is not specific enough for medical decisions in relation to diagnosis of a disease state or monitoring of treatment. This is probably not surprising when the chemical structures of steroids are examined. Measurements of single and multiple steroids using GC-MS are available both as reference techniques *(28)* and for specific clinical measurements *(29)* some such methods are described in detail in other chapters.

The range of steroids in urine can also be examined by GC-MS. Thus, urine steroid profiling by GC-MS has proved over 30 yr to be an effective analysis for investigation of patients with adrenocortical and certain gonadal disorders. This profile is usually based on analysis of steroids as MO-TMS derivatives and is described in detail elsewhere *(30–32)*. Steroids have been used in sports and in veterinary medicine to enhance muscle development. In 1973, urine tests were introduced for steroids in a program of testing to detect

doping. For doping control in sports, anabolic steroids are analyzed as enol-TMS derivatives. In the early days of steroid abuse in sports, synthetic steroids were used. These were chemical modifications of testosterone, 19-nor testosterone (nandrolone), and 17-methyl testosterone. The urine metabolites of the synthetic steroids are reasonably easy to detect using GC-MS. When testosterone itself is abused it became more difficult to detect. Because urine samples are usually collected in sports after a competition or training episode, it is difficult to interpret concentrations of natural steroids in the context of drug abuse. In order to detect testosterone abuse, a ratio of testosterone to epitestosterone is determined by GC-MS although the test is open to manipulation by injecting epitestosterone in order to restore the T/E ratio to normal. Combustion isotope ratio MS is now used to examine the carbon 12 to carbon 13 ratio of testosterone (and androstenedione) metabolites (androstanediols) *(33)*. This ratio is characteristic for pharmaceutically derived steroid (commercially prepared from plant sterols) *(34)* and different from the carbon ratios in steroid produced in the body. This approach has also been used for detection of exogenous corticosteroids *(35)*.

GC high resolution MS and GC with tandem MS/MS has been used to increase the level of specificity of steroid analyses when at low levels. There is concern that lowered sperm counts of men may be owing to intake of oral contraceptives in drinking water and the steroid content of polluted water has been undertaken using GC/MS/MS *(37)*. Negative ion/GC-MS of pentafluorobenzyloxime/TMS derivatives has been used to increase sensitivity in order to detect steroids in aqueous and vitreous humour *(26)*, so-called neurosteroids *(25)*, and oestrogens in meat *(38)*.

3.2. Thyroid Hormones

Although thyroxine can be measured by GC-MS as a heptafluorobutyrate methyl ester derivative against a carbon-13 labeled internal standard *(39)*, the technique is not widely used. Application of the technology seems to have not extended beyond quality control *(28)*.

3.3. Melatonin

The analysis of melatonin has proved to be difficult. Immunoassays alone have lacked specificity in clinical trials of melatonin agonists and needed a preliminary liquid-chromatography step to improve performance *(40)*. Derivatives formed with pentafluoropropionic anhydride have been used for melatonin analysis by GC-MS *(41)* and this has proved suitable for checking the purity of drugs and pharmaceuticals and for monitoring plasma and saliva concentrations of melatonin in agonist studies.

3.4. Hormone-Related Issues: Diabetes

Cholesterol oxidation in the body may be influenced by free radicals. An increase in free radical production in diabetes is associated with an increase in circulating 7-oxocholesterol concentrations determined by GC-MS *(42)*. Microvascular complications of diabetes may be related to increased polyol pathway activity that can be revealed from measurements of urine myo-inositol, sorbitol, and fructose using GC-MS *(43)*. Prostacyclin production may also be involved in the complications of diabetes. An increase in prostacyclin in serum as measured by GC-MS with the administration of eicosopentanoic acid suggests that EPA may be an effective treatment *(44)*.

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4

An Introduction to PCR Techniques

Gill Rumsby

Summary

The polymerase chain reaction (PCR) is a method for the enzymatic amplification of DNA and has revolutionised our approach to both diagnostics and research, permitting the amplification of small amounts of genetic material, either derived from genomic (chromosomal DNA) or RNA via reverse transcription to cDNA. This chapter gives a primer of methods for the preparation of materials for PCR and some advice on the requirements for the PCR itself.

Key Words: Polymerase chain reaction (PCR); primers; DNA extraction; RNA preparation; reverse transcriptase PCR (RT-PCR); Taq polymerase.

1. Introduction

The polymerase chain reaction (PCR) was first described in the mid-1980s and is a method for producing multiple copies of a particular DNA sequence without the necessity for cloning into vectors. It would be fair to say that the technique has revolutionized molecular genetics research and diagnostics (from molecular archaeology to zoology) and the number of applications is constantly increasing. The method utilizes a heat stable enzyme, Taq polymerase, to synthesize DNA from a single-stranded template, the

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specificity of the reaction determined by the choice of two oligonucleotide primers, which hybridize to the DNA template on opposite strands and act as primers for the polymerase enzyme. The final reaction product obtained after 25–35 cycles of denaturation, primer annealing, and extension, can be visualized as a single band of DNA on an agarose gel and contains in the region of 10^{12} copies/100 µL reaction—an increase of sensitivity of approx 10^7 copies over the starting material.

A large number of applications and adaptations of this technique have now been described including reverse transcription (RT)-PCR, mutation-specific amplification techniques, and *in situ* PCR. All PCR procedures require genetic material as the primary template, either genomic (chromosomal) DNA or complementary DNA (cDNA) that is prepared by reverse transcription of messenger RNA (mRNA). Genomic DNA is more complex because it contains exons (i.e., coding DNA) and introns (i.e., noncoding DNA), the latter are usually spliced out of the mRNA transcript to produce mature mRNA. The advantage of genomic DNA is that it is present in all nucleated cells and therefore can be readily obtained from either a blood sample or simple scrape of the buccal membrane on the inside of the cheek.

cDNA, derived by reverse transcription of mRNA, is less readily available because it often follows a tissue specific pattern of expression; for example, expression of the steroid 17-hydroxylase is restricted to the adrenals and gonads. In addition, differential splicing of mRNA may take place in a tissue-specific manner with the result that different protein products are synthesized from a single gene by alternative splicing of the mRNA. For example, calcitonin and calcitonin gene-related peptide are created by alternative splicing of the calcitonin gene on chromosome 11 ***(1)***. This restricted expression means that it is not always possible to obtain mRNA for analysis. However, it is known that illegitimate transcription occurs in all tissues to a greater or lesser extent and thus it may be possible to extract mRNA transcribed by any gene from cultured lymphocytes ***(2,3)*** thus simplifying the search for gene mutations. This

approach has proved invaluable in the investigation of a number of endocrine disorders, for example autosomal recessive hypoparathyroidism *(4)*.

As more subtle differences in expression are sought, there is a requirement to quantitate the amount of mRNA produced. This process is fraught with difficulties but recent PCR developments including real-time PCR have allowed progress in this direction. This process is too complex to be dealt with in this chapter but interested readers are directed to an excellent review on these procedures *(5)*.

2. Materials

2.1. Genomic DNA Isolation

1. Red blood cell lysis solution: 155 m*M* ammonium chloride, 10 m*M* potassium hydrogen carbonate, 1 m*M* ethylenediaminetetraacetic acid (EDTA).
2. Cell lysis solution: 25 m*M* EDTA, 2% SDS.
3. Protein precipitation solution: 10 *M* ammonium acetate.
4. Rehydration solution (TE buffer): 10 m*M* Tris-HCl pH 8.0, 1 m*M* EDTA.
5. Isopropanol.
6. 70% ethanol.
7. 50-mL screw cap polypropylene centrifuge tubes.
8. 50 m*M* sodium hydroxide.
9. 0.1 *M* Tris-HCl, pH 8.0.

2.2. RNA Isolation

1. Guanidine thyiocyanate reagent, e.g., RNA Isolator (Genosys) (based on guanidine thiocyanate method *[6]*).
2. Isopropanol.
3. Diethylpyrocarbonate-treated water (*see* **Note 1**).
4. 75% ethanol.
5. Chloroform.
6. Microcentrifuge.
7. 1.5-mL microcentrifuge tubes.

2.3. Reverse Transcription

1. mRNA.
2. 50 μ*M* Oligo $dT_{12\text{-}18}$ primer or random hexamers.
3. Reverse transcriptase (MMLV or AMV).
4. dNTPs, containing 10 m*M* each deoxynucleoside triphosphate.
5. Magnesium chloride, 50 m*M*.
6. RNase inhibitor, e.g., RNasin (Promega), 40 U/μL.
7. 10X buffer: 200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl.
8. Nuclease-free water.
9. Water bath or thermal cycler.

2.4. Polymerase Chain Reaction

1. Working solutions (approx 50 μ*M*) of primers specific for product to be amplified.
2. dNTP stock solution containing 2.5 m*M* each deoxynucleoside triphosphate.
3. 10X polymerase reaction buffer supplied by manufacturer for enzyme to be used.
4. 25 m*M* magnesium chloride.
5. Nuclease-free water.
6. Mineral oil.
7. Taq polymerase 5 U/μL.
8. Thermal cycler.

2.5. 2% Agarose Gel

1. Agarose, electrophoresis grade.
2. Tris-borate with EDTA (TBE) buffer: 0.1 *M* Tris, 0.08 *M* borate, 5 m*M* EDTA.
3. Ethidium bromide, 10 mg/mL water.
4. Hot plate and magnetic stirrer or microwave.
5. Ultraviolet transilluminator.

2.6. Sample Loading Buffer (6X)

0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose (w/v) in water. Store at 4°C.

3. Methods

3.1. Genomic DNA Isolation From Blood

There are essentially three methods available for the isolation of genomic DNA: organic extraction with phenol/chloroform, salt precipitation, and selective binding to a solid phase matrix. The first method yields DNA of very high quality and purity, but has the disadvantage of using organic solvents and the inherent problems of disposal of these materials. The second, and probably the most popular method yielding high-quality DNA (described next), is one that utilizes differential precipitation of nucleic acids and proteins from aqueous solution using high salt solutions. Many commercial preparations utilize silica resin or other solid-affinity matrix for the selective binding of DNA, whereas RNA and proteins are removed by washing with high salt solutions and ethanol. These are relatively expensive to use, do not produce high-molecular-weight DNA and do not have a very high yield. However, they have a role particularly in purification of PCR products prior to sequence analysis. The most readily available source of DNA is blood collected into EDTA (*see* **Note 2**) as an anticoagulant but any tissue can be used (*see* **Table 1**).

1. Add 3 mL blood to 9 mL cold red blood cell lysis solution. Stand 15 min inverting occasionally. Spin 2000*g* for 10 min.
2. Pour off supernatant, leaving 100–200 µL residual liquid.
3. Vortex vigorously to resuspend (*see* **Note 3**).
4. Add 3 mL Cell Lysis solution: pipet up and down to lyse fully. At this point samples are stable for approx 18 mo at room temperature.
5. Add 1 mL protein precipitation solution. Vortex vigorously.
6. Centrifuge at 2000*g* for 20 min to pellet the proteins.
7. Pour supernatant into a clean tube containing 3 mL isopropanol.
8. Invert isopropanol tube 50 times to precipitate DNA (should be visible as fine, white strands).
9. Spin 2000*g* for 3 min. Carefully pour off supernatant.
10. Add 3 mL 70% ethanol. Invert tubes several times to wash DNA strands.
11. Spin 2000*g* for 5 min.

Table 1
Approximate DNA Yields From Various Tissues

Tissue	DNA yield
Blood (5 mL)	15–50 µg
Blood spot	0.5–1 µg
Chorionic villus biopsy (small frond)	1–3 µg
Buccal cells (single mouth rinse)	0.1–1 µg
Hair root	10–200 ng

12. Drain tube on absorbent paper and air dry for approx 15 min.
13. Resuspend in 250 L TE buffer.
14. Dilute an aliquot 1/10 and use 2–5 µL for each PCR reaction.

3.2. Isolation of DNA From Mouthwash Samples

1. Rinse mouth with tap water while scraping the inside of the mouth with the teeth for approx 20 s. Collect into 50-mL sterile polystyrene container with a screw lid top or into 50-mL sterile polypropylene tube. Store at 4°C until isolation.
2. Centrifuge for 10 min in 50-mL polypropylene centrifuge tubes and discard supernatant. 400 µL of 50 m*M* sodium hydroxide is added and the tube boiled for 10 min. After cooling, 200 µL of 0.1 *M* Tris-HCl, pH 8.0 is added.
3. Transfer specimen to a numbered, 1.5 mL sterile tube and centrifuge for 1 min at 12,000*g* to pellet the debris. Decant the supernatant into a fresh, labeled tube and store frozen.

3.3. RNA Isolation

As mentioned in the introduction, mRNA from nonhousekeeping genes tends only to be expressed in reasonable quantities in a tissue-specific manner. If appropriate target tissues are available, the following procedures can be used for isolation of mRNA. It should be noted that mRNA is particularly labile (*see* **Note 4**) and attention should be paid to the following housekeeping rules:

1. Samples for analysis should be frozen at –80°C immediately after collection and kept frozen until analysis (*see* **Note 5**).

2. Gloves should be worn at all times.
3. All glassware, e.g., homogenizers should be washed, rinsed in DEPC-treated water and ethanol before use.
4. All tips and plasticware should be baked for 2 h at 80°C before use.

Most methods are based on denaturation of the proteins (and therefore of ribonuclease) by guanidine thiocyanate followed by precipitation of mRNA by ethanol. These reagents are best purchased ready-made so that health risks associated with their preparation are minimized.

1. Homogenize tissue (50–100 mg) in 1 mL RNA Isolator (**Note 6**).
2. Incubate 5 min at room temperature (*see* **Note 7**).
3. Add 0.2 mL chloroform/mL RNA Isolator used.
4. Stopper tubes and shake thoroughly for 15 s or until emulsified.
5. Incubate at room temperature for 2–15 min.
6. Centrifuge 12,000*g* for 15 min at 4°C after which time the mixture will separate into two phases. RNA is in the upper (aqueous) phase. Transfer aqueous phase to fresh tube. Ensure no interphase proteins are carried over.
7. Precipitate RNA by adding 0.5 mL isopropanol/mL RNA Isolator used initially. Stand at room temperature for 5–10 min.
8. Spin at 12,000*g* for 10 min at 4°C, RNA forms a gelatinous pellet on bottom and sides of tube.
9. Remove supernatant and discard. Wash pellet once with 1 mL 75% ethanol by inverting tube several times.
10. Spin 5000*g* for 5 min at 4°C. Remove as much ethanol as possible.
11. Air dry for 5–10 min. Dissolve in 50 µL sterile water or 1X TE buffer.
12. Measure OD of 5 µL aliquot at 260 nm using quartz cuvetes (*see* **Note 8**).

3.4. Reverse Transcription

There are a number of kits available for the purpose of converting mRNA into cDNA which include either Moloney murine leukaemia virus (MMLV) or Avian myeloblastosis virus (AMV) reverse transcriptase. AMV is more robust and can help eliminate problems with secondary structure. However, MMLV has significantly less RNase H

activity, which degrades the RNA strand of RNA:DNA hybrids and is therefore a better choice if full-length cDNA products are required. All reactions must be done in the presence of ribonuclease inhibitors, for example RNasin.

The type of primer used can be either oligo dT, which primes off the 3' poly A tail on the mRNA, random hexamers, or a specific primer for the mRNA required. Oligo dT obviously gives a preference to the 3' ends of mRNA.

1. For each reaction:

50 m*M* $MgCl_2$	2 µL
10X buffer	2 µL
10 m*M* dNTPs	2 µL
RNase inhibitor	0.5 µL
Reverse transcriptase	0.25 µL
50 m*M* hexamers	1 µL
RNA	0.5–1.0 µg
Nuclease-free water	to a final volume of 20 µL

2. Incubate at room temperature for 10 min, then 42°C for 15 min, followed by 99°C for 5 min, and 4°C for 5 min.
3. Use 10 µL of the final reaction mix from **step 2** in a 50 µL PCR reaction.

3.5. Polymerase Chain Reaction

The specificity of the PCR reaction is largely the result of the primers and a number of programs are available (*see* **Note 9**) for the design of such oligonucleotides. Factors that need to be considered are as follow:

1. Primer length—ideally between 18 and 30 nucleotides and with similar melting temperatures (T_m) (*see* **Note 10**). The annealing temperature, which should be between 55 and 75°C is approximately T_m –5°C.
2. %GC content: between 35 and 65%.
3. Secondary structure: regions of secondary structure within the primer should be avoided.
4. Primer ends should be checked for self-complimentarity, especially at the 3' ends, try to avoid Gs and Cs at the 3' ends as this may increase the chance of forming primer artifacts.

For analysis of cDNA, primers should be selected that cross intron–exon boundaries ensuring that contaminating genomic DNA sequences will not be amplified. Ideally, selected sequences should be run through BLAST (http://www/ncbi.nlm.nih.gov/BLAST/) to ensure that they are unique and do not hybridize with any other sequences in the genome. Once the sequence has been selected the primers can be synthesized quite cheaply by any number of commercial enterprises. Purification by high-performance liquid chromatography (HPLC) should be considered if the same primers are to be used for sequencing purposes, otherwise it is not necessary. On receipt, primers are dissolved in TE buffer at an appropriate concentration (*see* **Note 11**), e.g., 50 μ*M* and an aliquot taken into an appropriately labeled tube. The master (stock) tube should then be kept for long term storage in case of contamination. Primer concentrations between 0.1 and 1 μ*M* can be used in the reaction (*see* **Subheading 3.5.1.**).

It is good to get into good housekeeping habits at the outset as PCR tends to spawn requirements for huge numbers of primers with very similar names. To avoid unnecessary confusion, set up an Excel file containing primer sequence, identifier, concentration of stock solution, site on gene, and so on.

3.5.1. Optimization of Reaction Conditions

A number of variables need to be tested to create a reaction with high specificity and yield of product and these include concentration of magnesium (0.5–3 m*M*), primers (0.1–1 μ*M*), and dNTPs (*see* **Note 12**) (20–200 μ*M*) as well as thermocycling parameters. Primers and dNTPs should be titrated together. A reduction in primer and dNTP concentration can improve specificity of the reaction by discouraging nonspecific annealing and primer dimer formation. Any reduction in dNTP should be accompanied by an equimolar decrease in magnesium concentration because dNTPs form a soluble complex with magnesium and there will therefore be an increase in the amount of free magnesium available if nucleotide concentration falls. The amount of template used is between 10^2 and 10^5 copies (*see* **Note 13**). The amount of Taq polymerase used

will depend on the length of product to be amplified and can range from 0.25 to 2 U/25 µL reaction. Excess enzyme may lead to non-specific amplification and unnecessary expense. Activity of the polymerases is dependent on Mg^{2+} as cofactor and it is important that this cation is precisely controlled. Taq polymerase is known to suffer from infidelity of replication because of a lack of proofreading ability. If high fidelity is required, other enzymes such as polymerase isolated from *Pyrococcus furiosus* (Pfu) can be used.

The very sensitivity of PCR means that one must be careful to avoid contamination of reagents and samples with either cloned DNAs or PCR products. Physical separation of pre- and post-PCR procedures including use of separate pipets and use of filter tips is essential. Premixing all reagents and enzyme minimizes pipetting, whereas adding the DNA last and sealing the tube minimizes the potential for contamination. Each assay should be set up with appropriate reagent blanks, i.e., contains all reagents but not DNA to check for reagent contamination.

Temperatures of the cycles will vary from machine to machine and from template to template. An initial denaturation phase is necessary to fully denature the template, which may be complex if genomic DNA, and destroy any proteases or nucleases present in the DNA sample. If performed before addition of enzyme this can be up to 5 min at 94°C, if however, the enzyme is already present in the reaction tube, a shorter initial denaturation time of 2 min should be used to avoid destruction of the enzyme. The subsequent annealing temperatures and times are determined empirically although a starting point would be the calculated annealing temperature of the primers (*see* **Note 14**) and increasing the annealing temperature by 2°C increments if increasing specificity is required. Time of annealing can range from 10 to 30 s.

Most commonly used Taq polymerases can extend at a rate of 50 nucleotides per second so extension times at 72°C can be fairly short for PCR products of 400 basepairs (bp) or less. For longer PCR products it may be necessary to increase the extension time at each cycle to compensate for the increased viscosity of the reaction and the decrease in enzyme. The extension reaction is followed by a

short denaturation step of 15–30 s at 93°C before the annealing and extension reactions are repeated. The number of cycles used varies between 25 and 50 with most diagnostic work using in the region of 35 cycles. There is an initial exponential phase of amplification until approx 10^{12} copies are reached. After this point, the reaction slows down and plateaus. Increasing the number of cycles does not necessarily improve product yield and may increase the nonspecificity of reactions. A final extension step of 5 min at the end of the reaction can often increase the yield of full-length PCR products.

3.5.2. Procedure

1. Write up work sheet.
2. Number up the tubes.
3. Thaw out all reagents, mix well and keep on ice.
4. Prepare master mix containing the following:

	Final concentration
10X polymerase buffer	1X
dNTPs	20–200 µ*M*
$MgCl_2$	0.5–3 m*M*
Forward primer	0.1–1 µ*M*
Reverse primer	0.1–1 µ*M*
Taq polymerase	0.25–1 U/tube

5. Add master mix to each tube including reagent blank tube.
6. Overlay with drop of mineral oil (not necessary if thermal cycler has heated lid).
7. Add DNA, stopper each tube after addition.
8. Spin tubes briefly in microfuge to ensure all contents are in the bottom of the tube.
9. Load into thermal cycler and run on suitable program.

3.6. Agarose Gel Electrophoresis

PCR products from 50 to 1300 bp can be run out on 2% agarose and > 1300 bp on 0.8% agarose in TBE. Addition of ethidium bromide (*see* **Note 15**) to the gel allows visualization of the bands on an UV light box. Small PCR products less than 100 bp may require

electrophoresis on 10% polyacrylamide gel electrophoresis to resolve them clearly from primers. These gels are best stained with silver.

For a 10 × 10 cm 2% agarose gel:

1. Add 1 g agarose to 50 mL TBE buffer.
2. Bring to a boil on hotplate with stirring until agarose has dissolved.
3. Cool slightly before adding ethidium bromide to a final concentration of 0.5 µg/mL.
4. Pour into gel tray with sealed ends and containing sample combs. Allow to set for approx 30 min.
5. When set, remove combs and end pieces, place gel tray plus gel in buffer tank. Add TBE buffer to cover.
6. Add 1 µL of sample loading buffer to 5 µL of each PCR sample (*see* **Note 16**) and load into each well.
7. Load approx 100 ng of molecular size marker into an empty well.
8. Run at 100 mA for 10–15 min before examining on an ultraviolet transilluminator (*see* **Note 17**).

3.7. Analysis of PCR Product

Ensure that the product amplified is the size expected by running appropriate size markers on the gel (*see* **Note 18**). A degree of skepticism is always useful as the brightest band is not always the one you want. Primers and nucleotides can be removed from PCR products if purified DNA is required for subsequent sequence analysis, by adsorption of the DNA to silica filters, followed by elution with water. If more than one product is present, the required band can be cut from the agarose gel with a new scalpel blade before solubilization of agarose and DNA clean up with glass milk.

3.8. Mutation Detection

A number of methods based on PCR are available for mutation detection including single-strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis, and heteroduplex analysis both by electrophoresis and HPLC. Each method has its share of good and bad points in terms of sensitivity and cost and the choice is largely dependent on facilities. We have success-

fully used SSCP with silver staining for mutation screening *(7)* but the sensitivity of this method is poor. With the ready availability of automated sequencing it may be preferable to go straight for sequence analysis of the PCR product. However, it should be noted that heterozygote detection is not always reliable on PCR sequencing and in some cases nonamplification of alleles occurs, which can lead to false or at least only partial results. Any sequence variant identified must be verified on a fresh PCR product either by restriction enzyme digestion, when the sequence change inserts or destroys a recognition sequence, or by repeat sequence analysis to exclude the possibility of PCR artifacts.

Having identified a sequence change, one needs to determine whether it is in fact pathological or simply represents natural DNA variation that occurs between individuals. The latter are known as polymorphisms which, by definition, occur in more than 1% of the population. These often have no functional consequence because they either do not alter the protein reading frame, or lead to a conservative change in sequence or occur within unimportant intronic sequences. However, there are an ever-increasing number of examples where particular polymorphisms may actually have some functional impact, for example, genetic variants of the drug-metabolizing enzyme CYP2A6. Possibly the first thing one needs to do is confirm that the sequence change is restricted to patients with the disease, tracks with the disease in family studies and is not present in at least 50 normal individuals preferably matched for ethnic group. However, the definitive proof that a mutation is pathological is to express the protein in vitro. This approach is relatively routine now for enzymes and receptors and a number of expression vectors are available into which the mutant cDNA can be cloned (or a mutant cDNA can be generated by site-directed mutagenesis of the normal cDNA).

The finding of splice variants in cDNA should be treated with initial caution as, in our experience, RT-PCR can identify apparently mis-spliced mRNA species that, on further investigation, are also present in controls. Whether these variants have any functional significance is not clear. They may simply reflect the sensitivity of PCR and the preference for amplification of smaller templates.

4. Notes

1. Prepared by adding 10 µL diethylpyrocarbonate to 10 mL water and autoclaving. Addition of DEPC should be carried out in fume hood.
2. EDTA is the preferred anticoagulant, heparin may interfere with later procedures.
3. This step is essential, yields are very reduced if it is not done.
4. Long-term storage of mRNA is probably best done in the form of cDNA.
5. Degradation of mRNA may occur under these conditions but template will probably still be adequate for PCR. If one needs higher quality mRNA for Northern blot analysis, consideration should be given to collecting the samples directly into guanidine thoicyanate with immediate preparation of the mRNA.
6. Can be scaled up.
7. Can store in this form at –70°C for at least 1 mo.
8. An absorbance of one at 260 nm = 40 µg RNA/mL.
9. See http://www.hgmp.mrc.ac.uk/GenomeWeb/nuc-primer.html.
10. T_m can be roughly calculated using the following formula 4(G+C) + 2(A+T) +8°C to allow for both DNA species, i.e., the template and the primer, being in solution.
11. Concentration of oligonucleotide can be calculated from OD260 nm where 1 OD unit = 33 µg/mL. To convert µg/mL to nmol/mL use the following formula µg/mL oligonucleotide × 1/330 × 1/N × 1000 where N is the number of bases in the oligonucleotide and 330 is the average molecular weight of a nucleotide.
12. The four dNTPs, i.e., dATP, dCTP, dTTP, and dGTP should be present in equal concentrations.
13. For a single copy gene 100 ng DNA will contain 1.4 × 105 copies.
14. Annealing temperature is T_m –5°C.
15. Ethidium bromide is a potent carcinogen, Always wear gloves, dispose of gels by incineration.
16. To save time and tubes, simply dot 1 µL of the sample buffer onto parafilm, mix with 5 µL of the PCR product and load directly into the well.
17. Ensure UV eye and skin protection is used. 10 ng is about the minimum one can see with ethidium bromide staining.
18. ØHaeIII digested DNA is a useful size marker for PCR products up to 1 kb. Other markers such as 100 bp ladders are also available.

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5

Methods for the Investigation of Thyroid Function

Christine R. Squire

Summary

The commonly used laboratory investigations for the diagnosis and management of thyroid disorders are the thyroid hormones, thyroxine and triiodothyronine and the pituitary hormone, thyrotrophin (TSH). Simple, readily available radioimmunoassay methods for the measurement of the thyroid hormones and an immunochemiluminometric assay for TSH are described. Variations in total thyroid hormone levels owing to changes in thyroid hormone-binding proteins can be assessed by the measurement of free thyroxine. An equilibrium dialysis method is described. Methods for the measurement of TSH receptor antibodies and for thyroid peroxidase and thyroglobulin antibodies used in the assessment of autoimmune thyroid disease are given.

Key Words: Thyroxine; triiodothyronine; thyrotrophin (TSH); thyroid autoantibodies.

1. Introduction

After the widespread introduction of radioimmunoassay (RIA) in the 1970s, the mainstay of thyroid investigation has been measurement of the two main hormones of the thyroid gland—thyrox-

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ine (T_4) *(1)* and triiodothyronine (T_3) *(2)*. The measurement of total thyroid hormones depends on the inhibition of binding of the hormones to their binding proteins *(3)*. In the methods described, this is achieved by blocking the binding sites with anilino-1-naphthalene-sulphonic acid (ANS). Double antibody RIA with polyethylene glycol (PEG) precipitation is used. The primary antibodies are sheep polyclonals raised by immunization with T_4-bovine serum albumin (BSA) or T_3-BSA conjugates and the methods are extremely robust and free from interference except, very rarely, from endogenous T_3 or T_4 autoantibodies *(4)*. The second antibody is donkey anti-sheep (DAS). Antibodies and labeled antigens are commercially available.

The later introduction of thyrotrophin (thyroid-stimulating hormone [TSH]) assay to the repertoire allowed assessment of the pituitary-thyroid axis. The use of monoclonal antibodies (MAbs) and nonisotopic labels led to improvements in sensitivity of TSH methods and allowed better discrimination between euthyroid and hyperthyroid TSH levels *(5,6)*. The present generation of immunometric TSH assays reliably measure concentrations less than 0.01 mU/L *(7)*. MAb assays are subject to interference from heterophilic antibodies *(4,7)* but this is minimized by addition of mouse serum or IgG fractions. The method described *(8)*, is a "third-generation" chemiluminometric assay. A "sandwich" is formed between mouse MAb to TSH immobilized on polystyrene beads, TSH, and an affinity purified goat polyclonal antibody to TSH conjugated with an acridinium ester. The chemiluminescent signal is triggered by hydrogen peroxide and sodium hydroxide in a luminometer. The light emitted is proportional to the concentration of TSH in the sample.

Free T_4 assays have increasingly become accepted as a superior indicator of thyroid status because they should, theoretically, be independent of changes in iodothyronine-binding proteins, which influence total hormone measurements. However, the validity of these assays has been challenged since their introduction *(8–10)*. Despite modifications in technique, interference and differences between methods are still evident *(11–13)* particularly in patients with changes in T_4-binding capacity owing to binding inhibitors or iodothyronine autoantibodies *(14,15)*. The technique commonly used as a reference

method for free T_4 estimation is equilibrium dialysis of undiluted serum followed by RIA of T_4 in the dialysate. Dialysis cells ***(16)*** and a sensitive T_4 RIA method are available in kit form.

Autoimmune disease is a common cause of thyroid dysfunction. Three different thyroid autoantibodies are important in the diagnosis and management of autoimmune thyroid disease; TSH receptor antibodies (TRAb) ***(17,18)***, common in Graves' disease, thyroid peroxidase, (TPO), and thyroglobulin (Tg) antibodies, associated with thyroid destruction ***(19)***. In the TRAb assay described ***(20)***, inhibition by the antibody of the binding of ^{125}I-labeled TSH to detergent solubilized TSH receptors is measured. The calibrators, which contain biologically active TSH, have been standardized in the receptor assay against MRC LATS standard B and 1 unit of calibrator is approximately equivalent to 1 U of MRC LATS B standard.

TPO and Tg autoantibodies are generally measured by enzyme-linked immunosorbent assay (ELISA) assays ***(21)***. However, direct assays have the advantage of high sensitivity, precision, and specificity, and do not suffer from interference from other autoantibodies ***(22)***. The same serum diluent can be used for both Tg and TPO autoantibody assay. This is incubated with highly purified preparations of ^{125}I-labeled Tg and TPO and the complexes formed are precipitated by addition of solid phase protein A. High sensitivity can be obtained by extending the incubation.

2. Materials

2.1. Thyroxine Radioimmunoassay

1. Stock phosphate buffer: 0.5 *M*, pH 7.4. Store at room temperature.
2. Assay buffer: phosphate buffer 0.05 *M*, pH 7.4 containing 1 g/L sodium azide. Store at 2–8°C.
3. PEG precipitant: 4% PEG 6000 containing 0.1% Triton X-100 in 0.05 *M* phosphate assay buffer. Mix and store at 2–8°C.
4. Tracer diluent buffer: 4 mg/mL ANS in 0.05 *M* phosphate assay buffer. Prepare freshly for each assay.
5. ^{125}I-T_4 tracer, (Skybio Ltd, Wyboston, Beds. UK): reconstitute tracer (220 kBq) with 1 mL distilled water (*see* **Note 1**). Aliquot for immediate use and store the remainder frozen at –20°C.

6. Working tracer: dilute the reconstituted tracer 1:25 with tracer diluent (200 µL added to 5 mL of tracer diluent is sufficient for 100 tubes). Prepare freshly for each assay (*see* **Note 2**).
7. Stock sheep anti-T_4 (first antibody; Skybio Ltd): reconstitute the vial with 5 mL distilled water, allow to stand for 30 min, mixing at 10-min intervals. Use immediately or aliquot and store remainder frozen at –20°C.
8. Working first antibody: dilute the reconstituted Anti-T_4 1 in 10 with 0.05 *M* phosphate assay buffer. Prepare freshly for each assay.
9. Stock DAS second antibody (Skybio Ltd): reconstitute the vial with 5 mL distilled water. Allow to stand for 30 min, mixing at 10-min intervals. Use immediately or aliquot and store frozen at –20°C.
10. Working DAS: dilute the reconstituted DAS second antibody 1 in 5 with 0.05 *M* phosphate assay buffer. Prepare freshly for each assay.
11. T_4 standards may be obtained commercially (Skybio Ltd) and reconstituted according to manufacturer's instructions or prepared in thyroid hormone free plasma/serum (*see* **Notes 3** and **4**).
12. Quality control (QC) material.

2.2. Triiodothyronine Radioimmunoassay

1. Stock phosphate buffer: 0.5 *M*, pH 7.4. Store at room temperature.
2. Assay buffer: phosphate buffer 0.05 *M*, pH 7.4, containing 1 g/L sodium azide. Store at 2–8°C.
3. PEG precipitant: 4% PEG 6000 containing 0.1% Triton X-100 in 0.05 *M* phosphate assay buffer. Mix and store at 2–8°C.
4. Tracer diluent buffer: 2 mg/mL ANS in 0.05 *M* phosphate assay buffer. Prepare freshly for each assay.
5. ^{125}I-T_3 tracer (Skybio Ltd): reconstitute tracer (220 kBq) with 1 mL distilled water (*see* **Note 1**). Aliquot for immediate use and store the remainder frozen at –20°C.
6. Working tracer: dilute the reconstituted tracer 1:25 with tracer diluent (200 µL added to 5 mL of tracer diluent is sufficient for 100 tubes). Prepare freshly for each assay (*see* **Note 2**).
7. Stock sheep anti-T_3 (first antibody; Skybio Ltd): reconstitute the vial with 5 mL distilled water, allow to stand for 30 min, mixing at 10-min intervals. Use immediately or aliquot and store remainder frozen at –20°C.
8. Working first antibody: dilute the reconstituted Anti-T_3 1 in 10 with 0.05 *M* phosphate assay buffer. Prepare freshly for each assay.

9. Stock DAS, second antibody: reconstitute the vial with 5 mL distilled water. Allow to stand for 30 min, mixing at 10-min intervals. Use immediately or aliquot and store frozen at –20°C.
10. Working DAS: dilute the reconstituted DAS second antibody 1 in 5 with 0.05 *M* phosphate assay buffer. Prepare freshly for each assay.
11. T_3 standards may be obtained commercially (Skybio Ltd) and reconstituted according to manufacturer's instructions or prepared in thyroid hormone-free plasma/serum (*see* **Notes 3** and **9**).
12. QC material.

2.3. TSH Immunochemiluminometric Assay

1. TSH-third generation kit (cat. no. 60-4100, Nichols Institute Diagnostics, San Juan Capistrano, CA). Store unopened kit at 2–8°C.
2. Trigger set (cat. no. 60-4050).
3. Bead dispenser capable of delivering 6-mm beads.
4. Horizontal rotator capable of maintaining 180 ± 10 rpm.
5. Saline wash solution: dilute 50 mL of saline wash concentrate with 1450 mL distilled or deionized water. Store at room temperature.
6. Automatic tube washer or 2-mL repeating dispenser and bead retaining decanting rack.
7. Luminometer (Berthold Lumat or equivalent).
8. Luminometer performance controls (cat. no. 60-4075).
9. QC sera.

2.4. Free Thyroxine by Equilibrium Dialysis

1. Nelson dialysis cells (cat. no. 30-0652, Nichols Institute Diagnostics, San Juan Capistrano, CA). The dialysis cell has two components, a vial and a dialysis membrane cylinder. The latter consists of two plastic sleeves, a dialysis membrane and a compressible 0 ring. The membrane cylinder fits firmly into the dialysis vial. Store at 2–8°C.
2. Hepes dialysis buffer with 0.1% sodium azide and antibiotics (cat. no. 30-0650).
3. Rack to hold dialysis cells.
4. T_4 RIA kit (cat. no. 40-2211), which includes:
   a. Anti-T_4 antibody coated tubes.
   b. ^{125}I-T_4 (370 kBq) in Hepes buffer with 0.1% sodium azide (*see* **Note 1**).

c. T_4 standards: reconstitute each vial with 6.0 mL distilled or deionized water, (*see* **Note 15**). Stable at 2–8°C for 8 wk after reconstitution.
d. Wash solution: mix vial (turbidity is normal) and add 50 mL wash concentrate to 450 mL distilled or deionized water Stable at room temperature for 6 mo.
e. QC sera: reconstitute each vial with 1.5 mL distilled or deionized water (*see* **Note 15**). Stable at 2–8°C for 8 wk after reconstitution.

2.5. Thyroid Autoantibody Assays

2.5.1. TSH Receptor Antibody Assay

1. Ice bath or insulated box containing cool packs to keep reagents cold (0–4°C) on the bench.
2. TRAb assay kit (RSR Ltd, Pentwyn, Cardiff, UK). Store unopened kits at 2–8°C. The kit contains:
   a. TSH receptors: immediately before use, reconstitute each vial with 1.3 mL cold (0–4°C) assay buffer. Vortex at maximum speed for 5 s. Never allow the temperature of reconstituted receptors to exceed 4°C (*see* **Note 19**).
   b. ^{125}I-labeled TSH: immediately before use, reconstitute each vial (9 KBq) with 2.7 mL cold (0–4°C) assay buffer and mix gently (*see* **Note 1**). Keep at 0–4°C prior to use.
   c. Negative serum and control sera: reconstitute in distilled water according to manufacturer's instructions. Stable at 2–8°C for 2 mo.
   d. Standards: reconstitute with 0.5 mL distilled water. Mix gently. Stable at 2–8°C for 2 mo.
   e. Precipitator solution: 16.5% PEG.
   f. Microfuge.

2.5.2. Thyroglobulin Autoantibody Assay

1. Direct TgAb assay kit (RSR Ltd). Store at 2–8°C. The kit contains:
   a. Assay diluent: 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.5, 5 g BSA and 1 mL Tween-20/L
   b. ^{125}I-labeled Tg: reconstitute each vial (40 KBq) with 2.6 mL assay diluent, (*see* **Note 1**). Stable at 2–8°C for 2 mo.
   c. Tg antibody standards: calibrated against MRC 65/93. Stable at 2–8°C for 2 mo.

d. Solid phase protein A: reconstitute with 2.6 mL assay diluent. Mix on a vortex mixer for 10 s and check that the suspension is uniform. Store at 2–8°C for up to 2 mo or at –20°C for 6 mo.

2.5.3. Thyroid Peroxidase Autoantibody Assay

1. Direct TPOAb assay kit (RSR Ltd). Store at 2–8°C. The kit contains:
   a. Assay diluent: as TgAb assay.
   b. ^{125}I-labeled TPO : reconstitute each vial (40 KBq) with 2.6 mL assay diluent (*see* **Note 1**). Stable at 2–8°C for 2 mo.
   c. TPO antibody standards: calibrated against MRC 66/387. Stable at 2–8°C for 2 mo.
   d. Solid phase protein A: as TgAb assay.

2.6. General Materials

1. Precision pipets, 25–1000-µL.
2. Adjustable or graduated pipets, 1–6-mL.
3. Repeating pipets, 50–500-µL.
4. Repeating dispenser, 2–3-mL.
5. Single and multi-vortex mixers.
6. Parafilm/cling film or equivalent for covering tubes.
7. Distilled or deionized water.
8. Timer.
9. 37°C ± 0.5°C temperature controlled water bath or dry heat incubator.
10. γ-Spectrometer.
11. Centrifuge capable of 2000*g* and preferably refrigerated.
12. Micro-balance.

3. Methods

3.1. Total Thyroxine Radioimmunoassay

We have used antibodies and tracer from Skybio Ltd for total thyroid hormone assays but complete diagnostic kits are available from the same supplier.

1. Allow all reagents to reach room temperature before use. Mix well.
2. Prepare working Anti-T_4 first antibody, DAS, and tracer.

3. Label sufficient 12 × 75-mm plastic tubes in duplicate for nonspecific binding (NSB), total activity (TC), standards, test, and QC samples (*see* **Note 5**).
4. Pipet 25 µL standard, test, or QC serum into the bottom of the tubes (*see* **Note 6**).
5. Prepare two tubes with zero calibrator to determine NSB.
6. Add 50 µL ^{125}I-T_4 working tracer to all tubes (including TC) with a repeating pipet.
7. Add 100 µL Anti-T_4 first antibody to all tubes except NSB and TC with a repeating pipet.
8. Add 50 µL DAS second antibody to all tubes except TC with a repeating pipet.
9. Vortex well (*see* **Note 7**).
10. Cover with cling film and incubate at room temperature for 2 h.
11. Add 500 µL PEG solution to all tubes except TC tubes. Vortex well and centrifuge at 2000*g* for 30–40 min.
12. Decant over a sink designated for radioactive waste into running water. Place the inverted tubes on a wad of absorbent material and allow to drain for a few minutes. Blot (or aspirate the tube necks) to ensure that all remaining liquid is removed before reinverting.
13. Count the radioactivity in all tubes for at least 1 min using a γ-spectrometer.
14. Data reduction: subtract NSB from all results, calculate B/Bo (cpm test/cpm zero standard) for each standard, QC, and sample. Plot a standard curve of B/Bo vs concentration for each standard and interpolate results for QC and test samples (*see* **Note 8**).

3.2. Total Triiodothyronine Radioimmunoassay

1. Allow all reagents to reach room temperature before use. Mix well.
2. Prepare working Anti-T_3 first antibody, DAS, and tracer.
3. Label sufficient 12 × 75-mm plastic tubes in duplicate NSB, TC, standards, test, and QC samples (*see* **Note 5**).
4. Pipet 50 µL standard, test, or QC serum into the bottom of the tubes (*see* **Note 6**).
5. Prepare two tubes with zero calibrator to determine NSB.
6. Add 50 µL ^{125}I-T_3 working tracer to all tubes (including TC) with a repeating pipet.

7. Add 100 µL Anti-T_3 first antibody to all tubes except NSB and TC with a repeating pipet.
8. Add 50 µL DAS second antibody to all tubes except TC with a repeating pipet.
9. Vortex well (*see* **Note 7**).
10. Cover with cling film and incubate at room temperature overnight.
11. Continue with the protocol outlined in **Subheading 3.1., step 11**.

3.3. TSH Immunochemiluminometric Assay

1. Store the kit at 2–8°C upon receipt. Allow reagents to come to room temperature before use and mix by gentle inversion. Reagents from different kit lot numbers should not be combined or interchanged (*see* **Note 10**).
2. Label sufficient 12 × 75-mm borosilicate glass or plastic tubes (*see* **Note 11**) in duplicate for standards, test, and QC samples.
3. Pipet 200 µL of standard, test, or QC sample directly to the bottom of each tube.
4. With a repeating pipet, add 100 µL of acridinium ester-labeled antibody to the bottom of the tubes. Vortex each tube gently and avoid foaming.
5. Ensure that the beads have reached room temperature. Tilt the rack to avoid splashing and gently add one antibody-coated bead to each tube using a bead dispenser (*see* **Note 12**).
6. Incubate on a horizontal rotator at 180 ± 10 rpm for 2 h at room temperature.
7. Wash the beads three times with an automatic tube washer using 2 mL working saline solution. Alternatively, aspirate the reaction mixture, add 3 mL of working saline solution to all tubes, and aspirate fluid, repeat the last two steps three times (*see* **Note 13**).
8. Prepare the luminometer with trigger solutions according to the manufacturer's instructions.
9. Count each tube for 2 s in the luminometer (*see* **Note 14**).
10. Data reduction: the luminometer can be programmed to plot mean relative light units (RLU) for each pair of standards against concentration of TSH on a logarithmic scale using a cubic spline curve fit. Test and QC samples are interpolated from the curve.

3.4. Free Thyroxine Equilibrium Dialysis Assay

3.4.1. Dialysis Procedure

1. Separate the dialysate vial and membrane cylinder.
2. Label sufficient membrane cylinders and vials for test and QC samples.
3. Pipet 2.4 mL of Hepes dialysis buffer into each dialysate vial and reinsert the membrane cylinder (*see* **Note 16**).
4. Pipet 200 µL of test or QC sample into the membrane cylinders. Do not dialyze the standards.
5. Seal the dialysis cells with parafilm or cling film and place in a rack.
6. Incubate at 37°C ± 0.5°C for 16–18 h. Use an accurately calibrated dry heat incubator or water bath (*see* **Note 17**).
7. When incubation is complete, take the cells out of the incubator and immediately remove the membrane cylinders.
8. Allow the dialysates to cool to room temperature before beginning the RIA assay. Dialysates of serum can be stored for up to 10 d at 2–8°C in the original dialysate vial. Do not transfer to another container. Seal with parafilm before storage.

3.4.2. Radioimmunoassay Procedure

1. Allow reagents to come to room temperature before use and mix by gentle inversion.
2. Label Anti-T_4-coated tubes in duplicate for each standard, test, or QC sample.
3. Label two 12 × 75-mm polystyrene tubes for TC tubes.
4. Pipet 800 µL of each standard into appropriately labeled tubes.
5. Pipet 800 µL of the dialysate for each test and QC sample into appropriate tubes.
6. Using a repeating pipet, add 50 µL of ^{125}I-T4 solution to all tubes.
7. Vortex all tubes. Cover with cling film and incubate for 3 h at 37°C ± 0.5°C.
8. Wash all tubes (except TC) twice by dispensing 2.0 mL of working wash solution into each test tube and completely aspirating or decanting the liquid from each tube (*see* **Note 18**).
9. Count each tube in a γ-spectrometer for 1 min and record the counts.

10. Data reduction: as for total T_4 RIA, plot a standard curve of B/Bo vs concentration for each standard on semi-log paper and interpolate results for QC and test samples (*see* **Note 8**).

3.5. Thyroid Autoantibody Assay

3.5.1. TRAb Assay

1. Storage and preparation of serum samples: sera should be assayed soon after separation or stored (preferably in aliquots) at –20°C. 0.2 mL is sufficient for one assay. Do not use plasma in the assay (*see* **Note 20**). When required, thaw sera at room temperature and mix gently to ensure homogeneity. Centrifuge the serum prior to assay (preferably for 5 min at 10,000–15,000*g* in a microfuge) to remove any particulate matter.
2. Pipet 50 µL of each standard, test, and control serum into the appropriately numbered duplicate tubes.
3. Pipet 50 µL of negative serum into two numbered tubes for control receptors.
4. Reconsitute the TSH receptors and place in ice bath or cool box.
5. Pipet 50 µL of cold TSH receptors into all the tubes except the control receptor tubes and TC tubes.
6. Pipet 50 µL of control receptors into the control receptor tubes.
7. Mix the tube contents by shaking the tube rack vigorously for about 10 s or by placing each tube on a vortex mixer for about 1 s. Incubate for 15 min at room temperature (20–25°C).
8. During this first incubation step, reconstitute each vial of ^{125}I-labeled TSH and place in the ice bath.
9. Add 100 µL of ^{125}I-labeled TSH to each assay tube and to two empty TC tubes. Mix by shaking the tubes for about 10 s (or 1 s on a vortex mixer). Cover the tubes and incubate for 2 h at room temperature (20–25°C) or for 1 h in an air incubator at 35°C. Use of a water bath is not recommended.
10. Using a repeating pipet, add 2.0 mL of cold (2–8°C) precipitator solution to all tubes (except TC) and mix each tube 10 times quickly on a vortex mixer set at maximum speed (the tube contents must be homogeneous at this stage).
11. Centrifuge all the tubes (except TC) at 1500*g* for 30 min at 4°C and aspirate the supernatants (decanting can be used but aspiration is preferable).

12. Count all tubes for 2 min in a γ-spectrometer.
13. Prepare a calibration curve by plotting ^{125}I-labeled TSH bound against standard concentrations (*see* **Note 21**) and interpolate test serum and QC values (*see* **Note 22**).

3.5.2. Thyroglobulin Autoantibody Assay

Details are given for the standard assay procedure with sensitivity less than 0.3 U/mL (*see* **Notes 23** and **24**).

1. Allow the reagents to reach room temperature.
2. Dilute all serum samples 1 in 20 in assay diluent (e.g., 50 µL serum plus 950 µL assay diluent). Do not dilute standards.
3. Pipet 50 µL TgAb standard or diluted serum in duplicate into appropriately labeled 12 × 75-mm plastic tubes.
4. Pipet 50 µL of ^{125}I-labeled Tg into each tube.
5. Pipet 50 µL of ^{125}I-labeled Tg into two empty tubes for TCs.
6. Vortex or mix by shaking the tubes for a few seconds.
7. Incubate for 1 h at room temperature.
8. Mix the protein A suspension (*see* **Note 25**) and pipet 50 µL of the homogeneous suspension into each tube (except TC).
9. Incubate 1 h at room temperature.
10. With a repeating pipet, add 1 mL of assay diluent; mix briefly on vortex mixer and centrifuge at 1500*g* for 30 min at 4°C.
11. Aspirate or decant the supernatants.
12. Count all tubes for 1 min in a γ-spectrometer.
13. Data reduction: prepare a calibration curve by plotting cpm directly or as a percentage of the total radioactivity originally added against standard concentrations. Antibody concentrations in the test sera are then interpolated (*see* **Note 26**).

3.5.3. Thyroid Peroxidase Autoantibody Assay

Details are given for the standard assay procedure with sensitivity less than 0.3 U/mL (*see* **Note 24**).

1. Allow the reagents to reach room temperature.
2. Dilute all serum samples 1 in 20 in assay diluent (e.g., 50 µL serum plus 950 µL assay diluent). Do not dilute standards.

3. Pipet 50 µL TPOAb standard or diluted serum in duplicate into appropriately labeled 12 × 75-mm plastic tubes.
4. Pipet 50 µL of ^{125}I-labeled TPO into each tube.
5. Pipet 50 µL of ^{125}I-labeled TPO into two empty tubes for TCs.
6. Continue with the procedure outlined in **Subheading 3.5.2.**, **step 6** of the TgAb assay.

4. Notes

1. Disposable rubber gloves should be worn when handling isotopes. All local regulations for the safe handling and storage of radioactive materials should be followed.
2. 50 µL of T_4/T_3 tracer should yield 20,000–30,000 cpm.
3. To prepare thyroid hormone free plasma: filter a fresh plasma or serum pool and add activated charcoal (Norit PN.5) at 4% w/v. Mix continuously for 20 min on a magnetic stirrer then incubate at 37°C for at least 30 min, mixing occasionally. Centrifuge the mixture at 1500*g* for 20 min to remove the bulk of the charcoal. Transfer to clean tubes and re-centrifuge for 60 min at 1500*g*. Charcoal "fines" will still be present, so freeze the plasma overnight, allow to thaw and the "fines" will appear in the fibrin deposits. Centrifuge for 30 min at 1500*g* and transfer the supernatant into a clean container. Add sodium azide at 0.1%. Test the plasma for thyroid hormone clearance before use.
4. To prepare T_4 standards: using L-T_4 (MW 776.9) that has been stored in the dark in a dessicator at room temperature, accurately weigh 15–20 mg. Note the exact weight for the dilution. Transfer to a stoppered tube and add enough 0.13 *M* NaOH in 70% ethanol solution to give a 1 mg/mL solution. It is important that the NaOH/ethanol is at room temperature before addition. Allow to stand at room temperature overnight, until dissolved. Check the concentration of the primary stock standard by spectrophotometry. T_4 has a molar absorbency coefficient of 6200 $mol^{-1}cm^3$ at 325 nm. The solution is stable for several years when stored at 2–8°C in the dark. Dilute the primary stock 1 in 10 with ethanol (99–100% w/v) in a volumetric flask to give a 0.1 mg/mL secondary stock standard. Using a positive displacement pipet, take 250 µL of secondary stock into a 100-mL volumetric flask and dry down at 37°C under nitrogen. Make up to 100 mL with stripped plasma. Mix and incubate at 37°C for 30 min. Allow to reach

room temperature and equilibrate overnight at 4°C. Bring to room temperature while mixing before assaying to check value. This standard is equivalent to 322 nmol/L T_4. Serially dilute a 25 mL aliquot with stripped plasma to produce the required calibration range, e.g., 322, 161, 121, 80.5, 40.3 nmol/L. Aliquot and freeze. Stable for 1 yr.

5. Serum is the sample of choice, but lithium heparin plasma may be used. Grossly haemolysed or lipaemic samples should be avoided. Frozen samples should be thawed, mixed well, and centrifuged before use.
6. Pipet sample into the bottom of the tube, first ensuring that the outside of the tip is dry.
7. If sample or reagents do become spread on the tube surface then centrifuge briefly before the vortex step.
8. A suitable computer data reduction program may be used, e.g., 4 parameter logistic ***(23,24)***.
9. To prepare T_3 standards: using 3,3'5' L-triiodothyronine (MW 651) which has been stored in a dessicator at –20°C, accurately weigh 15–20 mg. Note the exact weight for the dilution. Transfer to a stoppered tube and add enough HCl/ethanol (25 mL *M* HCl mixed with 50 mL ethanol [99.7–100%]) solution to give 1 mg/mL. It is important that the HCl/ethanol is at room temperature before addition. Stand at room temperature overnight, until dissolved. The solution is stable for several years when stored at 2–8°C in the dark. Dilute the primary stock 1 in 1000 with ethanol (99–100% w/v) in a volumetric flask to give a 1 ng/mL secondary stock standard. Using a positive displacement pipet, take 250 µL of 2°C stock into a 100-mL volumetric flask and dry down at 37°C under nitrogen. Make up to 100 mL with stripped plasma. Mix and incubate at 37°C for 30 min. Allow to reach room temperature and equilibrate overnight at 4°C. Bring to room temperature while mixing before assaying to check value. This standard is equivalent to 7.68 nmol/L T_3. Serially dilute a 25 mL aliquot with stripped plasma to produce the required calibration range. Aliquot and freeze. Stable for 1 yr.
10. The presence of any particulate matter in the liquid TSH reagents may indicate deterioration of the kit.
11. The use of borosilicate glass or plastic tubes is recommended owing to their low luminescence background and NSB. Store glass tubes in the dark.
12. Close the bottle of antibody-coated beads immediately after use. Do not remove the desiccants.

13. It is essential that thorough washing of the beads is carried out. If performing this step manually, add the wash from a repeating dispenser with some force and ensure that the bead is completely dry after aspiration.
14. Since the entire chemiluminescent reaction is completed in 2 s, each bead can be read only once. It is, therefore, important that luminometer performance checks are carried out before use.
15. Allow reconstituted vials to stand for 30 min, then mix thoroughly by gentle inversion.
16. Always handle the cells with care. Any damage to the membrane may cause leakage of serum proteins and an elevated result.
17. It is essential to maintain a steady 37°C ± 0.5°C during the dialysis incubation. The cells may need to be weighted to keep the sample below water level in a water bath.
18. The RIA washing step is an important step in the assay procedure. Thorough and complete aspiration of the wash solution is essential. An automatic tube washer may be used.
19. Reconstituted TSH receptors are unstable, therefore use only sufficient vials for immediate use in the assay. Discard any excess reagent.
20. Subsequent freezing and thawing or increases in storage temperature must be avoided. Incorrect storage of serum samples can lead to loss of TRAb activity. Do not use grossly haemolysed or lipaemic serum samples.
21. Nonspecific tracer binding in the assay should be less than 15%. Tracer binding to receptors in the presence of negative serum should be more than 25%.
22. TSH concentrations greater than 100 mU/L may interfere in the assay. Sera with unusually low or unusually high protein concentrations give erroneous results.
23. Increased sensitivity of the Tg antibody assay can be achieved by carrying out the standard assay procedure (2 × 1-h incubation) at 37°C rather than room temperature. Maximum sensitivity is achieved by carrying out the first incubation (diluted serum plus labeled Tg) overnight (18 h) at 37°C and the second incubation for 1 h at 37°C.
24. The assay time of either the TgAb or TPOAb assay can be reduced by adding diluted serum, tracer and protein A suspension together, incubating for 30 min at room temperature followed by addition of diluent and centrifugation. This procedure can be used as a rapid

screening assay and has a sensitivity of 3 U/mL for Tg and 1U/mL for TPO antibodies.

25. Settling of protein A suspension occurs on standing and the bottle must always be mixed end-over-end gently for about 20 s immediately prior to use. It is not necessary to mix the bottle while taking aliquots for the assay.
26. Values of greater than 0.3 U/mL can be considered definitely positive for Tg antibody and TPO autoantibody. Although low levels of autoantibody indicate that the process of thyroid autoimmunity is occurring, this is not necessarily associated with abnormalities of thyroid function.

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6

Assays for LH, FSH, and Prolactin

Michael J. Wheeler

Summary

In the clinical laboratory the reproductive hormones are probably the most commonly measured hormones after the thyroid hormones. More than 300 laboratories participate in the UK National External Quality Control Scheme. In addition, investigations into reproduction and fertility in humans and animals remains a major area of research. Illustrative methods are described for the three reproductive hormones (leutinizing hormone, follicle-stimulating hormone, prolactin). Radioimmunoassay, immunoradiometric assay, and enzyme assays are described to give a wide choice of assay formats. There are many commercial assays available and illustrative ones are described. Possible interferences are discussed and procedures for investigating their presence and removal are given.

Key Words: Leutinizing hormone (LH); follicle-stimulating hormone (FSH); prolactin; heterophilic antibodies; macroprolactin; immunoassay.

1. Introduction

The reproductive peptide hormones, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin are secreted from the anterior pituitary. Secretion of LH and FSH are stimulated by gonadotrophin-releasing hormones from the hypothalamus

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and inhibited by negative feedback control by the reproductive steroids from the gonads and the inhibins *(1)*. Prolactin is under the control of dopamine that acts by inhibition. Increased prolactin secretion results from anti-dopaminergic drugs, stress, pituitary adenomas, pregnancy, and suckling. LH and FSH act on the gonads of both sexes stimulating steroidogenesis and gamete maturation. Although prolactin is involved in milk production in the breast, there is evidence that it also influences steroidogenesis in the gonad as well as the adrenal gland *(2–4)*. In vitro experiments suggest that there are optimum levels of prolactin for normal steroidogenesis and both low and high levels result in reduced steroidogenesis. Patients with a prolactin-secreting tumor of the pituitary often have reduced gonadal activity leading to low testosterone levels in men and oligomenorrhoea or amenorrhoea in women. Because of their involvement in reproductive function these three peptides are commonly measured in the investigation of infertility and gonadal dysfunction *(5)*.

2. Materials

2.1. Simple Radioimmunoassays for LH, FSH, and Prolactin

1. Standard preparations for the peptides may be obtained from the National Institute of Biological Control, South Mimms, UK.
2. Polyclonal antibody can be obtained from a number of companies, e.g., Cambio Ltd, Cambridge, UK.
3. Peptides for iodination may be obtained from the National Institute of Biological Control, South Mimms, UK. Methods for iodination have been described in Chapter 1.
4. Donkey or goat anti-rabbit immunoglobulin (IgG) (Guildhay Ltd, Guildford, UK).
5. Nonimmune rabbit serum (Guildhay Ltd).

2.2. Shortened Radioimmunoassays for LH, FSH, and Prolactin

1. Standards, tracer, and antibodies as **Subheading 2.1.1.**
2. Polyethylene glycol (PEG) 6000 (Sigma-Aldrich Company Ltd, Poole, Dorset, UK).

2.2.1. Human FSH IRMA

Kit supplied by Diagnostics Products Corporation, Los Angeles, CA, contains:

1. Monoclonal antibody (MAb)-coated polystyrene assay tubes (*see* **Note 1**).
2. Liquid iodinated FSH polyclonal antibody reagent.
3. Bottles of calibrator at seven concentrations in liquid form.
4. Concentrated buffered saline (*see* **Note 2**).

2.2.2. Human LH ELISA

Kit supplied by IBL Immuno-biological Laboratories, Hamburg, Germany and contains:

1. 12 × 8 well break apart strips.
2. 6 calibrators including a zero calibrator.
3. Liquid anti-LH antiserum conjugated to horseradish peroxidase.
4. Liquid substrate solution containing tetramethylbenzidine .
5. Liquid stop solution: 0.5 *M* sulfuric acid

2.2.3. Rat EIA for Prolactin

Kit supplied by Société de Pharmacologie et d'Immunologie, France and contains:

1. A 96-well plate coated with mouse anti-rabbit IgG.
2. Rat prolactin tracer.
3. Rat prolactin standard.
4. Assay buffer.
5. Concentrated wash buffer.
6. Ellman's Reagent.
7. Tween-20 detergent.
8. Quality control (QC) sample.

2.3. Use of Blocking Tubes to Remove Heterophilic Antibody Interference

1. Tubes supplied by SkyBio Ltd, Wyboston, England.

2.3.1. PEG Precipitation Method for Investigating the Presence of Macroprolactin in Samples

1. PEG 6000 (Sigma-Aldrich Company Ltd).

2.3.2. Column Chromatography Method for Investigating the Presence of Macroprolactin in Samples

1. Sephacryl S 300 (Pharmacia Ltd, Milton Keynes, UK).

3. Methods

Although there have been significant changes in immunoassay technology, there has been remarkably little change in the values produced by these assays. LH is perhaps the exception because immunometric assays have been associated with a decrease in the reported LH concentration in blood of about 30%. Nevertheless the old radioimmunoassays (RIAs) produced results similar to the immunometric assays used today and were capable of similar sensitivities if optimized correctly.

3.1. Radioimmunoassays for LH, FSH, or Prolactin

3.1.1. Simple Radioimmunoassays for LH, FSH, or Prolactin

An example of a simple RIA used 30 yr ago, which is still valid today is as follows:

1. 100 µL standard/sample.
2. 100 µL antibody dilution.
3. Incubate overnight at 4°C.
4. 100 µL radioactive LH solution.
5. Incubate overnight at 4°C.
6. Add 500 µL second antibody solution.
7. Incubate overnight at 4°C.
8. Centrifuge, aspirate, and count the radioactivity in the precipitate.

This assay, therefore, takes 4 d before results are produced. The introduction of PEG 6000 into the separating reagent reduced the separation stage to 30 min or less. Sac-cell (IDS Ltd, Tyne and Wear, England), which has second antibody attached to cellulose particles, has a

similar purpose. Using Sac-cell as precipitating reagent saves having to optimize a second antibody-separating reagent. This latter reagent contains donkey or goat anti-rabbit or anti-mouse antibody (depending on the animal species used to raise the primary antibody) and PEG 6000. PEG 6000 is usually used at a concentration of 8% but the two other reagents have to be optimized. Further experimentation and the availability of polyclonal antibodies with higher affinity showed that the incubation steps could be shortened without compromising the clinical utility of the assays. Together these modifications shortened the assay considerably.

3.1.2. Shortened Radioimmunoassays for LH, FSH, or Prolactin

1. 100 µL sample + 200 L assay buffer + 100 L antibody solution.
2. Incubate overnight at 4°C.
3. 100 µL iodinated hormone.
4. Incubate at room temperature for 6 h.
5. 500 µL separating solution (PEG second antibody solution).
6. Incubate at room temperature for 15 min.
7. Centrifuge at 1500*g* for 20 min.
8. Decant supernatant and count for 60 s.

The availability of MAbs and a solid phase sandwich technique led to the development of noncompetitive or reagent excess assays. In the sandwich the two antibodies are raised against two different epitopes of the antigen increasing the specificity of the assay. One antibody, the capture antibody, is bound to a solid phase and the other antibody is iodinated or conjugated to an enzyme. In the latter case, after the final incubation, substrate is added to give a color, chemiluminescence, or fluorescence. Other labels are also used, for example the rare earth chelates europium and terbium. Today commercial assays use either two MAbs or a polyclonal and a MAb.

Both MAbs and polyclonal antibodies can be purchased from several suppliers and it is relatively simple to prepare tracer. Details for the latter have been given in the Chapter 1.

Automated immunometric assays using reagent excess, controlled incubation at 37°C and precision timing and engineering under sophisticated software and has allowed manufacturers both to automate the assays and reduce the assay time dramatically. Such assays on the Bayer Advia Centaur now take only 9 min. The automated assays for LH, FSH, and prolactin are nonradioactive, mostly chemiluminescent, immunometric assays and virtually every clinical laboratory measures them on a daily basis. Therefore, it is not difficult to find a laboratory willing to measure small to medium numbers of samples. The cost of an assay is approx £1 to 1.50, although a laboratory is likely to charge at least twice this amount (much more for commercially financed studies). Therefore, it is less expensive and may be more practical to set up an in-house assay or buy a commercial kit. Manual assays using radioactive, enzyme, fluorescent, and chemiluminescent are available, although the first two are more common.

Human assays cannot be used to measure these hormones in lower vertebrates, such as rats and mice, because there are significant differences. Specific rat assays are available commercially and one is described in **Subheading 3.2.** The main problem with animal work is the limited amount of blood available if animals are not to be sacrificed. As the assays require 50–100 µL serum and each sample should be analyzed in duplicate at least 250 µL of blood is required for each hormone.

3.2. Example of Currently Available Commercial Manual Assays

The following assays are described to show the variety of assays available.

3.2.1. Human FSH IRMA

The method is a solid phase immunoradiometric assay. Monoclonal anti-FSH antibody is coated on the inside of a polystyrene assay tube. Sample is added and the endogenous FSH is bound to

the immobilized MAb. Iodine-125 labeled polyclonal anti-FSH antibody is added and a sandwich is formed with the endogenous FSH at the center. Excess reagents are washed away and the radioactivity bound is counted. The amount of radioactivity is proportional to the amount of hormone present in the sample.

1. Label tubes in duplicate for total, calibrators, QCs, and patient samples.
2. Pipet 100 µL of calibrator, QC, and patient sample into appropriate tubes (*see* **Note 3**).
3. Add 100 µL iodinated FSH to all tubes.
4. Shake for 60 min on a rack shaker.
5. Decant off liquid and add 2 mL wash buffer.
6. Wait 2 min and repeat the process.
7. Decant the liquid as completely as possible.
8. Count the radioactivity in the tubes for 1 min.
9. Read off concentrations from the calibration curve (*see* **Notes 4** and **5**).

3.2.2. Human LH ELISA

The assay is microtiter based with a MAb directed against the β-subunit of LH coated onto the inside of the wells. Patient sample is incubated in the wells and the LH becomes bound to the bound antibody. A second antibody directed against the α-subunit of LH and conjugated to horseradish peroxidase is then added. A sandwich is formed with the LH at the center. The amount of conjugate bound is determined by adding a substrate for the enzyme. The color that develops is proportional to the concentration of LH.

1. The desired number of microtiter wells is fixed into the holder supplied (*see* **Note 6**).
2. 25 µL standard, control, and patient sample are added to the appropriate wells (*see* **Note 7**).
3. 100 µL enzyme conjugate is added to all the wells.
4. Mix the plate well for 10 s.
5. Seal with adhesive foil and incubate for 30 min at room temperature.
6. Shake out contents and wash five times with distilled water.
7. 100 µL substrate solution are added to all wells.

8. Incubate for 10 min at room temperature.
9. Add 50 µL stop solution to all wells and mix.
10. Read at 450 × 10 nm with a microtiter plate reader within 10 min of adding stop solution (*see* **Note 8**).

3.2.3. Rat EIA for Prolactin

Rat prolactin has been labeled with acetylcholinesterase (AChE). This tracer competes with prolactin in the sample for binding sites of a specific rabbit anti-rat prolactin antiserum. The complex formed binds to mouse monoclonal anti-rabbit antibody bound to the wells of a microtiter plate. After washing, Ellman's Reagent is added to the wells followed by AChE that reacts with the Ellman's Reagent to form a yellow color. The intensity of the color is proportional to the amount of prolactin present in the sample. Both serum and plasma samples may be used.

1. Add 100 µL assay buffer to the blank wells (*see* **Note 9**).
2. Add 50 µL assay buffer to the maximum (zero) binding wells.
3. Add 50 µL of each standard in duplicate to the standard wells.
4. Add 50 µL QC sample to the two QC wells.
5. Add 50 µL of each sample in duplicate.
6. Add 50 µL AChE tracer to each well.
7. Add 50 µL rat prolactin antiserum to all wells except the blank wells.
8. Incubate overnight at room temperature.
9. Tip out the well contents and shake.
10. Wash the wells five times with 300 µL reconstituted wash buffer.
11. Add 200 µL Ellman's Reagent to each tube.
12. Incubate in the dark at room temperature for about 90 min (*see* **Note 10**).
13. Read the developed color in a plate reader at a wavelength between 405 and 414 nm.
14. Construct a standard curve and read the concentrations of the samples from it (*see* **Note 11**).

The previous methods demonstrate the simplicity of the methods available for the measurement of these peptides. The immunoradiometric assay is quickest and most simple because no color development is required. However, arrangements have to be in place

for the storage and disposal of radioactivity. Immunometric assays are faster than competitive assays because they use reagent excess enabling shorter incubation times.

3.3. Problems Encountered in the Measurement of LH, FSH, and Prolactin

Despite the sophistication of modern assays, they are all prone to interference *(6,7)*, which can give rise to erroneous and misleading results *(8)*. In the most serious cases such results can lead to misdiagnosis and wrong treatment. Some of the causes of interference and the methods to detect interference are given next.

Heterophilic antibodies: all assays that use MAbs are subject to interference by heterophilic antibodies. These latter antibodies may bind to either or both of the MAbs interfering with the binding of the monoclonal to the hormone. Ismail et al. *(9)* have recently reported such interference in the LH, FSH, and TSH assays used in their laboratory. They found interference could occur in any of the three assays but not necessarily all three at once. Such interference may be investigated in three ways *(10)*.

1. Suspicious samples may be sent away to another laboratory for analysis in a different assay system. However, Ismail et al. *(9)* found one case where two assays for TSH gave suspicious results and this could also occur in assays for other hormones.
2. Dilution of sample in assay diluent, hormone-free serum, or zero calibrator. When there are interfering proteins or IgGs present there is frequently, but not always, lack of parallelism. At least two dilutions should be carried out and preferably three.
3. Removal of interference from heterophilic antibodies by using heterophilic blocking tubes.

3.3.1. Use of Blocking Tubes to Remove Heterophilic Antibody Interference

1. Holding the tube upright, gently tap the bottom of the tube on a hard surface to bring the blocking reagents to the bottom of the tube.
2. Uncap the blocking tube and add 500 µL serum to the bottom of the tube.

Table 1
Examination of Interference in LH and FSH Assays

Case	LH IU/L FSH IU/L	Post-blocking	Mean on dilution
50/F	11.0	21.5	21.5
	99.0	105	106
42/F	84.0	7.6	90.3
	4.4	7.9	4.6

3. Cap the blocking tube and invert five times to mix the sample with the blocking reagent.
4. Incubate at room temperature for 60 min.
5. Assay the treated sample along with a pretreated aliquot and compare the results.
6. Where the results are significantly different, interference may be considered present.

Table 1 shows the results in two samples when the LH and FSH assays were tested for interference. Greater interference was found in a TSH assay where 25 samples showed interference with only 9 showing an abnormality in both the dilution study and after incubation in heterophilic blocking tubes. Five showed interference after dilution but not after blocking and 11 showed interference after blocking but not in the dilution study. The authors also showed that interference could result in both higher or lower results. Two lessons may be learned from this paper that, although it focuses mostly on TSH assays, is still applicable to LH, FSH, and prolactin assays. First, when investigating interference in these assays, more than one test should be carried out and second, interference is unpredictable and any suspicious results should be investigated thoroughly. The authors give a clinical case where one patient, who turned out to be normal, was investigated over 2.5 yr, had 15 consultations, 77 laboratory tests, a CT scan, and inappropriate drug treatment.

Crossreaction: there is very little crossreaction with similar peptides when using MAbs in immunometric assays. LH, FSH, TSH, and

human chorionic gonadotropin (hCG) all have an α- and a β-subunit. The α-subunit is common in all four hormones, whereas the α-subunit is different in each and confers biological activity. LH and hCG are similar structurally and have similar biological activities. Immunoassays for LH that employed polyclonal antibodies used to have a significant crossreaction with hCG. Nearly all current immunometric assays have no detectable crossreaction with hCG. This was investigated in 2002 by the UK National Quality Assessment Scheme for LH. They found that a sample containing about 16,000 IU/L hCG gave a LH result of approx 5 IU/L in the DPC Immulite assay, 7 IU/L in the DPC Immulite 2000 assay, 5.5 IU/L in the Bayer Immuno-1 assay, and 1.5 IU/L in the Abbott AxSym and IMx assays. All other assays found undetectable LH concentrations in this sample. The previous sample would be equivalent to about 2–4 wk after the first missed period. At 8 wk hCG levels may reach 200,000 IU/L in singleton pregnancies, leading to a further 10-fold increase in the LH result reported by the previous assays. The crossreaction of the Immulite hCG assay has also been reported by Vivekanandan et al. ***(11)***. It is therefore important to be aware of such crossreaction otherwise there may be confusion in the interpretation of results.

Macroprolactinaemia: three main forms of prolactin circulate in the body: monomeric (MW 23 kD), big (MW 50–60 kD), and big, big prolactin (MW 150–170 kD) ***(12)***. The latter form has been shown to be bound to an IgG antibody and is termed macroprolactin. Although it shows biological activity in vivo, it is thought to be too big to enter cells or bind to receptors, and therefore in vitro it is not biologically active ***(13)***. However, some recent reports have questioned this ***(14)***, although it is unclear whether the prolactin level still remains above the reference range after treatment with PEG (described next). It is now apparent that all assays are affected by the presence of macroprolactin, although some assays detect it more readily than others.

The concentration stated previously, where macroprolactin is investigated, varies between laboratories but a large proportion use 700 mIU/L as the cut-off level. The presence of macroprolactin has been investigated in two ways. One is by precipitation of the

macroprolactin with PEG 6000 ***(15)***, and the other by the more laborious gel filtration on a column of Sephadex ***(16)***.

3.3.2. PEG Precipitation Method for Investigating the Presence of Macroprolactin in Samples

1. Prepare a solution of 25% PEG 6000.
2. Add 200 L PEG solution to 200 L patient serum (*see* **Notes 12** and **13**).
3. Mix well and centrifuge at 1500*g* for 30 min.
4. Measure the prolactin concentration in the treated sample.
5. Correct for dilution (*see* **Note 14**) and calculate the percentage of prolactin (*see* **Note 15**).

As suggested previously, some assays detect macroprolactin more commonly, e.g., Wallac DELFIA, but this does not mean that such an assay will always give a higher result than an assay that detects macroprolactin less frequently, e.g., Bayer Advia Centaur. **Figure 1** shows a chromatographic profile of a sample that gave a result of 1800 mIU/L (reference range for normals < 500 mIU/L). In the Wallac DELFIA assay, the prolactin was only 700 mIU/L. After PEG precipitation the Bayer Advia Centaur assay gave a result of 70 mIU/L and after column chromatography the Wallac DELFIA assay gave a result of 50 mIU/L.

3.3.3. Column Chromatography Method for Investigating the Presence of Macroprolactin in Samples

The gel chromatography used in the large number of studies has varied. The one given here was used in the study of Fahie-Wilson and Soule ***(16)***.

1. Prepare a 1.5 × 50 cm column of Sephacryl S-300.
2. Apply 1 mL of serum to the top of the column and run in.
3. Elute with 10 m*M* Tris-HCl buffer pH 7.0 containing 140 m*M* sodium chloride, 1.25 m*M* calcium chloride, and 0.5 m*M* magnesium chloride at a flow rate of 0.5 mL/min.
4. Discard the first 30 mL.
5. Collect 1 mL fractions of the next 40 mL.
6. Measure the prolactin in each fraction.

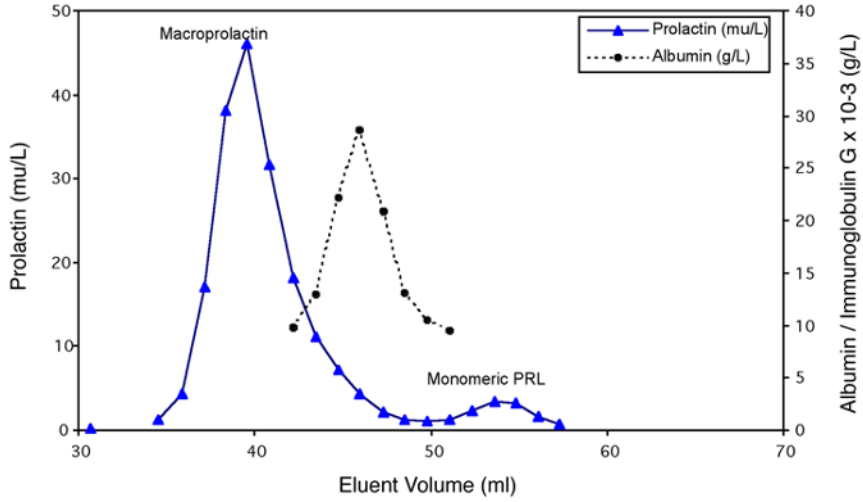

Fig. 1. Separation of macroprolactin and monomeric prolactin in a patient sample on Sephacryl Gel.

A typical profile is given in **Fig. 1**. As mentioned previously some methods detect macroprolactin more than other methods. For example, the Wallac DELFIA method detects macroprolactin more frequently than the Bayer Centaur method, which at first was thought to be free from interference. There are some samples, however, which contain macroprolactin and result in higher results in the Bayer method compared to the Wallac method. **Figure 1** was such a sample. The Bayer Centaur result for this sample was 1800 mIU/L, but was only 700 mIU/L when measured by the Wallac DELFIA method. After PEG precipitation the Centaur method gave a result of 70 mIU/L and the DELFIA method 50 mIU/L. Therefore the detection of macroprolactin is both method and sample dependent.

4. Notes

1. 100, 200, and 500 tube kit sizes are available.
2. The working wash solution is prepared by diluting 40 mL of the concentrate with 400 mL of distilled water.

3. Method specifies use of serum only.
4. Material should be pipeted directly to the bottom of the tubes.
5. Intra-assay precision is quoted as <4% and inter-assay precision as <6%. Quoted analytical sensitivity is 0.06 IU/L.
6. The kit is supplied with 8 × 12 well strips that can be snapped so that only the required number of wells is used.
7. Manual pipetting should be completed within 5 min.
8. The within-assay imprecision is quoted as <7% and the between-assay precision as <10%. Crossreaction with hCG is 2.5% which means that high LH levels will be reported in pregnant women.
9. An example of a well set-up is shown in the kit insert. The first well of each row has been left empty. This seems rather odd and any user should be aware that edge effects might occur.
10. No clear instruction for the last incubation is given, although it is suggested that an orbital shaker should be used if available. An example of typical results is given following a 90 min incubation at room temperature on an orbital shaker. Presumably development of the color would take longer without shaking.
11. The reported intra-assay precision is <10% and the inter-assay precision <14%.
12. Some methods may be affected by PEG solution.
13. It is good practice to rerun the undiluted sample at the same time as well as a diluted QC.
14. Owing to the change of matrix in the diluted sample a correction other than two may have to be applied. For example, Bayer recommend a correction factor of 2.7 following studies involving gel chromatography.
15. The percentage of macroprolactin chosen as significant varies between centres between 40 and 60%. When there is below 40% recovery, most studies have found no significant monoprolactin. When there is above 60% recovery, there is no significant amount of macroprolactin. When there is between 40 and 60%, there is usually a preponderance of macroprolactin but not always.

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7

Adrenocorticotrophic Hormone and Related Peptides

Les A. Perry

Summary

This chapter describes how adrenocorticotropin (ACTH) can be measured in biological fluids. The principle can be applied to similar peptide hormones. Following an introduction of the principle of the method, details are given on sample collection and storage, and materials required. Detailed descriptions are given for the methodology with a "Notes" section that gives practical tips on the more complex or difficult steps in the analytical process. A brief bibliography lists peer reviewed articles relevant to the development and application of this method.

Key Words: Adrenocorticotropin (ACTH); immunoassay; radioiodination.

1. Introduction

Adrenocorticotrophic hormone (ACTH) is a peptide secreted by the anterior pituitary. Secretion is stimulated by corticotrophin-releasing hormone (CRH) produced by the hypothalamus. ACTH stimulates the adrenal cortex to synthesize cortisol. ACTH production is controlled by a feedback mechanism of cortisol on the hypo-

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thalamus and the anterior pituitary. ACTH can be secreted ectopically by some tumors. ACTH measurement is an important marker in the differential diagnosis of Cushing's syndrome where the resulting hypercortisolism is owing to either a pituitary tumor (Cushing's disease), ectopic ACTH production, or an adrenal adenoma/carcinoma *(1)*.

Measurement of small peptide hormones in biological fluids is used in research and routine clinical biochemistry. Small peptide hormones whose concentrations in body fluids are at or below the minimum detection limits of a fully optimized radioimmunoassay (RIA) can be concentrated by extraction with particulate adsorbents. The commonly used adsorbents are particulate silicates (e.g., Fuller's earth, glass microbeads). More convenient, ready-prepared and disposable mini-columns have been developed (most notably Sep-Pak columns from Waters Associates). These have been used for the extraction and concentration of a wide variety of steroid and small peptide hormones *(2)*. This extraction procedure can also improve the specificity of an assay by separating the ligand from interfering proteolytic enzymes.

RIAs can suffer from lack of sensitivity, frequent radioiodination (although the use of non-isotopic labels is increasing), limited assay size, and a long turn-around time (less important with research). Many modern assays for ACTH and related peptides now use monoclonal antibodies in a "two-site" immunometric format. By using antibodies to two distinct epitopes on the ligand, the assay will measure only those molecules that contain both epitopes. This approach does overcome some of the disadvantages experienced with RIAs. One disadvantage of immunometric assays is that they may become too specific—especially in some clinical situations. For example, some ACTH-secreting tumors secrete fragments that are bioactive but do not react in an immunometric assay *(3)*.

The procedure described next for ACTH has also been used to measure growth hormone-releasing hormone (GHRH) *(4)*, CRF *(5)*, and β-endorphin *(6)* in human and animal plasma and tissue culture extracts. This ACTH assay uses an antibody raised in sheep to the N-terminus of the ACTH molecule and detects intact ACTH 1-39

and its larger forms 22K pro-ACTH and proopiomelanocortin (POMC). This is of great clinical significance because specific immunoradiometric assays (IRMAs) will not detect these larger forms and can thus lead to errors in diagnosis, particularly those with the ectopic ACTH syndrome.

The assay uses an initial extraction procedure followed by a late addition of tracer RIA. ACTH is extracted from plasma onto powdered glass (Vycor), then washed and eluted into acetone. The acetone extract is evaporated and the residue reconstituted in assay buffer. Standards, quality controls (QCs), and patient samples (at a minimum of four doubling dilutions) are incubated with antiserum for 24 h, then further incubated for 48 or 72 h following the addition of ^{125}I-ACTH. Dextran-coated charcoal suspension is used for the separation of antibody-bound and free hormone. The radioactive counts recorded in the charcoal pellet are inversely proportional to the concentration of ACTH in the tube *(7)*. This assay takes 4 d to complete and is considered to be a reference method for plasma ACTH measurement in the United Kingdom.

1.1. Specimen Type, Collection, and Storage

The process of sample collection should ensure that the material that goes into the assay tube is as near identical to what was present in the circulation of the patient. Normally, analyte stability is not a problem. However, some analytes are degraded rapidly in serum or plasma by endogenous enzymes unless steps are taken to prevent this. The principal means is by rapid separation of the plasma in a refrigerated centrifuge following when the plasma is "snap-frozen" then stored and transported frozen. The sample is then only thawed on the day of analysis. For some analytes the addition of enzyme inhibitors (e.g., trasylol) and/or reducing agents (to minimize the oxidation of methionine residues as on ACTH *[8]*) may also be required to further reduce the rate of degradation.

All syringes, pipetes, and containers used in the collection and storage of ACTH samples must be plastic because ACTH is adsorbed onto glass.

The patient does not need to be fasted prior to sample collection. It is important when collecting samples that the following criteria are satisfied:

1. The patient must sit in a quiet area with a canula inserted at least 30 min prior to sample collection (*see* **Note 1**).
2. The time at blood collection must be recorded (*see* **Note 2**).
3. ACTH samples are collected into a chilled lithium heparin tube, cold centrifuged immediately, and the plasma separated and flash frozen. Samples are stored and transported to the laboratory frozen (*see* **Note 3**).

The assay requires a minimum of 1.2 mL plasma, although usually 3 mL plasma is used (*see* **Note 4**).

If a sample arrives in the lab thawed, it is discarded. Partially thawed samples are acceptable provided the sample is immediately flash frozen. If provided samples remain frozen, they are stable for at least 6 mo.

Haemolysed samples are not suitable for ACTH analysis because the result is rapidly lowered ***(9)***. Icteric and lipaemic samples should be avoided wherever possible.

2. Materials

1. Sterile saline (sodium chloride injection BP; 9 g/L).
2. Vycor (50 mg/mL). Weigh 2 g activated Vycor into a disposable 100-mL plastic pot (*see* **Note 5**). Measure 40 mL sterile water with a measuring cylinder and add to the Vycor. Continuously mix using a magnetic flea on a magnetic stirrer until required for the assay.
3. 20% Human serum albumin (HSA).
4. Donor Horse Serum.
5. 2-Mercaptoethanol.
6. Charcoal (decolorizing purified).
7. Dextran T-70.
8. Acetone (60 % v/v). In a 50-mL stoppered glass measuring cylinder add 24 mL acetone followed by 16 mL water. Stopper, then **gently mix** by inverting the cylinder several times until the two solutions are thoroughly mixed.
9. Synthetic human ACTH (1–39) for standards and iodination.

10. ACTH antiserum raised "in house" in sheep against synthetic ACTH (Synacthen 1–24 ACTH) conjugated to thyroglobulin by the gluteraldehyde method.
11. C18 Sep-Pak column chromatography cartridge.
12. 0.5 *M* phosphate buffer, pH 7.4. Weigh 28.56 g disodium hydrogen orthophosphate and 6.75 g sodium dihydrogen orthophosphate into a 500-mL glass measuring cylinder and make up to 500 mL with sterile water then place on a magnetic stirrer and continue to mix using a magnetic flea until all the phosphate has dissolved. Cover with parafilm and mix by gentle inversion a few times. Check that the pH is 7.4 using a pH meter (*see* **Note 6**). Aliquot 10 mL into conical plastic tubes, stopper, and label with the reagent name and date of manufacture.
13. Assay buffer. Take aliquots of 0.5 *M* phosphate buffer (10 mL) and HSA (2.5 mL). Thaw using "hand hot" tap water (*see* **Note 7**) and transfer both aliquots to a 500-mL glass bottle then add 90 mL sterile water with a measuring cylinder. Ensure all reagents are thoroughly mixed. Transfer the reagent to a fume cupboard, then while continuously swirling by hand, **slowly** add 0.5 mL 2-mercaptoethanol using a micropipet (this is what is later referred to as "smelly buffer"). Transfer the buffer solution to a 150-mL disposable plastic screw-top pot and add five to six drops of phenol red (*see* **Note 8**).
14. Tracer: ^{125}I-ACTH *Caution*: Hazard Reagent

    This is an "in-house" manufactured reagent. The iodination protocol is:

    a. In a glass bottle, mix 99 mL methanol + 1 mL TFA (MeOH/TFA). Do in a fume hood.
    b. In a glass bottle mix 99 mL water + 1 mL TFA (water/TFA). Do in a fume hood.
    c. In 10-mL plastic tubes prepare 0, 30, 40, 50, 60, and 80% MeOH/TFA solutions diluted with water/TFA.
    d. Weigh 10 mg chloramine-T into a 10-mL conical tube then stopper until required.
    e. Prepare 10 mL 0.05 *M* phosphate buffer (dilute 0.5 *M* phosphate 10 times with sterile water).
    f. Prepare 2 mL 0.25 *M* phosphate buffer (diluting 0.5 *M* phosphate twice with sterile water).
    g. Take 5 mL "smelly buffer" saved from the previous day from the fridge and bring to room temperature.

h. Take iodination ACTH vial from freezer and bring to room temperature.
i. Number LP3 tubes 1–25.
j. Remove the syringe plunger from the syringe attached to the top of the Sep-Pak C18 cartridge. Using a Finn pipet, wash the cartridge with 3 × 0.6 mL 80% MeOH/TFA solution, followed by 3 × 0.6 mL 60% MeOH/TFA.
k. Then working in a well ventilated fume hood, add to the ACTH iodination vial 10 µL 0.25 *M* phosphate buffer followed immediately with 10 µL ^{125}I (1mCi).
l. Add the 10 mL 0.05 *M* phosphate buffer to the chloramine-T solution, mix thoroughly, and transfer 10 µL to the iodination vial. Secure the stopper, mix for 30–45 s.
m. Add 0.6 mL "smelly buffer," mix, then transfer to the Sep-Pak cartridge, and push through the cartridge. Collect the filtrate in a LP3 tube. Count the radioactivity in the tube and in the empty iodination vial.
n. Add 0.6 mL 0% MeOH/TFA solution, insert syringe plunger, and push solution through the column. Collect the filtrate into LP3 tube.
o. Remove plunger and repeat process twice more, each time collecting the filtrate into a new LP3 tube.
p. Repeat **steps n** and **o** using 30, 40, 50, and 60% MeOH/TFA solutions though with the 60% solution this is done a total of four times.
q. Repeat **steps n** and **o** using 80% MeOH/TFA solution until only background radioactive counts are being eluted (normally approx 8X).
r. Count the radioactivity in all fractions eluted with MeOH/TFA. The two fractions with the highest radioactivity (normally fractions 2 and 3) of the 60% MeOH/TFA solution are pooled into a 10-mL plastic screw top tube and made up to 5 mL with 60% MeOH/TFA solution then made up to 9 mL with "smelly buffer."
s. The tube is stoppered, mixed, and labeled appropriately, then stored at –20°C.
t. The tracer is tested in a RIA. The tracer batch is acceptable if the NSB is <3% and 0 standard binding is >60%. The tracer is replaced every 3 wk usually because the NSB is >12% or the 0 standard binding has fallen too low.

u. Calculate the yield of the iodinated ACTH by expressing the total radioactive counts for the two fractions from the 60% MeOH/TFA elution as a percentage of all the counts recorded for every tube. This can then be translated as a percentage of the 1mCi started with and hence a specific activity can be derived.

15. Standards. Prepared "in-house" according to the protocol described next:
    a. Assay aliquots.
        i. Make 100 mL buffer in a disposable plastic screw top container by mixing 10 mL 0.5 *M* phosphate buffer pH 7.4 (removed from freezer, allowed to thaw and ensure that all the phosphates have redissolved and thoroughly mixed), 2.5 mL human serum albumin and 90 mL sterile water. **This makes 102.5 mL buffer as 100 mL will be needed later.**
        ii. Make 10 mL 10 m*M* HCl, by adding 0.1 mL 1 *M* HCl to 9.9 mL sterile water in a container labeled with the reagent name and hazard note.
        iii. Weigh as near to 100 µg ACTH in a plastic LP3 cap on a microbalance (*see* **Note 9**). Record the actual amount dispensed. Transfer into a plastic LP3 tube by inverting a tube onto the cap, turn the tube into an upright position, and tap the ACTH powder down to the bottom of the tube. Dissolve in 10 m*M* HCl to achieve a master stock solution of 1 µg ACTH/2 µL (therefore if weighed 105 µg dissolve in 210 µL).
        iv. Weigh into a plastic container 100 g buffer using the top pan balance. Add 10 µL ACTH master stock solution to the buffer. Flush the disposable plastic tip several times with the buffer. This submaster standard is 5 µg ACTH/100 mL or 50 ng/mL (*see* **Note 10**).
        v. Aliquot (0.5 mL) with a plastic syringe ACTH standard into plastic LP3 tubes. Cap firmly and store at –20°C. The sub master standard is stable for 1 yr. The tubes are labeled with the analyte name, date, and concentration (*see* **Note 11**).
        vi. The calibration of new standards is checked at least twice against the old standards and, finally, patient results derived from the new and existing standards are also compared for agreement. Keep all records relating to calibration. A new

standard is prepared about 2 mo before the old standard time expires to allow sufficient time to review the new calibration. It is not uncommon for the standard to not be 40 ng/mL, this is because of the problems of trying to accurately weigh ACTH as it absorbs water.

b. Iodination aliquots.
   i. Prepare at the same time as the assay standards, using the remains of the master stock 1 mg ACTH/2 mL.
   ii. Pipet 10 µL master standard into plastic screw-cap iodination vials. Label each vial with the date and analyte and store at 20°C. Iodination ACTH is stable for 1 yr. Each aliquot contains 5 µg ACTH by weight, equivalent to 4 µg ACTH as the starting material is only 80% pure.

16. Quality control and assessment. Three internal QC pools are prepared "in house." QC 1 (approx 50 ng/L), QC 2 (approx 200 ng/L), and QC 3 (approx 20 ng/L). Each pool (500 mL) is prepared in "time expired blood transfusion plasma" by spiking with appropriate volumes of the sub master ACTH standard. Aliquots (125 × 3.5 mL) are dispensed into plastic stoppered tubes, labeled with the analyte name, QC pool name and date of manufacture, and stored at –20°C. One aliquot of each QC is taken per assay and measured at the beginning (immediately after the standards) of the assay. QC pools are renewed after 1 yr (*see* **Note 12**).
17. The following additional materials are required:
    a. Refrigerated centrifuge.
    b. Bench top centrifuge with suitable buckets to hold 10-mL plastic tubes.
    c. Vortex mixer.
    d. Rotary mixer.
    e. Magnetic stirrer.
    f. Glass graduated measuring cylinder (to measure 50 and 100 mL).
    g. Disposable plastic graduated pipets (5 and 10 mL) (*see* **Note 13**).
    h. Glass Pasteur pipets.
    i. Micropipets and disposable tips for 10–40, 40–200, 200–1000 µL dispensing.
    j. Micropipet and disposable tips for repeated 50, 100, 250, and 1000 µL dispensing.
    k. Cling film.

l. Polystyrene tubes and caps LP3 (75 × 10 mm).
m. 10-mL conical plastic tubes (stoppered).
n. 10-mL plastic round bottomed tubes (95 × 16 mm).
o. Volumetric flask (1000-mL).
p. Rack to hold 10-mL tubes.
q. Rack to hold LP3 tubes.
r. Disposable screw-top plastic containers (25- and 100-mL).
s. Glass bottles 2 × 500-mL.
t. Glass dispensers (2) 1000-mL to deliver 2 or 3 mL.
u. Vacuum pump suitable for attaching to water tap.
v. Heating block and sand bath.
w. Multi-channel manifold with tubing connected to nitrogen cylinders.
x. Hamilton MicroLab 900 (Reagent syringe size 1000 µL/ Sample syringe size 500 µL).
y. Multihead γ-counter.
z. Multicalc data reduction package on computer linked to the γ-counter.

3. Methods

The maximum number of samples that can be done in one assay is 35 (includes QCs).

3.1. Sample Preparation

Each assay has unextracted standards (assayed in triplicate), a horse serum blank, and the three QC samples (1, 2, and 3). Samples are numbered for the work list and kept frozen until ready to start the assay. The volume of sample used in the assay depends on the volume available and the expected result (*see* **Note 14**).

3.2. Assay Day 1

Involves extraction of ACTH from the plasma sample/QCs/standards using Vycor, reconstitution in assay buffer then setting up the first step of the late addition of tracer RIA. As only one extraction is performed on each sample (no replicate check on the extrac-

tion process). It is very important that great care is taken at all times during the assay.

1. Take one set of ACTH QCs (1, 2, and 3), 2 × 10-mL aliquots of horse serum and samples that need to be assayed and allow to thaw by placing in a rack in "hand hot" tap water.
2. All samples, horse serum aliquots, and QCs are thoroughly vortex mixed prior to commencing the assay. Patient samples (and occasionally a QC sample if particulate) are then spun at 1000*g* for 10 min at 4°C in the MSE Coolspin centrifuge to remove fibrin and other debris.
3. Label clearly the plastic assay conical extraction tubes (10-mL) with the assay sequence (1 = horse serum blank, 2 = QC1, 3 = QC2, 4 = QC3, 5 = patient sample, 6, 7, 8, and so on) as the numbers can be erased during the assay procedure.
4. Take one aliquot of ACTH standard from freezer and bring to room temperature (usually about 15 min).
5. Pipet QC/sample into the appropriately labeled plastic conical tubes using a 5-mL plastic disposable graduated pipet and bulb pipet holder: CHANGE THE PIPET AFTER DISPENSING EACH QC OR SAMPLE.

   The volume of sample actually taken **must** be accurately measured and recorded.
6. To each tube add 1 mL Vycor suspension with a 1-mL hand pipet (*see* **Note 15**).
7. Stopper tubes, vortex mix, and place on a circular rotator for 30 min (*see* **Note 16**).
8. Transfer tubes to bench top centrifuge and spin for 10 min at 1000*g*. After centrifugation aspirate the supernate to waste using the sink water vacuum.
9. Add 3 mL sterile water to each tube then vortex thoroughly to break up the pellet. **Do not restopper**. Transfer tubes to bench top centrifuge and spin for 10 min at 1000*g*. After centrifugation aspirate the supernate to waste.
10. Add 2 mL 1 *M* HCl from the dispenser to each tube, then vortex thoroughly to break up the pellet. Transfer tubes to bench top centrifuge and spin for 10 min at 1000*g* at room temperature. After centrifugation aspirate the supernate.

11. Add 1 mL 60% acetone to each tube using a new 10-mL graduated plastic pipet. Stopper each tube then vortex thoroughly to break up the pellet, then rotate for 30 min.
12. Transfer tubes to a bench top centrifuge and spin for 10 min at 1000*g*. After centrifugation transfer the supernatant to a plastic 10-mL round bottom tube using a separate glass Pasteur pipet for each sample.
13. Place the tubes in a sand bath at 60–70°C and evaporate the solvent to dryness under a stream of oxygen free nitrogen (*see* **Note 17**).
14. Label LP3 tubes (*see* **Note 18**).
15. When dry, remove the tubes from the sand bath, place in a rack on the bench, then add 0.5 mL assay buffer to each tube using a 200–1000-µL hand held micropipet.
16. Reconstitute the residue by vortex mixing the contents of each tube for 10–20 s (*see* **Note 19**). To ensure full reconstitution vortex all tubes, then repeat vortexing process.
17. Transfer 400 µL of reconstituted sample into the appropriately labeled LP3 tube "A" with a 200–1000-µL micropipet in order to prepare the doubling dilutions. Use a clean pipet tip for each sample/QC/standard dispensed.
18. Using a 200–1000-µL micropipet, to the four tubes labeled NSB add 250 µL assay buffer; while to the four tubes labeled ZERO add 200 µL assay buffer. Put to one side in the assay rack because these tubes will not be involved in the doubling dilution process.
19. Double dilute 200 µL vol in assay buffer (not the NSB or ZERO) using the Hamilton MicroLab 900 dilutor 20.
20. Prepare the antiserum solution (*see* **Note 20**).
21. Dispense 50 µL freshly prepared antiserum solution to all tubes (except NSB + total counts tubes). Vortex mix all tubes, cover with cling film. Incubate overnight at 4°C.

    Day 2: involves the addition of the tracer solution then leave to incubate for 48 h (*see* **Note 21**).
22. Prepare the tracer solution (*see* **Note 22**).
23. Remove assay tubes from the fridge.
24. Immediately, dispense 50 µL 125I-ACTH to all tubes including the four "total count tubes." Stopper the total count tubes. Vortex all tubes (except the total counts) cover with cling film and incubate at 4°C for 48 h.

    Day 4 ***(5)***: Involves the separation of the assay using dextran-coated

charcoal, counting the radioactivity in the charcoal pellet, and calculating the final result using the Multicalc data reduction package.

25. Prepare dextran-coated charcoal suspension about 30 min before use (*see* **Note 23**).
26. After incubation remove the cling film and add 200 µL dextran-coated charcoal suspension to every tube except the TC tubes (*see* **Note 24**). Thoroughly vortex mix the contents of each tube then transfer to a centrifuge and spin at 1500*g* for 15 min at 4°C. After centrifugation aspirate the supernate down the designated radioactive sink.
27. The total count tubes and assay tubes are transferred to the multi-well γ-counter. The residual radioactivity in each assay tube is counted until at least 10,000 counts have been recorded in the total counts tubes (100 s).
28. The calculation of the ACTH results is achieved using a data reduction program written by Wallac for use on the multi-well γ-counter and the Multicalc data reduction package. The program has to be informed of assay position number, sample volume extracted, and the number of dilutions performed. The program will then print a final ACTH concentration.

4. Notes

1. Failure to obtain blood at the first attempt may invalidate the results of subsequent venepuncture. In particularly anxious patients the ACTH concentration can rise to twice the upper limit of normal, which is why a sample 30 min after the insertion of a butterfly needle is recommended.
2. Plasma ACTH and concurrent serum cortisol concentrations have a circadian rhythm of secretion, so the time of sampling is important for interpretation.
3. Within 10 min of venepuncture, separate the plasma using a refrigerated centrifuge.
4. It is recommended that a "spare" sample is collected and stored in case the original sample thaws in transit or the assay fails. It is recommended that at least 10 mL of blood is collected.
5. Vycor is no longer commercially available, however, please contact the Immunoassay lab to discuss availability from our lab.

6. If the pH needs adjusting use a drop or two of the concentrated sodium hydroxide solution.
7. Mix until all the phosphate has dissolved. Remember to mix the HSA solution by gently inverting the stoppered tube several times.
8. Phenol red is to generate a pink colored solution. When added to the dried extracts the solution should remain pink, indicating the pH is still acceptable for the assay.
9. Ensure the air conditioning is not switched on while weighing takes place.
10. Equivalent to 40 ng/mL ACTH as the starting material is only 80% pure. ACTH purity should always be checked as it does vary with batches and taken into consideration when calculating the stock ACTH solution.
11. Tubes are prepared in advance. When the standard is dispensed the aliquots can be frozen immediately.
12. When a new pool is introduced, it should be run with the existing pool for at least seven assays in order to derive performance limits before the existing pool is exhausted or becomes time expired.
13. Do not use recycled washed pipets as the detergent impedes the performance of the assay—always use new pipets.
14. If a high result is expected use a smaller volume (usually not less than 2 mL), if a low result is expected then the maximum available sample should be taken (5 mL is the maximum). Occasionally, samples of less than 1 mL are analyzed only when a high result is expected.
15. Vycor is continuously mixing on a magnetic stirrer. It is easier to add if the end of the disposable tip has been cut off with a scalpel.
16. This stage must be reached within 1 h of thawing the samples.
17. This usually takes up to 1 h. It is important not to leave the residue to dry for too long. It can get too dry and become loose from the bottom of the tube and be lost.
18. Label LP3 tubes in the sequence:

total counts tubes	(in quadruplicate)
NSB tubes	(in quadruplicate)
ZERO binding tubes	(in quadruplicate)
standard	(in triplicate) A-H (i.e., 8 tubes)
horse serum blank	(as singleton) A-D (i.e., 4 tubes)
QC 1 pool	(as singleton) A-D (i.e., 4 tubes)
QC 2 pool	(as singleton) A-F (i.e., 6 tubes)
QC 3 pool	(as singleton) A-D (i.e., 4 tubes)

patient 1	(as singleton) A-D/F/H (i.e., 4/6/8 tubes depending on expected result)
patient 2	(as singleton) A-D/F/H (i.e., 4/6/8 tubes depending on expected result)

Continue until all tubes for patient samples have been labeled. The higher number of tubes are used when a high result is expected and therefore more dilutions are made.

19. The buffer should stay pink. If it turns yellow (a sign that the sample had not completely dried) adjust the pH with 1 *M* sodium hydroxide (minute amount [insert a wetted disposable pipet tip—ensure no volume dispensed] until solution turns pink).
20. Prepared on the day of use immediately before required. Working stock solution is diluted 20% with assay buffer in a disposable plastic screw top container. Make the required amount for the assay plus an extra 1 mL (usually 10–12 mL).

    Add 11.4 mL of assay buffer using a new disposable 10-mL graduated pipet into a disposable plastic screw top container. Transfer 0.6 mL working stock antiserum solution with a 200–1000-µL dispenser into the assay buffer. Secure the screw top lid and ensure the contents are thoroughly mixed.
21. The incubation can be left for 72 h if the separation step was to fall over a weekend.
22. This is prepared on the day of use immediately before addition. For the RIA, dilute the stock tracer in assay buffer to give 10,000 counts/100 s/50 µL (±5%). Usually involves the transfer of 15–20 µL of stock solution with a micropipet into a disposable plastic screw-top container with 12 mL assay buffer (measured with a new disposable 10-mL graduated pipet). Check the counts in 50 µL: count for 100 s then adjust as appropriate to give 10,000 counts/100 s/50 µL.
23. Take 0.5 *M* phosphate buffer (5 mL) and horse serum (30 mL) from freezer. Thaw in hand hot tap water and thoroughly mix at room temperature to ensure all the phosphate has dissolved and the horse serum is evenly mixed. Transfer the phosphate buffer to a 500-mL glass container and add 15 mL sterile water measured with a cylinder. Mix the reagents with a magnetic stirrer and flea. Meanwhile, weigh 0.375 g Dextran T-70 and add to the continuously mixing buffer solution. Weigh 1.5 g charcoal and add this to the mixing solution **only after the dextran has dissolved**. Add 30 mL donor horse serum to the charcoal solution and continue to mix on the

magnetic stirrer for a further 15 min. Transfer the dextran-coated charcoal suspension to a 100-mL plastic container with a small flea and continue to mix while the reagent is being added to each assay tube.

24. Mix the suspension a few minutes prior to addition to ensure all the charcoal is in suspension and continue mixing while the charcoal suspension is added to all tubes.

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8

Measurement of Glucocorticoids in Biological Fluids

Geoff Holder

Summary

The background to the physiological production of glucocorticoids is described as well as their concentrations in some biological fluids. Detailed methods are described for the collection, storage, and preparation of samples and for the immunoassay techniques for some common steroids in serum, urine, and saliva. Alternative methods (high-performance liquid chromatography, gas-liquid chromatography, Gas chromatography-mass spectrometry) that have greater specificity and/or sensitivity are also discussed, together with automated procedures able to analyse large numbers of samples rapidly. Information and references are presented on the analysis of less commonly measured glucocorticoids, including synthetic steroids such as dexamethasone and prednisolone.

Key Words: Chemiluminescence; dexamethasone; glucocorticoids; mineralocorticoids; prednisolone; radioimmunoassay; saliva; urinary-free cortisol.

1. Introduction

In humans, two adrenal glands lie near the kidneys. They are made up of two areas, the cortex and the medulla. These have different embryological origins. The cortex is divided into morpho-

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logically different areas of cells called the zona glomerulosa, zona reticularis, and zona fasciculata. Steroids produced by the cortex have a number of vital biological functions that are concerned with electrolyte balance (mineralocorticoids) and carbohydrate metabolism (glucocorticoids). This division is not absolute because many corticoids may have both activities. The zona reticularis and fasciculata are thought to act as a unit that under the stimulation of pituitary adrenocorticotrophic hormone (ACTH) produces the principal glucocorticoid, cortisol, as well as androgens. When there is a physiological deficiency, cortisol or synthetic steroids, such as prednisolone or dexamethasone, are used in replacement therapy. Steroids may be used in treatment for their anti-inflammatory properties.

There is a close structural similarity between glucocorticoids. This can lead to analytical problems. Often, it is necessary to have a specific endpoint to the analytical procedure or incorporate steps to separate compounds before detection. This may be further complicated by the low concentrations in some biological fluids. Steroids can be found in blood, urine, and saliva. Analysis in each of these fluids poses problems. Measurement in other media, such as tissue culture, may add further complications. The choice of method is affected by several factors including the type of sample to be analyzed, the steroid to be analyzed, the speed of analysis, the relative accuracy required, and the specificity of the method. Other factors, such as the invasive nature of some tests, can also influence the choice of procedure. **Table 1** shows the concentration of some common glucocorticoids in biological fluids.

Some of the original methods for measuring corticosteroids used fluorimetric or colorimetric endpoints. These often lacked specificity and required large volumes of sample. Immunoassays largely superseded these techniques and are probably the method of choice at present. Although reagents are available for in-house development of methods, many of the common steroids can be analyzed by commercial kits. Despite their ease of use, caution should be observed when analyzing samples for which the kit was not designed, such as from other species or in unusual matrices.

Table 1
Concentration of Glucocorticoids Found in Various Biological Fluids in Normal Subjects

	Serum/plasma total	Serum/plasma free	Urine	Saliva	Amniotic fluid
Cortisol	196–650 nmol/L *(8)*	16.6 ± 8.3 nmol/L *(9)*	<350 nmol/24 h	8.00–9.30 12.5 ± 4.9 nmol/L (SD) 22.00–23.30 3.0. ± 1.0 nmol/L (SD) *(10)*	17.9 nmol/L <20wk 38.3 nmol/L 28–37 wk *(11)*
Cortisone	40–84 nmol/L	—	—	—	—
11-Deoxycortisol	26–46 nmol/L at 9.00 h *(12)*	—	—	—	7.5 nmol/L throughout pregnancy *(11)*
Corticosterone	2.3–23 nmol/L *(13)*	—	—	—	—
21-Deoxycortisol	Male 199 ± 104 pmol/L Female 280 ± 79 pmol/L *(14)*	—	—	—	—

1.1. Measurement of Cortisol

Cortisol circulates in the blood bound to a specific binding protein, cortisol-binding globulin (CBG), as well as being weakly bound to other plasma proteins. The effect of this protein binding must be removed or neutralized to enable accurate measurement. Solvent extraction and addition of agents that compete or eliminate binding are commonly used, but heating of diluted plasma to denature CBG also works. Related problems exist with the measurement of other glucocorticoids, so the measurement of cortisol will be used to illustrate the techniques available.

2. Materials

2.1. Common

1. Positive displacement pipets and tips are necessary when measuring accurate volumes of nonaqueous solvents.
2. Dichloromethane is the most commonly used solvent for the extraction of cortisol. The purest grade available will reduce nonspecific interference from solvent residue. Extractions should be carried out in a suitable fume-cupboard. Tubes for the extraction should have necks wide enough to permit easy access to the liquid layers.
3. Any suitable cortisol antiserum can be used. Most have significant crossreaction with prednisolone. Use after testing the titer. It is best to make up a primary stock that is stored frozen in aliquots large enough for a single assay. On the day of analysis, an aliquot can be thawed then diluted in buffer ready for use.
4. Many forms of label have been employed in immunoassays, both isotopic and nonisotopic. Although manual methods with nonisotopic endpoints have been developed, they are more frequently used on automated systems. Radioactive labels may be tritium-based with measurement by liquid scintillation counting or a gamma label such as ^{125}I. These can be obtained from Amersham Biosciences, 1,2,6,7-3H Cortisol (cat. no. TRK407) and Cortisol-3-CMO-(2 ^{125}-Iodohistamine) (cat. no. IM 129), respectively.
5. Separation of antibody bound and nonbound label has been tried by most if not all methods used for other immunoassays. The most con-

venient tend to be solid phase: either tubes or antisera. Sac-Cel is a commercial solid-phase second antibody. Anti-Sheep/Goat immunoglobulin (Ig)G solid phase second antibody-coated cellulose suspension (Sac-Cel; IDS LTD cat no. AA-SAC2) is stored at 4°C. Bring suspension to room temperature and mix gently before use.

2.2. Serum and Plasma

Samples should be collected in plain tubes (no anticoagulant), allowed to clot, and the serum removed to a plain tube after centrifugation. Serum can be stored at 4°C until analysis although freezing at –20°C is best if this is delayed for more than 48 h.

2.3. Saliva

1. Samples can be collected by placing a tube against the lower lip and collecting 1–2 mL of saliva (*see* **Note 1**). It may help if the subject thinks of food. Avoid collecting samples soon after vigorously cleaning teeth. Patients with gum disease may have interference from bleeding in the mouth (*see* **Note 2**).
2. Samples should be centrifuged to eliminate solid matter. Mechanical devices are available for collecting directly from the salivary glands (*see* **item 3**).
3. Sarstedt Ltd supplies a tube device called a salivette. This has an inner 5-mL tube, with a hole in the bottom that contains a dental pad. This, in turn, fits inside a plastic conical tube. The cotton wool pad is placed in the mouth for about 2 min until it is saturated with saliva. It is then placed in the inner tube and the whole device placed in a freezer. This breaks down the mucin and liquefies the saliva. The tube can then be taken out of the freezer, the pad thawed, and the whole device centrifuged for about 10 min at 1750*g*. The saliva is pulled down through the hole in the inner tube into the conical tube. The inner tube and pad may be discarded.

2.4. Urine

1. A 24-h urine specimen should be collected into a clean, plain container with no preservative (*see* **Note 3**).
2. Store the container in a cool place during the collection period.

3. The total volume of the urine collection should be measured upon receipt and recorded with the date and time of collection.
4. Urine aliquots should be stored at –20°C upon receipt in the laboratory and thawed overnight at 4°C prior to analysis.

2.5. Other Biological Materials

These are best collected and frozen as soon as possible until analysis.

3. Methods

3.1. Serum or Plasma

3.1.1. Heat Denaturation

1. Accurately dilute 50 µL serum samples with 1 mL distilled water in a plastic tube.
2. Place the rack containing the diluted samples in a water bath at 60°C for 30 min.
3. Allow to cool at room temperature.
4. Suitable aliquots of the denatured sample can be used in specific radioimmunoassay (RIA) *(1)*. Higher temperatures and different dilutions of the serum have been used by Erkens *(2)* who heated the diluted samples at 80°C.

3.1.2. Solvent Extraction (Work in a Fume Cupboard)

1. Add 0.5 mL serum to a wide-necked screw-top glass tube.
2. Accurately dispense 5 mL cold dichloromethane to the tube (*see* **Notes 4** and **5**).
3. Screw on the lid.
4. Place the tubes on a vortex mixer for 10 min ensuring the two phases are mixed.
5. Allow the phases to separate for 10 min.
6. Aspirate the upper serum layer using a glass pipet attached to a water pump. Ensure that the waste is disposed of in compliance with local regulations.
7. Carefully remove 1 mL of the solvent phase to a suitable glass tube.

8. Place the tubes in a water bath at 45°C in a fume cupboard to evaporate the solvent. The rate of evaporation can be increased by gently blowing nitrogen or filtered air onto the surface of the liquid.
9. Add suitable buffer to the dry residue (*see* **Note 6**). Vortex mix and leave for at least 15 min.
10. Remix the extracts. Suitable aliquots of the extract can be used in specific RIAs.

3.1.3. pH

1. Accurately dilute 50 µL plasma samples or standards with 200 µL 0.125 *M* phosphate citrate buffer pH 4.0 containing 0.1% gelatin.
2. Add suitably diluted solid-phase antibody ***(3)*** diluted in pH 4.0 buffer.
3. Add 200 µL ^{125}I-Cortisol in pH 4.0 buffer.
4. Incubate 2 h then add 3 mL assay buffer.
5. Centrifuge 10 min, aspirate supernatant, blot quickly by inverting the tubes onto absorbent paper.
6. Count the tubes.
7. Values can be interpolated from the standard curve.

3.1.4. Displacement of Binding

1. Dilute 50 µL serum or standard with 200 µL 0.1 *M* phosphate buffer pH 7.4 containing 8-anilinonaphthalene sulfonic acid (5 g/L) and ^{125}I-Cortisol. Add 200 µL solid-phase cortisol antiserum ***(4)***.
2. Incubate 60 min at 20°C.
3. Wash three times with 0.9% saline.
4. Centrifuge, aspirate supernatant, and count the tubes.
5. Values can be interpolated from standard curve.

Other blocking agents such as 1% salicylate in the assay buffer have also been used ***(5)***.

3.2. Urinary-Free Cortisol

3.2.1. Radioimmunoassay

Although methods for UFC without solvent extraction have been described, interference from steroid conjugates in the urine is often

Table 2
Addition of Reagents to Dried Down Urine Extract

	Totals µL	NSB µL	STDS µL	Samples/IQC/ solvent blank
Standard inassay buffer	—	—	100	—
Assay buffer	—	200	—	100
Vortex mix all tubes and leave to stand at RT for 10 min before adding the next reagent.				
Antiserum	—	—	100	100
Label	100	100	100	100
Vortex mix all tubes and incubate for 1 h at 37°C in a water bath.				

a problem. Most UFC assays include extraction of the cortisol using dichloromethane (*see* **Note 7**). After evaporation (*see* **Note 8**) of the solvent the extract is analysed by RIA with specific antiserum and ^{125}I-Cortisol as a label. A solid phase second antibody (Sac-Cel) is used to separate free from bound hormone. The concentration of cortisol in the sample is inversely proportional to the radioactivity bound and is determined from a standard curve.

1. Bring the buffer standards, urines, and the appropriate IQCs to room temperature.
2. Pipet 250 µL of patient sample or IQC sample in duplicate into numbered 10-mL glass tubes. Include duplicate solvent blank tubes.
3. Add 5 mL of dichloromethane (in a fume cupboard) to all tubes using an appropriate solvent dispenser. Cap the tubes and vortex for 10 min. Allow the tubes to stand at room temperature for 10 min until the aqueous and solvent phases have separated fully.
4. Aspirate the upper aqueous phase to waste.
5. Using a positive displacement pipet, transfer 600 µL of each sample extract (solvent phase) into glass assay tubes. Wash the pipet tip several times with dichloromethane between pipetting samples. Evaporate to dryness (usually 20–30 min). Gentle heating in a water bath with pumped, filtered air or nitrogen will assist evaporation (*see* **Note 9**).
6. Dilute antiserum and tracer in buffer (0.05 *M* pH 7.0 sodium phosphate with 0.1% w/v gelatin and 0.1% w/v sodium azide) and then add the following reagents as shown in **Table 2**.

7. Remove the Sac-Cel from the refrigerator and place on the agitating mixer for 20 min.
8. Add 100 µL of Sac-Cel to all tubes except the totals. Ensure that the Sac-Cel remains in suspension by stirring with a magnetic stirrer during addition. Vortex mix the tubes and incubate at RT for 30 min.
9. Add 1 mL of wash solution (0.9% w/v saline [18 g NaCl in 2 L of distilled or deionised water] and 0.25% v/v Tween-20 [Merck, cat. no. 663684B]) to all tubes except the total counts. Do not remix. Centrifuge the tubes for 20 min at 1750*g*.
10. Aspirate the supernatant to waste taking care not to disturb the precipitate.
11. Count the assay tubes and plot the standard curve. Results for the urine can read off the curve using a suitable software package.
12. Discard the radioactive waste, solvent, and tubes according to the locally approved protocol.
13. The UFC concentration should be corrected for the 24-h vol to calculate the total excretion.

3.3. Steroid Extraction Methods

Removal of the steroid of interest from the endogenous sample for analysis may be necessary to improve sensitivity by concentration or specificity by elimination of contaminating materials. Thin layer chromatography, paper chromatography, Celite, or preparative high-performance liquid chromatography have all been used followed by measurement by RIA or UV absorption. Such techniques are not always suitable for large numbers of samples and require significant experience to yield useful results.

3.4. Automated Methods

Cortisol is so frequently measured for clinical purposes that, unlike many other glucocorticoids, automated methods have been produced. These mainly rely on immunoassay with a nonisotopic endpoint and accurate pipeting of reagents and incubation times that are under microprocessor control ***(6)***. Most of these machines can achieve a result rapidly with little manual intervention. Some of the methods currently available are listed in **Table 3**.

Table 3
Some Automated Systems for Cortisol Measurement

Instrument	Principle	Endpoint	Manufacturer
Abbott TDx	Fluorescence polarisation	Fluorescence	Abbott, Maidenhead
ACS 180 and Centaur	Paramagnetic particles	Chemiluminescence	Bayer,Newbury
Immuno I	Magnetic particles	Alkaline phosphatase	Bayer, Newbury
Access	Paramagnetic particles	Chemiluminescence	Beckman, High Wycombe
Immulite and Immulite 2000	Coated beads	Alkaline phosphatase	DPC, Llanberis
Advantage	Magnetic particles	Chemiluminescence	Nichols, Heston
Elecsys	Paramagnetic beads	Electrochemiluminescence	Roche, Lewes
Tosoh A1A	Magnetic microbeads	Kinetic Fluorescence	Eurogenetics Ltd, Redditch
Delfia	Coated microtiter plates	Time resolved fluorescence	Wallac

3.5. Highly Specific Methods

Methods such as high-pressure liquid chromatography (HPLC), gas liquid chromatography (GLC), and gas chromatography mass spectroscopy (GC-MS) can have a high specificity for glucocorticoids by exploiting the physico-chemical properties of the different compounds. They have a limited analytical capacity compared to immunoanalyzers or even manual RIA but may be able to quantify several steroids at the same time. Sample preparation is often complex and may involve hydrolysis of conjugated steroids and derivitisation to produce volatile compounds prior to injection or application in a machine. Some sample preparation methods are shown in **Table 4**.

It is difficult to generalize about assays for measuring glucocorticoids and some of the reagents such as 3H-labeled steroids that were available some years ago have been withdrawn. Nevertheless the following section contains advice that may assist in producing the best possible assay.

4. Notes

1. The viscous nature of saliva samples can be eliminated by freezing the samples at –20°C, thawing, and then centrifugation. The supernatant is usually a clear watery liquid.
2. If unexpected values are obtained it is important to be aware of the problems of gum disease or the effects of teeth cleaning. Damage to the gums can cause bleeding into the mouth with subsequent contamination of the specimen. Hemoglobin measurement has been used to detect such contamination.
3. Sometimes, routine laboratories specimens are collected and acidified for catecholamine measurement. These are not suitable for the measurement of UFC.
4. Use a pure extraction solvent to avoid solvent blanks. Solvent of Aristar or equivalent grade is preferred.
5. Use positive displacement pipets to dispense solvents. Air displacement pipets are frequently inaccurate when used with nonaqueous solvents.

Table 4
Examples of Methods Used to Measure Glucocorticoids.

	Method	Reference
Cortisone, serum	radioimmunoassay (RIA)	Wood *(15)*
Urine	Dilute 100 µL serum with 400 µL Tris-HCl buffer pH 7.5 containing bovine serum albumin (BSA) (5 g/L). Add 4.0 chloroform and extract for 4 min. Centrifuge for 10 min, then aspirate the aqueous phase to waste, and accurately transfer 1.0 solvent to a glass assay tube. Evaporate the solvent with an air stream. Add 100 µL diluted antiserum and 300 µL 125I labeled cortisone, vortex mix and incubate for 2 h at 37°C. Add 100 µL pre-mixed Sac Cel to each tube, vortex mix, and leave for 30 min. Add 1.0 mL of distilled water. Centrifuge, 3500 rpm, 4°C for 15 min. Aspirate the supernatant to waste. Count the assay tubes and calculate concentration.	
Dexa methasone	RIA 50 µL plasma diluted with charcoal stripped plasma. Chromatograph on Sephadex G25. Chromatograph on paper benzene:methanol:ethyl acetate H_2O 1:1:0.1:1. Dissolve extract in phosphate buffer/gelatin. Incubate overnight. 4°C with specific antibody, 3H label. Separate free from bound with dextran-coated charcoal.	Kream *(16)*

Prednisolone	HPLC 1.0 mL plasma + 0.1 internal standard. Extract with 7.0 mL dichloromethane. Centrifuge, discard plasma. Wash 3× with 2.0 mL 0.1*M* HCl, 0.1 *M* sodium hydroxide, deionised H_2O. Evaporate solvent under vacuum. Reconstitute with 0.75 mL methanol:H_2O (40:60 v/v) Inject 30 µL onto reverse phase C6 column. Detect at 254 nm.	Cheng ***(17)***
11 deoxycortisol	RIA 50 µL sample diluted with 450 µL 0.1 *M* citrate/0.2 *M* phosphate buffer, pH 4.0. Add 200 µL 125I labeled deoxycortisol and 250 µL antiserum. Incubate at room temperature for 1 h. Separate free from bound with 500 µL dextran-coated charcoal. Centrifuge, aspirate supernatant and count charcoal.	Perry ***(12)***
	Enzyme Immunoassay Extract 100 µL serum with 2 mL dichloromethane. Remove solvent and evaporate under a stream of air. Dissolve extract in 200 µL cyclohexane:benzene:methanol 6:2:1 v/v. Apply to Sephadex LH-20 column (0.8 × 5.7 cm). Elute with the same solvent and collect the fraction containing 11 deoxycortisol. Evaporate the solvent and reconstitute with 100 µL 0.05 *M* phosphate buffer pH 8.0 containing 0.5% normal rabbit serum, 0.25% BSA and alkaline phosphatase labeled 11 deoxycortisol.	Kobayashi ***(18)***

(*continued*)

Table 4 ***(continued)***

	Method	Reference
	Add 500 µL buffer containing diluted antiserum. Incubate at room temperature for 60 min. Add 100 µL second antibody. Incubate 16 h at 4°C. Add 2 mL deionized H_2O, centrifuge, aspirate and measure the enzyme concentration.	
Corticosterone	RIA Dilute 200 µL plasma with 500 µL H_2O. Extract with 5 mL hexane, centrifuge then discard solvent. Re-extract with 5 mL dichloromethane. Chromatograph the extract on 0.5 g Celite premixed with 250 µL ethylene glycol as the stationary phase. Wash twice with 3.5 mL ethyl acetate: isooctane 20:80. Elute with solvent by increasing polarity. The extract is analyzed using 3H label and dextran-coated charcoal separation of free from bound.	Sulon ***(19)***
21-Deoxycortisol	RIA Extract 0.5–3.0 mL plasma containing with dichloromethane. Chromatograph on Whatman No. 1 paper with isooctane:benzene:methanol:water 550:450:200:800 then with the same solvent in the proportion 750:250:100:300. Elute with 0.2% ethylene glycol/H_2O. Incubate 0.4 mL eluate with 3H label, antiserum at 4°C for 1.5 h. Separate free from bound with dextran-coated charcoal.	Milewic ***(14)***

6. When dissolving the residue ensure that all possible material is taken up into solution. There is some evidence that the concentration of protein in the aqueous solvent used to redissolve the residue can affect the amount taken up *(7)*. After adding the liquid, mix thoroughly and allow sufficient time for dissolving the material. Dissolved extracts can be stored at 4°C for up to 48 h.
7. A solvent:urine ratio great enough to ensure good extraction efficiency is required and allows the phases to separate easily. Ratios equal to or greater than 5:1 (Solvent:aqueous phase V/V) are recommended. Consider low speed centrifugation to ensure good separation if emulsions form. Be aware of solvent evaporation and use a closed tube if possible at this stage. This will improve accuracy and helps eliminate possible toxic effects of solvent vapor. Cooling the solvent before use also helps reduce evaporation.
8. Ensure that complete evaporation of the solvent phase occurs because small quantities may not be visible to the naked eye. It may be necessary to displace the remaining vapour from tubes using filtered air or nitrogen. Evaporation may be aided by raising the temperature but do not exceed about 40°C owing to possible oxidation or permanent "sticking" of the residue onto the tube.
9. After evaporation, extracted samples should not be left dry but reconstituted in buffer. Recoveries are often low and variable if extracts are not immediately reconstituted due to the steroid adhering strongly to the tube surface and not dissolving into the solvent.

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9

Urinary Steroid Profiling

Norman F. Taylor

Summary

Urinary steroid profiling provides quantitative information on the steroid biosynthetic and catabolic pathways. It is essential for identification of inborn errors of steroid metabolism, and useful in other disorders with altered steroid secretion. A general method is detailed. Steroids, mostly in the form of glucuronide and sulphate conjugates, are extracted using solid-phase cartridges, followed by enzymatic hydrolysis, re-extraction of freed steroids, formation of methoxime trimethylsilyl derivatives and analysis by sas chromatography and gas chromatography-mass spectrometry. Newborns excrete large quantities of sulphates, so conjugate separation by liquid gel chromatography is used prior to hyrolysis. Normal ranges for adults and children are given, together with advice on chromatogram evaluation and a summary of the profile findings in steroid disorders.

Key Words: Urine; steroids; metabolites; gas chromatography (GC); gas chromatography-mass spectrometry (GC-MS).

1. Introduction

Urinary steroid profiling by high resolution gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS) provides qualitative and semi-quantitative data on steroid excretion *(1–8)*.

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Table 1
Reference Ranges (μg/24 h)

Steroid	Adult male	Adult female
Androsterone	490–2570	< 1610
Aetiocholanolone	180–2424	< 2180
DHA	< 1750	< 800
11β-Oxoaetiocholanolone	< 1280	30–610
11β-Hydroxyandrosterone	370–1340	80–980
11β-Hydroxyaetiocholanolone	< 1540	20–650
16α-Hydroxy DHA	< 1480	< 515
Preganediol	< 1450	< 2430
Pregnanetriol	< 1563	< 890
Androstenetriol	< 1630	< 760
Tetrahydrocortisone	570–5700	370–3510
Tetrahydro-11-dehydrocorticosterone	< 660	40–410
Tetrahydrocorticosterone	10–410	< 360
Allo-Tetrahydrocorticosterone	80–570	< 422
Tetrahydrocortisol	310–2240	250–1510
Allo-Tetrahydrocortisol	190–2220	90–920
α-Cortolone	< 1520	140–1210
β-Cortolone + β-cortol	150–1590	< 1100
α-Cortol	60–700	70–340
Total 11-deoxy-17-oxosteroids	690–5220	< 3750
Total corticosterone metabolites	220–1410	110–1080
Total cortisol metabolites	3000–13,140	1370–8140

(continued)

The GC method is essentially unselective, the reported components being those excreted in greatest amounts (**Table 1**). This information provides a composite picture of the quantitatively major biosynthetic and catabolic pathways. This is not synonymous with biological importance: metabolites of cortisol, progesterone, corticosterone, and testosterone are readily detected, whereas those of oestradiol and aldosterone are not. However, because most steroidogenic enzymes have more than one role, in practice almost all known disturbances of steroid metabolism are amenable to investigation by GC-based profiling. GC-MS provides additional speci-

Table 1 (*continued*)

Steroid	Child 2–6	Child 7–10	Child 11–16
Androsterone	< 52	< 337	<1947
Aetiocholanolone	< 55	< 368	< 1526
DHA	< 7	< 21	< 147
11-Oxoaetiocholanolone	< 95	< 208	< 450
11β-Hydroxyandrosterone	< 215	65–305	< 1114
11β-Hydroxyaetiocholanolone	< 88	< 200	< 399
16α-Hydroxy DHA	< 50	< 100	< 150
Pregnanediol	< 51	< 102	< 624
Pregnanetriol	< 59	< 189	< 724
Androstenetriol	< 23	< 80	< 231
Tetrahydrocortisone	190–1218	543–1655	973–2393
Tetrahydro-11-dehydrocorticosterone	40–196	30–226	6–290
Tetrahydrocorticosterone	5–101	17–109	< 254
Allo-tetrahydrocorticosterone	76–292	88–292	127–423
Tetrahydrocortisol	88–444	312–576	263–1363
Allo-Tetrahydrocortisol	87–587	184–732	159–1535
α-Cortolone	23–363	182–570	372–1104
β-Cortolone + β-cortol	54–426	178–534	247–907
α-Cortol	8–180	60–192	98–362
Total 11-deoxy-17-oxosteroids	< 104	< 704	< 3511
Total corticosterone metabolites	175–535	217–525	203–863
Total cortisol metabolites	683–2979	2107–3611	2737–7041

ficity and sensitivity and overcomes the limitations mentioned previously. Preparation methods are identical.

Inborn errors of steroid metabolism may be investigated using one or more plasma assays, but it is recommended that one profile analysis be performed on every subject with a possible inborn error because confusion between types of deficiency may occur. Several disorders require lifelong treatment so that a definitive identification is efficient in terms of both patient welfare and cost.

Profiling has special relevance to the perinatal period: suspected inborn errors of steroid metabolism may need to be identified rapidly post partum to anticipate life-threatening steroid deficiency and, in the case of sexual ambiguity, to establish the sex of rearing as early as possible. There are marked differences in steroid metabolism at this stage of life and immunoassays developed for children or adults may give discrepant results owing to the presence of crossreactants.

Steroid metabolites are excreted in the urine mainly as free compounds and as glucuronide and sulfate conjugates. All forms may be extracted together using Sep-Pak C18 (ODS silica) cartridges (Waters). This is a reversed phase solid-liquid partition chromatography system. The material absorbs organic compounds in a limited polarity range, which includes steroids, from the aqueous solution. Infants less than 90 d old excrete substantial amounts of 3β-hydroxy-5-ene-steroid sulfates that overlap in retention time on GC analysis with metabolites of cortisol and cortisol precursors, which are predominantly free and glucuronide-conjugated. These groups are separated by prior liquid-gel chromatography on Sephadex LH-20.

Extracted steroids, with or without conjugate separation, are then subjected to conjugate hydrolysis using Helix pomatia digestive juice. This preparation contains glucuronidases that cleave glucuronides, and sulfatases that cleave most 3-sulfates, but may not cleave 17- and 21-sulfates. Following hydrolysis, freed steroids are extracted using Sep-Pak cartridges as before.

Three internal standards are used for the GC analysis to identify steroids of interest by their position and for quantification: 5α-androstane-3α,17α-diol, stigmasterol, and cholesterol butyrate. The first one elutes before the naturally occurring steroids; cholesterol butyrate elutes after them. Stigmasterol is included because it is relatively difficult to silylate and its peak height, in comparison with the other standards, provides a check on the completeness of the derivative. An aqueous wash step is used prior to formation of derivatives to remove polar contaminants: these may retain water or consume the reagents, leading to incomplete reaction.

Methyl oxime-trimethylsilyl ether (MO-TMS) derivatives are used. These render the steroid more stable, more volatile, and greatly

reduces ionic interactions, all of which are advantageous for GC and GC-MS analysis. The ketones are oximated first and then the hydroxyls are silylated.

For GC, a column coated with OV-1 is used. It is nonselective, separating steroids in order of increasing size and polarity. The order of elution depends on the phase type and is independent of column wall composition and conditions of temperature and flow. The GC is fitted with a split–splitless injection port, normally used in the splitless mode. An automated sampler enables extended runs of samples to be set up.

Use of 24-h urine collections is preferable, but random specimens will enable the identification of inborn errors. A short collection may be unavoidable in babies when a diagnosis is not clearly established and treatment cannot be delayed. Reliable 24-h collections may be made from infants using disposable diapers.

Once the sample has been hydrolyzed, it is desirable to retain a portion of this steroid extract in case accidents occur during derivatization and to enable different derivatization methods to be used. The extract is stored in precisely measured volumes of ethanol in screw-cap glass vials at room temperature. In this form, most steroids are stable for long periods, but there is a greater risk of breakdown than for steroids in frozen urine or as derivatives. Derivatives are stable if kept in cyclohexane, and do not show breakdown even after years of storage at room temperature.

2. Materials

2.1. Method for Children Older Than 12 Weeks and Adults

2.1.1. Urine Extraction

1. Sep-Pak C18 cartridges (Waters). The “Classic” type have double female luer ends.
2. Methanol.
3. Glass syringes (10-mL).
4. Plastic universal tubes.
5. Peristaltic pump.
6. Water bath.

2.1.2. Hydrolysis of Conjugates

1. Stock acetate buffer 5 *M*, pH 4.6, is prepared by mixing 78.8 mL glacial acetic acid with 152.8 g sodium acetate trihydrate and making up to 500 mL with distilled water. Check pH using a meter and adjust if necessary.
2. Working acetate buffer (0.5 *M*): prepared by diluting the stock buffer 1:10.
3. L-Ascorbic acid, sodium salt.
4. Helix pomatia (enzyme) (BioSepra, France).
5. Ethanol.
6. Screw cap glass vials.

2.1.3. Addition of Internal and Calibration Standards and Purification

1. Internal standards: three internal standards, 5α -androstane-3α,17α-diol, stigmasterol, and cholesterol butyrate, are made up at a concentration of 1 mg/mL in ethanol. The solutions are stored in Teflon-lined septum-sealed screw-capped vials (Pierce) at 4°C. A working mixture of internal standards is made up at a concentration of 100 µg/mL.
2. Calibration standards: stock solutions of commonly occurring urinary steroid metabolites are made as for the internal standards. Portions of these, representing equal quantities of steroid, are combined with the same quantities of the three internal standards. The derivatized mixture is then used for calibration of the GC integrator or GC-MS system.
3. Ethyl acetate.
4. Sodium bicarbonate (8%): weigh out 8 g of solid sodium bicarbonate ($NaHCO_3$) and dissolve in 100 mL of distilled water.

2.1.4. Derivatization

1. Methoxyamine hydrochloride (MO): prepared as a 2% solution in pyridine. Store at room temperature in the fume cupboard.
2. Trimethylsilylimidazole (TSMI or TSIM): ready to use. Store at 4°C.
3. Pyridine: distilled and stored over sodium hydroxide.
4. Cyclohexane: if provided from a company other than Rathburn Ltd, whatever the grade, it will probably need to be washed

with decolorizing charcoal packed into a column and then double distilled.

2.2. Method for Infants Younger Than 12 Weeks

1. Chloroform.
2. Sephadex LH-20 (Pharmacia).
3. Sodium Chloride.

3. Methods

3.1. Method for Children Older Than 12 Weeks and Adults

3.1.1. Urine Extraction

1. Sep-Pak cartridges (*see* **Note 1**) are primed by passing through 2 mL of methanol using a glass syringe and then, using a different syringe, with 2 mL distilled water.
2. Mix the urine by inversion and pipet 10 mL, if sample quantity allows, into labeled plastic universal tubes (*see* **Note 2**).
3. Rinse the intake tubes for the peristaltic pump lines (*see* **Note 3**) with distilled water and pump distilled water through the lines. Place the primed Sep-Pak cartridges onto the exit ends of the pump lines.
4. Insert an intake tube into each sample. Switch on the pump to pass the urine through the Sep-Pak cartridges into a waste container. Particulates in the urine sometimes block the intake point or accumulate in angles in the lines. Agitate the intake tubes or twist the lines to dislodge these.
5. Once all the urine has passed through, wash down the walls of the universal containers with approx 1 mL water and pass through the Sep-Pak cartridge. Add a further 1 mL water and pass through. Allow the pump to push air through the cartridges to remove excess water. Take cartridges off one-by-one and proceed to **step 6** (*see* **Note 4**).
6. Place the Sep-Pak on to the end of a glass syringe, add 4 mL of methanol to the syringe, and immediately place over a large glass centrifuge tube. Slowly push the methanol through the Sep-Pak in a dropwise manner. It should take at least 20 s to complete the elution. If the sinter is partly blocked and methanol is being pushed past the plunger, wrap a tissue around the syringe barrel to avoid any methanol running down the outside and contaminating the sample. Add

more methanol until 4 mL, judged by eye, of eluate has been collected.
7. Place the tubes in a 60°C water bath and evaporate under nitrogen. Begin drying the samples with the jets at the top of the tubes and lower them as the tube walls dry.
8. Process the Sep-Pak cartridges for storage or reuse by passing through in the reverse direction water, methanol, water and methanol (2 mL each), using glass syringes.

3.1.2. Hydrolysis of Conjugates (see ***Note 5***)

1. Pipet 5.0 mL of 0.5 *M* acetate buffer into each of the evaporated extracts (for urine volumes other than 10 mL, adjust *pro rata*). Sonicate each tube.
2. Add 200 mg sodium ascorbate to each tube or adjust *pro rata* (*see* **Note 6**). This can be measured approximately by using a spatula. Vortex the tubes gently.
3. Add five drops of Helix pomatia juice (or adjust *pro rata*) to each tube and rock the tubes gently to mix.
4. Cap each of the tubes with foil and incubate in a water bath set at 37°C for 64 h. Longer periods are permissible.
5. After the incubation is complete, extract the freed steroids from the sample using the Sep-Pak cartridge extraction procedure as used for urine (*see* **Subheading 3.1.1.**).
6. Place the tubes in a 60°C water bath and evaporate to dryness under nitrogen.
7. Add one drop water to the dry residue and vortex to wet it before accurately adding 1 mL ethanol, then sonicate to resuspend. Transfer to a screw-cap glass vial, add a second 1 mL ethanol, sonicate again, and transfer.

3.1.3. Addition of Internal Standards and Purification

1. Add 25 µL (for urine volumes other than 10 mL, adjust *pro rata*) of working standard solution to silanised test tubes.
2. Add 500 µL (or adjust *pro rata*) of extract to the tube.
3. Evaporate the solvent in a 60°C block.
4. Cool tube and add 1.0 mL ethyl acetate (*see* **Note 7**).
5. Add 200 µL of 8% aqueous $NaHCO_3$ and vortex for at least 10 s to

extract the residues into the aqueous layer. Leave the tube to stand for a few moments and finally give the sides a tap with a fingernail to dislodge any droplets.

6. Take off the bottom aqueous layer with a glass Pasteur pipet by lowering the pipet until it is just below the surface of the aqueous layer and then aspirating while lowering it to keep it just below the surface. This avoids droplets of the aqueous layer sticking to the outside of the pipet and falling back when it is withdrawn. Retain the pipet. Discard the aqueous layer.
7. Add 200 µL of water and vortex. Leave the tube to stand until the aqueous layer is clear, give the sides a tap and, using the retained pipet blotted on a tissue, take off the bottom layer as stated previously. Repeat the water wash once again. After settling, take off the top layer with a fresh pipet and dispense into a stoppered Quickfit test tube.
8. Evaporate the ethyl acetate in a 60°C block.

3.1.4. Derivatization

1. Ensure the residue from **Subheading 3.1.3.** is *completely dry*. Take the tube from the block and before cooling add seven drops of 2% methoxyamine solution. Stopper the tube, sonicate, and then shake the tube and place the in the 60°C block for 1.5 h. Alternatively, leave at room temperature overnight (*see* **Note 8**).
2. Add seven drops of trimethylsilylimidazole (TSIM) to the tube. Shake to mix. Incubate in a 100°C block overnight.
3. After the incubation, evaporate the mixture in a 60°C block for approx 10 min or until the volume is constant. This will be about half the original volume. This removes the volatile pyridine and leaves the relatively involatile TSIM behind.
4. Add 1 mL of cyclohexane at the moment of removal from the nitrogen jet, washing down the tube walls by rotating the pipet or tube (*see* **Note 9**).
5. The TSIM is removed by adding 500 µL of water and vortexing. Centrifuge the tube if the top layer is cloudy. Remove the bottom layer with a glass Pasteur pipet and discard to waste. Repeat this step with a further 500 µL of water. Take a fresh pipet and remove the top layer and transfer to an injection vial. Ensure that no water is accidentally transferred. If it is, the cyclohexane can be transferred to a

second injection vial, leaving the water behind (*see* **Note 10**).

6. If it is intended to concentrate the derivative or have it in a known volume, the solvent may be evaporated and cyclohexane added again. If this is done, the solvent must be added at the same time as the tubes are removed individually from the nitrogen jets. Any delay in solvent addition will destroy the derivative.

*3.1.5. GC/GC-MS (*see ***Note 11)***

A suitable temperature program is: inject at 100°C; increase at 20°C/min for 4 min to 180°C; increase at 2.5°C/min to 275°C; hold for 10 min (*see* **Note 12**).

3.2. Method for Infants Younger Than 12 Weeks

Term infants in the first 3 mo of life and preterm infants up to an equivalent postconceptional age excrete large amounts of 3β-hydroxy-5-ene steroids which are sulfate conjugated ***(9)***. These obscure smaller amounts of clinically significant components (e.g., metabolites of cortisol precursors and cortisol), which are glucuronide conjugated or unconjugated. Conjugates are separated and both fractions are subjected to Helix pomatia hydrolysis. Many components are very polar. For this reason the washing of the extract prior to derivatization is omitted.

After **Subheading 3.1.1.** proceed as indicated next.

3.2.1. Conjugate Separation

1. This method uses 0.9 g Sephadex LH-20. The gel may be kept in the solvent mixture of chloroform:methanol 1:1 saturated with NaCl, or it may be weighed out, adding solvent and allowing it to swell for 30–60 min. The volume occupied by weighed-out gel may be marked on the column for future use of preswollen gel.
2. Rinse a column (*see* **Note 13**) with 1 mL of fresh chloroform:methanol 1:1 solvent.
3. Leaving the tap open, pipet the gel into the column. Rinse the column walls with solvent to dislodge any adhering gel. Allow the gel to settle and top up to the mark. If too much gel is added, add a little solvent to resuspend the gel and remove.

4. Wash the gel with 1 mL of solvent. The gel must not be left to dry, but can be left up to 1 h without damage.
5. Place a glass centrifuge tube under the column. Ensure the dried extract of urine is free from water and dissolve the residue with 0.2 mL of solvent, using shaking and brief sonicating. Apply the sample to the column using a Pasteur pipet, allowing it to drain into the gel.
6. Repeat twice, using 0.2 mL of solvent, allowing each portion to drain into the gel before adding the next. If the column flow is prevented by a crust of solids on top of the gel bed this may be broken up gently with a Pasteur pipet.
7. Repeat using 0.4 mL of solvent, followed by 5.0 mL. Collect the combined eluates (6.0 mL). This is the free (neutral) and glucuronide fraction.
8. Change to a fresh tube. Add 7.0 mL of methanol and collect the eluate. This is the sulfate fraction.
9. Evaporate the solvents under nitrogen in a water bath 60°C.
10. Proceed to hydrolysis (*see* **Subheading 3.2.**).
11. Remove the LH-20 from the columns while still damp with solvent by blowing out with nitrogen. This is accumulated and periodically washed for reuse (washing in a Buchner funnel with deionised water, ethanol, and acetone and drying). Allow the column to dry and clean it for reuse by washing in reverse using a tube connected to a tap followed by rinsing with distilled water and acetone.

3.2.2. Derivatization

Proceed as for **Subheading 3.1.3.**, but add solutions directly to a stoppered Quickfil test tube and omit washing **steps 4–8**.

3.3. Evaluation of GC and GC-MS Data

A valid chromatogram should show peaks for the three internal standards at positions that have been previously established. Peak heights for S and CB should be similar. If S is low relative to CB or even absent the derivative is poor or has failed completely. A repeat using another portion of the urine extract should be made. If there is no extract left, the existing derivative may be remade by repeating the silanization using TSIM.

If using GC as the primary analyzer, the major peaks may be identified by comparing their positions with those in a chromatogram of a standard mixture copied onto an acetate sheet. If the positions of the flanking internal standards are matched, the upslopes of all the peaks eluting between them should match in position within less than the width of a line. GC-MS may be used for full scan analysis, with use of a spectrum library, preferably user-established, for steroid identification. Extracted ion plots may be used for quantification, with higher sensitivity for ion trap than quadrupole instruments. Identification and quantification at higher sensitivity (in the case of quadrupole instruments) of target compounds is achieved with selective ion monitoring.

The identification of additional steroids produced in abnormal traces is best done by comparing the profile with profiles previously obtained in the same condition. This requires some expectation of what the condition is, based on the clinical information supplied and on prior experience. GC retention times show little variation from year to year so that direct comparison of archived results with new results can be helpful. Crucial to this is the evaluation of the whole pattern and not just individual peaks ***(10)***. Identification of a disorder should be consistent with all elements of the chromatogram. The possibility that the patient has received treatment that has not been disclosed should always be considered. Those with major effects are ACTH, glucocorticoids, and enzyme inhibitors, such as metyrapone. The most common situation where this occurs is in infants with salt wasting or hypoglycaemic crisis, who may have been given steroids without this being recorded in the notes.

3.4. Interpretation of Results and Summary of Profile Findings in Steroid Enzyme Deficiencies

1. 3β-Hydroxysteroid dehydrogenase deficiency: There are increased metabolites of 17α-hydroxypregnenolone and DHA with low/absent androgen, corticosterone, and cortisol metabolites.
2. 5α-Reductase deficiency: There is an increased ratio of 5b:5α reduced metabolites of androgens, corticosterone, and cortisol metabolites.
3. 11β-Hydroxylase deficiency: There are increased metabolites of 11-deoxycortisol and androgens. 11-Deoxycorticosterone (DOC) metabo-

lites are much increased but detection of this requires MS. Corticosterone and cortisol metabolites are low or absent.

4. Corticosteroid-11-dehydrogenase (11HSD II) deficiency (apparent mineralocorticoid excess): There is an increased ratio of cortisol:cortisone metabolites with decreased adrenal steroid levels.
5. Corticosteroid-11-oxoreductase (11HSD I) deficiency (apparent cortisone reductase deficiency): There is a decreased ratio of cortisol:cortisone metabolites with increased adrenal steroid levels.
6. 17α-Hydroxylase deficiency: There are increased metabolites of progesterone, DOC, and corticosterone with an absence of androgen and cortisol metabolites.
7. 21-Hydroxylase deficiency: 17α-Hydroxyprogesterone and 21-deoxycortisol metabolites are increased with low cortisol metabolites. The salt wasting and simple virilizing forms cannot be distinguished.
8. 17,20-Lyase deficiency: Androgen metabolites are absent.
9. 21,22-Lyase deficiency/StAR protein deficit. All steroids are absent.
10. 18-Oxidation defect (corticosterone methyl oxidase defect, hypo-aldosteronism): There are increased corticosterone metabolites and, on GC-MS analysis, decreased or normal tetrahydroaldosterone, the major metabolite of aldosterone.
11. Pseudohypoaldosteronism (congenital aldosterone unresponsiveness): There are increased corticosterone metabolites and, on GC-MS analysis, increased tetrahydroaldosterone.
12. Placental sulfatase deficiency: In maternal urine steroid sulfates are increased; oestriol is low. No differences from normal are seen in the urinary steroids from individuals with steroid sulfatase deficiency, but plasma cholesterol sulfate is elevated.
13. Cytochrome P450 oxoreductase deficiency: This resembles a combined 17α- and 21-hydroxylase deficiency, with increases of metabolites of corticosterone and 17α-hydroxyprogesterone with relatively low cortisol metabolites.

3.5. Interpretation of Results and Summary of Profile Findings in Steroid Associated Clinical Pathologies

1. Congenital adrenal hypoplasia: In the "Anencephalic" form in infancy 3β-hydroxy-5-ene steroids are absent. In other forms all steroids are absent.
2. Steroid-producing tumors: Adrenocortical tumors usually give rise to distinctive profiles. Some adrenocortical tumors do not secrete

steroid. Occasionally the profile mimics a partial enzyme deficiency. Gonadal tumors may give rise to notable increases in androgen metabolites, but are more often suggested by increased testosterone or oestradiol in plasma together with urinary metabolites within the normal range.

3. Precocious puberty/virilization: Androgen excess owing to partial enzyme deficiencies or tumors is readily investigated. Precocious increases in adrenal androgen synthesis (premature adrenarche) may be indicated by increases in steroid sulfate excretion. The relative proportions of androgen and other steroid metabolites provide an indication of "biochemical" pubertal stage for comparison with the clinical assessment.
4. Hirsutism in women: As in **Subheading 3.5.3.**, specific causes of androgen excess may be identified. After excluding these, in about 50% of cases, adrenal steroid excess is demonstrable. Among possible causes are mild glucocorticoid resistance and enhanced cortisol clearance as a result of change in relative activities of 11HSD I and 11HSD II.
5. Cushing's syndrome: If because of ACTH excess, all steroids are increased. The relative proportions depend on the duration of excess and the rate at which ACTH levels are rising. Cortisol metabolites are increased relative to androgen metabolites, but less so if excess stimulation is long term. The more acute the increase, the more cortisol metabolites are increased relative to cortisone metabolites and the more free cortisol is increased relative to cortisol metabolites. Excretion of tetrahydrocortisol (5b-reduced) is high relative to that of *allo*-tetrahydrocortisol (5a-reduced). Adrenocortical tumors usually produce a similar pattern but with additional, unusual metabolites.
6. Hypertension: Profiling is only appropriate after aldosterone and renin assay. Inborn errors of steroid metabolism associated with hypertension (deficiency of 11β-hydroxylase, 17α-hydroxylase, and 11HSD II) are readily distinguishable. Dexamethasone-suppressible hyperaldosteronism is identifiable by 18-hydroxycortisol assay. It may be possible to distinguish Conn's syndrome from bilateral adrenal hyperplasia on the basis of a relative change of tetrahydro-11-dehydrocorticosterone, but further experience is needed to verify this.

4. Notes

1. Sep-Pak cartridges may be reused up to 10 times if well washed.
2. The capacity of Sep Pak cartridges is very high with near total recovery. One cartridge can retain steroids from up to 100 mL of urine *(11)*. However, retention of the very polar steroids found in samples from infants is compromised if very dilute samples are extracted. This is overcome by extracting on a peristaltic pump at slow flow-rates. Commercially available batch extraction devices based on vacuum are not recommended because flow through individual cartridges is difficult to control.
3. Pump lines are chosen to give a flow of 0.15 mL/min. A glass haematocrit tube with flame polished ends is inserted into the intake end. The outflow end is connected to the Sep-Pak via a cut-down disposable pipette tip. Alternatively, extraction can be carried out with a glass syringe. Urine must be pushed through dropwise.
4. Avoid loss of sample identity at this stage by transferring sample labels one by one. The pump lines may be color-coded at each end to help prevent misidentification.
5. Prior extraction rather than direct hydrolysis in urine is used because enzyme inhibitors, e.g., phosphates and sulfates, are present in the sample in varying amounts.
6. Some steroids, most notably DHA, pregnenediol, and pregnenetriol, undergo changes during incubation with Helix pomatia. Addition of sodium ascorbate to the hydrolysis greatly improves recovery of these labile compounds. Alternative, fast procedures, such as incubation at 55°C for 3 h, does not improve recoveries of these steroids. Recovery of other steroids is also more erratic, so careful evaluation is advisable.
7. Residue after evaporation of steroid extracts contains material, usually brown in color, which dissolves readily in water, but is insoluble in organic solvents. This probably represents a small proportion of the material present in the original sample or in the enzyme preparation, e.g., proteins, urinary pigments, which, although they may be much more polar than the steroids, are still carried through the method. When a lot of this material is present, it may be more difficult to make a complete derivative or the derivative breaks down during analysis. It is useful to wash all the samples except those from infants. These usually do not require purification and they contain more polar steroids that would be lost during washing.

8. All ketone groups (except at position C11) react readily under these conditions. Different oximes (e.g., ethyl and benzyl) may be used. The longer the alkyl chain of the oxime, the higher the retention time. Methyl oximes are most commonly used: the others can be used selectively to permit separation of an overlapping ketonic compound. *Syn* and *anti* forms of the oxime are probably formed in all cases but are only separable when in certain positions. Most 3-oxo-4-ene compounds form double peaks, e.g., testosterone, androstendione, progesterone, and cortisol. Others include 16α-hydroxy-DHA and 16α,18-dihydroxy-DHA.
9. Silylated derivatives are rapidly degraded on contact with air because of hydrolysis by moisture. If derivatization reagents are evaporated under nitrogen, cyclohexane must be added to the dry residue immediately the tube is removed from the nitrogen jet. If a derivative dries up on storage it is always destroyed but it may be resilanised.
10. Although most derivatization methods use reagents that may be evaporated under nitrogen, TSIM is very involatile. It is essential that it be removed from the derivative since damage to the GC column would result from injecting it. Removal of TSIM is achieved by adding cyclohexane to the reaction mixture and then washing with water. Paradoxically, contact with water in this way does not cause degradation of the derivative.
11. The GC flame ionization detector responds to any organic compound that elutes off the column and burns in the flame. It is important to avoid all contact with potentially dirty surfaces including the hands and to use glass wherever there is contact with solvents. Gloves other than polythene should not be used. The most common contaminants are hydrocarbons and phthalates (plasticizers). Do not use hydrocarbon-containing materials, such as Parafilm or stopcock grease.
12. OV-1-coated columns that are not chemically bonded provide excellent separation of the major steroid metabolites. Bonded phase OV-1 columns give slightly different retention times from unbonded columns for certain compounds: two important steroids (tetrahydrocortisol and *allo*-tetrahydrocorticosterone) overlap. Bonded phase columns are more stable and can be rejuvenated by washing. Whichever is chosen, consistent use of one phase simplifies identification by providing consistent elution order. The temperature program incorporates a *cold trap*. When injected at 100°C, the steroid deriva-

tives are immobile, so that the sample is concentrated as a narrow band at the front of the column. The temperature is rapidly raised to 180°C and then increased at a slower rate to achieve the analytical separation.

13. Custom-made glass columns consist of a reservoir 25 mm diameter, 100 mm long, a barrel 10 mm diameter and 120 mm long, and a Teflon tap with glass sinter.

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10

Measurement of Aldosterone in Blood

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Summary

A detailed method for measuring aldosterone in serum/plasma is described. Aldosterone is first extracted into dichloromethane to improve the specificity of the assay. A radioimmunoassay is performed on the dried extract using an antibody raised in rabbits to aldosterone conjugated at position C3, and iodinated aldosterone. Details are given for optimizing conditions for the second antibody, raised to rabbit immunoglobulins, and carrier protein used in the separation of the antibody bound fraction from the free aldosterone at the end of equilibration. Details are provided in additional notes, for steps in the assay that could be problematic.

Key Words: Mineralocorticoid; solvent extraction; radioimmunoassay; second antibody separation; specificity.

1. Introduction

Aldosterone is the most potent mineralocorticoid in humans. It is secreted by the zona glomerulosa of the adrenal cortex and acts to promote sodium ion re-absorption in exchange for potassium and hydrogen ion excretion in the distal renal tubules. Aldosterone is important in the maintenance of arterial blood pressure in the sodium deplete state.

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The renin–angiotensin system is a major regulatory mechanism of aldosterone secretion. In order to investigate abnormalities in blood pressure and/or disorders of sodium and potassium homeostasis, it is helpful to assess the integrity of the renin–angiotensin–aldosterone axis. This requires measurement of one of the components of the renin–angiotensin system and aldosterone. In isolation, aldosterone concentrations are difficult to interpret, but may be useful in assessing adrenal response to stimulation.

Many methods have been described for the measurement of aldosterone in blood, including double isotope-derivative techniques *(1,2)*, gas chromatography/mass spectrometry *(3)*, tandem mass spectrometry *(4)*, and immunoassy *(5,6)*. Although high-performance liquid chromatography (HPLC)-tandem mass spectrometry is cited as a reference method *(7)* and is potentially the most accurate technique available at present, the method of choice for measuring large numbers of small volume samples is radioimmunoassy (RIA).

Commercial assays are available for measuring aldosterone directly in unextracted plasma or serum, but aldosterone circulates at low concentrations (pmol/L) and there are problems of sensitivity and accuracy with the direct assays. There is an increasing awareness of nonspecific interference in direct immunoassay of steroids as well as for other analytes *(8,9)*. The majority of these can be overcome for steroid analysis by prior solvent extraction from the plasma. An additional problem in the assay of aldosterone is that many of the patients under investigation have renal problems. In patients with chronic renal failure, polar metabolites of aldosterone are present in high concentrations and crossreact with antibodies, especially those raised to aldosterone conjugated at C3 *(10)*. A prior solvent extraction leaves these polar metabolites in the plasma, greatly enhancing the specificity of the assay.

This chapter describes a method that includes an initial extraction step with dichloromethane. An RIA is performed on the dried extract using an antibody raised in rabbits against aldosterone-3-(*O*-carboxymethyloxime)-bovine serum albumin (BSA) conjugate, and a label of aldosterone conjugated to iodinated histamine, also at

position C3. Separation of the antibody bound from unbound aldosterone is achieved by using a second antibody raised in sheep against rabbit immunoglobulins (Ig)G to precipitate the first antibody. Rabbit carrier serum is included to ensure that there is sufficient rabbit IgG for optimum precipitation that is further enhanced by the addition of polyethylene glycol. After centrifugation, the supernatants are decanted to waste and the precipitate containing the bound fraction is counted in a γ-counter. The aldosterone concentrations in the unknowns are determined by interpolation of the counts bound on a standard curve prepared with solutions of known aldosterone concentration.

Aldosterone can also be measured in urine by a modification of this method. Only about 2% of aldosterone is excreted in the free form. The term urinary aldosterone is used to describe the 18-glucuronide conjugate of aldosterone, which accounts for about 10% of all aldosterone urinary metabolites. The 18-glucuronide is acid hydrolysed at pH 1.0 and 30°C for 4.5 h to liberate aldosterone, which can then be extracted into dichloromethane (DCM) and assayed using the same RIA procedure as outlined previously. An RIA technique has also been described for the assay of aldosterone in saliva ***(11)***.

2. Materials

2.1. Solvent Extraction

1. Glass tubes (75 × 12 mm) and suitable racks (*see* **Note 1**).
2. Pipets, e.g., Oxford type with tips; repeating pipettor, e.g., Eppendorf Multipette and combitips; bottle dispenser, e.g., Oxford Laboratories Pipettor.
3. Internal quality control material: Lyphocheck Hypertension Markers (Bio-Rad) are available at low, medium, and high concentrations (C1, C2, C3) and are supplied freeze dried. Reconstitute in 2 mL distilled water for a minimum of 20 min before use. Prepare a plasma pool at an aldosterone concentration of 400–600 pmol/L from outdated blood bank plasma as an additional control (PP). These controls should be aliquoted and stored frozen.

4. DCM, high-performance liquid chromatograph (HPLC) grade (**Caution:** toxic by inhalation: irritating to skin and eyes) (*see* **Note 2**).
5. Vortex mixer (preferably a multivortex mixer suitable for mixing 50 tubes at a time if available).
6. Dry ice/technical methanol freezing bath.
7. Vacuum oven heated to 35°C attached to an electric compressor pump (*see* **Note 3**).
8. Phosphate-buffered saline (PBS), pH 7.4: 2.58 g $NaH_2PO_4.H_2O$, 14.4 g $Na_2HPO_4.2H_2O$, 18 g NaCl, 0.2 g thiomersal (**Caution:** poisonous by inhalation and skin contact). Dissolve by stirring in about 1 L of distilled water in a conical flask over a magnetic stirrer. Make up to 2 L. Check pH and store at 4°C.
9. Assay buffer, 0.1% BSA in PBS: dissolve 1 g BSA in 1 L PBS. Store at 4°C.

2.2. Radioimmunoassay

1. Crystalline aldosterone (Sigma-Aldrich).
2. Aldosterone standard solutions: store and maintain during use at 4°C, ethanol, assay buffer, and standard solutions.
3. Primary stock A, 100 µg/mL: weigh out 1 mg aldosterone using a 6-place balance. Dissolve and make up to 10 mL in ethanol at 4°C.
4. Secondary stock B, 1 µg/mL: pipet 100 µL stock A into 10-mL volumetric flask and make up to volume with ethanol at 4°C.
5. Tertiary stock C, 25 ng/mL: pipet 250 µL stock B into 10-mL volumetric flask and make up to volume with assay buffer at 4°C.
6. Working standards: pipet the following volumes of stock C into 25-mL volumetric flasks and make up to volume with assay buffer at 4°C; 0, 20, 50, 100, 200, 400, 800, 1500, and 3000 µL to give concentrations of 0, 2, 5, 10, 20, 40, 80, 150, and 300 pg/100 µL, i.e., 0, 55.6, 139, 278, 556, 1112, 2224, 4170, and 8340 pmol/L, labeled 0, A–H. Once working standards have been checked, store at 4°C in approx 5 mL aliquots in screw-top glass vials.
7. Rabbit carrier serum (RCS): (cat no. RS015, TCS Biologicals Ltd., Buckinghamshire). Store at –20°C. For use, dilute 1 mL with 500 mL of PBS. Mix well and store in polypropylene bottles in aliquots of 50 mL at –20°C. Thaw an aliquot as required. Once thawed, store and use at 4°C until finished.

8. [^{125}I] Aldosterone label (**Caution**: follow normal safety precautions for handling radioactive material).
9. Stock label: (DPC, cat no. TAL2) received freeze dried, 111 KBq/vial: reconstitute in distilled water (usually about 30 mL) to give a solution containing between 10,000 and 15,000 cpm/100 µL.
10. Working dilution: check activity in 100 µL of stock. Calculate the volume of label required for batch (100 µL/tube). Take sufficient stock, which when diluted to give an adequate volume for the batch, will give approx 7000 cpm/100 µL.
11. First antibody (AB1) raised to aldosterone conjugated at C3, (*see* **Note 4**). Store undiluted serum at –20°C.
12. Primary dilution, 1/20: dilute to 1/20 in saline and store in 1 mL aliquots at –20°C.
13. Secondary dilution 1/500: dilute a 1 mL aliquot of primary dilution to 1/500 in PBS. Store in 1 mL aliquots at –20°C.
14. Working dilution, 1/20,000: thaw and store an aliquot of secondary dilution at 4°C. As required for batch (100 µL/tube), dilute a suitable volume with assay buffer to give a working dilution of 1/20,000.

2.3. Second Antibody Separation

1. Second antibody (AB2) raised in sheep against rabbit IgG: store stock second antibody at –20°C (*see* **Notes 5–7**).
2. Add 50 mL of stock AB2 plus 0.5 g sodium azide (**Caution:** toxic) to 500 mL defibrinated horse serum. Test performance in the assay (*see* **Note 7**) and then store in 50 mL aliquots at –20°C. Thaw and store a 50 -mL aliquot at 4°C and use as required.
3. 10% polyethylene glycol: dissolve 100 g PEG 6000 in 1 L PBS. Store at 4°C.

3. Method

3.1. Solvent Extraction of Aldosterone

1. Label in duplicate a set of glass assay tubes (75×12 mm) (set A), to be used for extraction, in the order C1, C2, C3, PP (QCs), samples 1-n (maximum 32), C1, C2, C3, PP.
2. Label in duplicate a second set of tubes (set B), to be used for RIA in the order T (total counts), nonspecific binding (NSB, *see* **Note 8**), 0 (zero standard), A-H (remaining standards), C1, C2, C3, PP, 1-n (unknowns), C1, C2, C3, PP.

3. Pipet 100 µL of controls and unknown samples into set A tubes.
4. Dispense 2 mL DCM from a bottle dispenser into each tube (*see* **Note 2**).
5. Vortex mix (preferably on multivortex mixer at speed 25) for 2 min.
6. Freeze aqueous/plasma (upper) layer in a dry ice/methanol bath and decant the DCM into the corresponding control and sample tubes in set B (*see* **Note 9**). Discard tubes in set A.
7. Balance the NSB and standard tubes in set B with 2 mL DCM from dispenser (*see* **Note 10**).
8. Evaporate the solvent from the set B tubes by placing them in a vacuum oven at 35°C (takes about 1–2 h) (*see* **Notes 11** and **12**).
9. Add 200 µL assay buffer to dried extracts of controls and samples. Vortex mix for 1 min and leave for at least a further 30 min to allow the dry residue to dissolve. Vortex mix again before proceeding.

3.2. Radioimmunoassay

1. Place rack of tubes in crushed ice/water bath for following steps.
2. Add 100 µL aldosterone standard solutions zero, A to H into appropriate tubes.
3. Add assay buffer to equalize the final volumes in the RIA tubes: to standards zero, A to H, 100 µL to NSB, 300 µL
4. Add 100 µL RCS to all tubes in set B apart from total tubes.
5. Add 100 µL [^{125}I]aldosterone working dilution to all set B tubes.
6. Add 100 µL AB1 working dilution to all tubes except totals and NSB.
7. Vortex mix for 15 s. Cover tubes with cling film and incubate at 4°C overnight (*see* **Note 13**).

3.3. Second Antibody Separation

1. Add 100 µL of AB2 to all tubes except totals.
2. Add 500 µL 10% PEG to all tubes except totals.
3. Vortex mix for 15 s and then incubate in the ice bath for at least 15 min, but not more than 1 h.
4. Towards the end of the incubation period, cool the centrifuge to 4°C.
5. Centrifuge at 1900*g* for 15 min at 4°C.
6. Decant supernatant down a sink designated for radioactive waste and keep the tubes inverted over absorbent paper to drain. Blot dry on fresh absorbent paper and turn upright (*see* **Note 14**).

7. Count the pellets containing [^{125}I]aldosterone bound to AB1 in a suitable γ-counter (*see* **Note 15**).
8. Using a data reduction package, such as Multicalc or manually, construct a standard curve of counts bound vs log aldosterone concentration in pmol/L. Read the values for the controls and unknowns by direct interpolation of the counts bound on the standard curve.

4. Notes

1. The system of tubes we use are made by Denley (*see* **Note 2**, Chapter 11).
2. Steps involving DCM extraction should be carried out in a fume cupboard.
3. The vacuum pump should be vented into a fume cupboard.
4. The AB1 in use is not commercially available but antibodies raised to aldosterone conjugated at C3 are available. The titer would have to be established and the assay validated with the antibody chosen.
5. AB2 used is dependent upon what animal was used to raise AB1. Antibodies raised to a wide range of animal IgG are readily available.
6. It is also possible to use dextran-coated charcoal for the separation of bound and free fractions.
7. The level of carrier serum and AB2 must be optimized for the assay. Carrier serum is necessary because the high titer of the first antibody results in an inadequate concentration of IgG to precipitate and form a pellet. However, if the concentration of carrier serum is too high it may displace AB1 from AB2 binding sites and prevent complete precipitation of the AB1 bound steroid. Optimal conditions can be established. Prepare a range of dilutions of carrier serum in assay buffer (1/200, 1/400, 1/800, 1/1600). Prepare a range of dilutions of AB2 in horse serum such that 100 µL contain 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 µL AB2. For each dilution of carrier serum, prepare a series of tubes containing AB1 and labeled aldosterone (comparable to zero points of the standard curve). Add the dilution of carrier serum and incubate under normal conditions. At the end of the incubation period, add 100 µL of each dilution of AB2 in duplicate and 500 µL 10% PEG. Mix, incubate, and centrifuge using standard conditions. Decant and count the pellets. Plot graphs of % bound for each dilution of carrier serum against amount of AB2/tube in l. Optimal dilutions of carrier serum are reached when the binding curves become

superimposed. The amount of AB2 is optimal when the binding reaches a plateau. For reasons of economy, choose the least amount of AB2 that achieves maximum binding on the plateau.

8. NSB tube is not a true measure of NSB, which can only be achieved by the addition of excess antigen (dangerous in a routine assay because of possible cross-contamination) to an incubation of AB1 and label. The NSB tubes in this assay do not have AB1 present. The counts in this tube at the end of the assay give a measure of the counts carried down in the precipitate in the absence of binding to AB1. Typically these are less than 2% of total counts.
9. Using the size of tube and volume of sample described previously, the aqueous layer forms a ring on top of the DCM, allowing the solvent to be poured off through the hole in the middle. We freeze a rack of 50 tubes at a time in a tray of dry ice/methanol. Blot the outside of the tube before decanting to prevent the freezing mixture contaminating the DCM extract.
10. It is important to ensure that an equivalent solvent residue is present in all tubes. Any interference with binding is then present in standards as well as unknowns.
11. If a vacuum oven is not available, the solvent can be evaporated under nitrogen, but aldosterone is a relatively labile steroid and should not be heated to above 40°C or left dried onto the glass at a raised temperature once the solvent has been removed. Also, great care must be taken to avoid cross-contamination of samples.
12. If using a vacuum oven, ensure that the bottoms of the tubes are in contact with the shelf of the oven, otherwise the evaporating time will be extended considerably.
13. Incubation times between 14 and 22 h do not affect the results in our assay.
14. Take care that the decanted tubes are not placed on the draining paper. It is easy to contaminate the outside with radioactivity.
15. Ensure that all radioactive waste is disposed of through correct channels.

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11

Measurement of Plasma Renin Activity

Marion L. Cawood

Summary

Measurement of angiotensin 1, released in plasma by the action of endogenous enzyme renin on endogenous substrate, angiotensinogen is described. Details are given for the generation of angiotensin 1 from plasma using controlled conditions of pH and temperature. A radioimmunoassay to quantify the generated material is then described using anti-angiotensin 1 antibody and iodinated angiotensin 1 as label. Separation of the antibody-bound fraction from the free is achieved using dextran-coated charcoal. Problems of cryoactivation of prorenin and the labile nature of angiotensin 1 are highlighted. Additional notes describe steps in the assay that are critical.

Key Words: Angiotensinogen; angiotensin-converting enzyme; angiotensin 1; prorenin; cryoactivation; radioimmunoassay; dextran-coated charcoal.

1. Introduction

The renin–angiotensin system plays an important role in the regulation of aldosterone secretion and consequently the retention of sodium and maintenance of arterial pressure, particularly in the salt deplete state.

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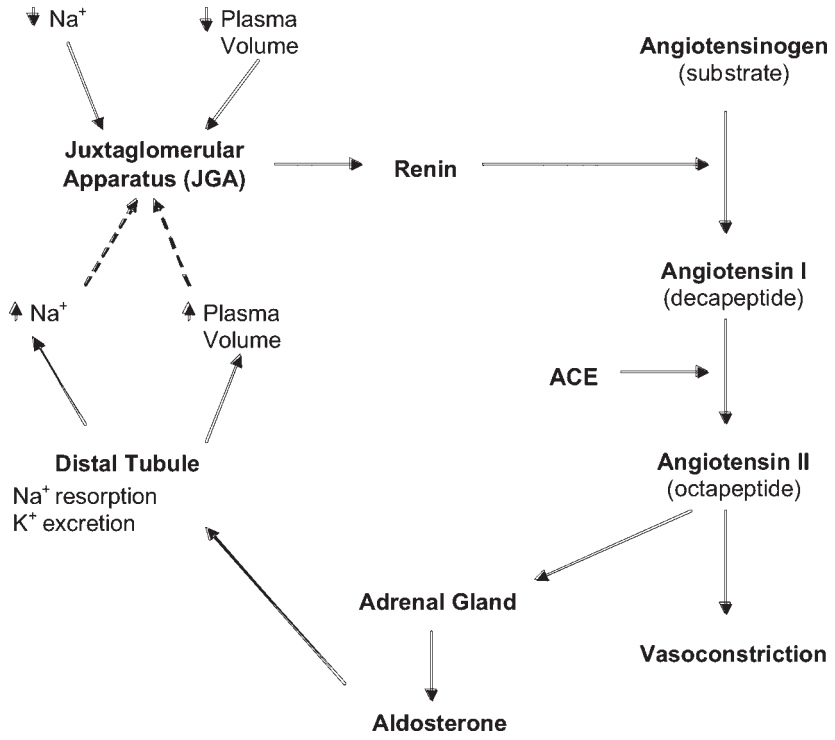

Fig. 1. The role of the renin–angiotensin system in the control of aldosterone secretion.

Renin is a proteolytic enzyme which acts on its substrate, angiotensinogen to release a decapeptide, angiotensin I (AI). Angiotensin-converting enzyme (ACE) acts upon AI to produce an octapeptide, angiotensin II (AII) (**Fig. 1**). AII has a very powerful pressor action but also stimulates the secretion of aldosterone from the adrenal cortex. Because AII is extremely labile and has a half-life of 1–2 min in the circulation it is not an easy analyte to measure. However, apart from very rare instances, angiotensinogen and ACE are present in excess and, therefore, the amount of AII produced is proportional to the concentration of renin in blood. An alternative approach is to assess the efficiency with which the plasma sample produces angiotensin. The sample is incubated under rigorously controlled conditions of pH, temperature, and time. Endogenous

renin acts upon endogenous substrate to produce AI. The conversion of AI to AII is blocked by inhibitors of the reaction. Accumulated AI is measured by radioimmunoassay (RIA). The yield of AI is highly dependent upon the pH and temperature chosen for the generation step. We have chosen to maximize yield at pH 5.5 and 37°C ***(1,2)***. This method measures plasma renin activity (PRA) and the results are expressed in terms of mass of AI produced per volume of plasma in unit time, e.g., pmol/mL/h. PRA usually reflects renin concentration but in rare cases in which either the enzyme or substrate are defective, high concentrations of renin are seen when PRA is low. Conversely, there are some treatments, e.g., with estrogen, which increase PRA but do not affect renin concentration.

A complication in the measurement of renin mass or activity is the presence, in the circulation, of an inactive form of renin, prorenin. The inactive form circulates in blood at about 10× the concentration of active renin, rising to as high as 100× in some patients with kidney damage resulting from diabetes ***(3)***. Although there is no evidence of conversion of the inactive form to active renin in the circulation in vivo, cryoactivation of prorenin has been demonstrated in vitro in liquid samples kept at low temperatures. The conversion occurs maximally at 4°C ***(4–6)***. The degree of cryoactivation is very variable between samples, but could be misleading in samples with low active renin concentration in which incorrect handling could result in anomalous normal or even high values of active renin or PRA. The opposite effect can be seen in PRA values if samples are left for prolonged periods at ambient temperature. The action of renin on angiotensinogen continues in the blood sample. Since the PRA assay utilizes endogenous angiotensinogen, falsely low estimates would result if the sample became substrate deplete before the controlled generation step. In order to minimize both of these effects, ethylenediaminetetraacetic acid (EDTA) or lithium heparin samples should be taken into syringes and tubes and transported to the laboratory at ambient temperature. The plasma must be separated from the cells within 1 h and the plasma frozen rapidly to –20°C. The sample must be maintained in the frozen state until immediately prior to assay when it should be thawed rapidly at 22–25°C.

Commercial assays are available for the measurement of active renin and these have been reviewed recently *(7)*. We have chosen to measure PRA because; the active renin assays are not sensitive enough to measure the low concentrations found in suppressed conditions, such as primary hyperaldosteronism; in conditions of high prorenin but low active renin, even a low crossreaction of prorenin with the antibody could result in misleadingly normal/high values *(3)*; and results could be misleading if either enzyme or substrate is defective. PRA is more representative of production of angiotensin and consequently the level of stimulation to the adrenal.

2. Materials

2.1. Generation of Angiotensin I

1. Shaking water bath equilibrated to exactly 37°C (*see* **Note 1**).
2. Large polystyrene tubes (75 × 12 mm), small polypropylene tubes (64 × 11 mm), and suitable racks (*see* **Note 2**).
3. Tepid water bath at 22–25°C.
4. Pipets, Oxford type; repeating pipettors, e.g., Eppendorfs Multipette; Dispenser bottle, e.g., Oxford Laboratories Pipettor.
5. Internal quality control material: Lyphocheck Hypertension Markers (Bio-Rad) are available at three levels and are supplied freeze-dried. Reconstitute in 2 mL distilled water for a minimum of 20 min before use. Once reconstituted, the solutions are stable at 4°C for up to 21 d or can be frozen in aliquots to be thawed once for each batch.
6. Stock inhibitor solution: dissolve 660 mg disodium EDTA, 120 mg 8-hydroxyquinolone hemisulphate in 10 mL distilled water. Store in plastic container at ambient temperature in the dark for up to 2 wk (*see* **Note 3**).
7. Acetate buffer, pH 5.5: dissolve 34.45 g anhydrous sodium acetate in about 800 mL distilled water. Add 4.58 mL glacial acetic acid (corrosive; harmful irritating vapor), mix well and adjust pH to 5.5 (*see* **Note 4**). Add 200 mg neomycin sulfate and make up volume to 1 L. Store indefinitely at 4°C.
8. Working inhibitor solution: immediately before required, prepare a 1/10 dilution of stock inhibitor in acetate buffer at ambient temperature.

9. Tris/bovine serum albumin (BSA) buffer, pH 8.5: dissolve 12.12 g Tris (hydroxymethyl)aminomethane in about 800 mL distilled water. Add 8 mL of 2 *M* HCl and adjust pH to 8.5 with additional HCl. Add 500 mg BSA (Sigma, Fraction V, cat no. A-802) and allow to dissolve. Make volume to 1 L with distilled water. Mix well but avoid frothing. Store and use at 4°C for up to 2 wk.

2.2. Radioimmunoassay of Angiotensin I

1. Trays of crushed ice/water. All reagents are prepared in ice baths, towards the end of the 3-h generation step.
2. RIA diluent: dilute 5 mL of working inhibitor solution to 100 mL Tris/BSA buffer.
3. AI primary stock standard (*see* **Note 5**): reconstitute the contents of an ampoule in 450 µL Tris/BSA buffer to give a concentration of 1 µg/50 µL. Dispense 50 µL aliquots into polypropylene tubes (64 × 11 mm) in a dry ice/methanol bath so that they freeze immediately (*see* **Note 6**). Store at –20°C.
4. AI secondary stock solution: dilute 1 aliquot of primary stock to 10 mL with Tris/BSA buffer in the designated 10-mL volumetric flask. Mix well avoiding frothing and transfer immediately to a universal container. Dispense 100 µL aliquots into small tubes in a dry ice/methanol bath. Store at –20°C.
5. AI working standards: dilute 1 aliquot of secondary stock to 5 mL in RIA diluent in the designated volumetric flask to give a concentration of 200 pg/100 µL. Mix well and immediately transfer about 3 mL to a polystyrene tube (75 × 12 mm). Double dilute this solution in polystyrene tubes with RIA diluent (1 mL + 1 mL) to give a series of working standards ranging from 200 to 3.125 pg/100 µL. The zero standard is RIA diluent.
6. Antiserum (*see* **Note 7**): dilute 100 µL neat antiserum to 1/200 in Tris/BSA buffer and store 0.5 mL aliquots frozen at –20°C. Prepare a working dilution on the day of assay by transferring quantitatively, 1 aliquot of 1/200 dilution to a universal container with 21 mL Tris/BSA buffer.
7. Stock [^{125}I] AI (**Caution:** follow normal safety precautions for handling radioactive material): (3-[^{125}I]iodotyrosyl^{4})-Angiotensin I(5-L-isoleucine) (Amersham UK, cat no. IM 176) supplied freeze

dried in vials containing 10 µCi/vial, specific activity approx 2000 Ci/mmol. Store at 4°C prior to reconstitution. Reconstitute in 1.2 mL ice-cold Tris/BSA buffer. Mix well and keep in an ice-bath while dispensing 50 µL aliquots into small tubes. Freeze rapidly and store at –20°C until required.

8. Working dilution of label: calculate volume of label required for batch of tubes (100 µL/tube) and pipet slightly more than that volume of Tris/BSA buffer into a universal container. Transfer 50 µL aliquot of stock label into the buffer and mix. Count 100 µL of this of working dilution in a suitable γ-counter. If necessary adjust counts by adding further aliquots of stock or Tris/BSA buffer to give about 5000 cpm/100 µL.

2.3. Charcoal Separation

1. Refrigerated centrifuge (*see* **Note 8**).
2. Dextran-coated charcoal (DCC): prepare DCC on day of separation, about 30 min before required. Add 1.0 g charcoal (activated) and 0.1 g dextran (mol.wt. 60,000–90,000, Sigma) to 100 mL ice-cold Tris/BSA buffer in a plastic beaker containing a magnet. Place beaker in an ice tray over a magnetic stirrer and mix gently for 20–30 min before use (*see* **Note 9**).
3. γ-counter preferably connected to a data reduction package, e.g., Multicalc.

3. Method

3.1. Angiotensin I Generation

1. Label a set of polystyrene tubes (75 × 12 mm) in duplicate for the generation stage of QCs and samples.
2. Check that the temperature of the shaking water bath is at exactly 37°C.
3. Remove samples and reconstituted QC aliquots from the freezer and thaw rapidly in the water bath at 22–25°C. Mix well.
4. Without delay, pipet in duplicate, 100 µL of QC or sample into the appropriately labeled polystyrene tube (*see* **Note 10**).
5. Add 100 L freshly prepared working inhibitor solution to each tube.
6. Transfer the tubes to the shaking water bath and incubate at 37°C for exactly 3 h.

7. After 3 h, stop the generation of AI by placing the tubes in an ice tray and adding 1.8 mL of ice-cold Tris/BSA buffer from the dispenser to each tube with sufficient force to ensure mixing. This solution is referred to as the generate. Because the standard curve has a relatively narrow working range, samples with high renin activity (above 5 pmol/ml/h) must be diluted 1/10 with RIA diluent before assay (*see* **Note 11**).

3.2. RIA of Angiotensin I

1. During 3-h incubation, prepare RIA diluent and use it to prepare working AI standards.
2. Prepare working dilutions of antiserum and [^{125}I]AI in Tris/BSA buffer.
3. Label a set of small polypropylene tubes in duplicate as follows: T (totals); CB (charcoal blank) (*see* **Note 12**); AI standards 0–200 pg; QC1 and QC 2; samples G1, G2 to Gn; QC1 and QC2, RG1 and RG2 (*see* **Note 13**) followed by samples requiring 1/10 dilution and QC2 and QC3 at 1/10 dilution and, if the batch contains more than 100 tubes, the standard curve again at the end.
4. Place rack of tubes in ice tray.
5. Pipet 100 µL of RIA diluent into tubes labeled CB and 0 pg standard. Pipet each standard into the appropriately labeled tube.
6. Pipet 100 µL of each generate and 1/10 diluted generate into appropriate tubes.
7. Add 100 µL Tris/BSA buffer into CB tubes and 100 µL working dilution of antiserum into all other tubes.
8. Add 100 µL working dilution of [^{125}I]AI to all tubes.
9. Vortex mix all tubes for 10 s.
10. Return tubes to ice tray and incubate for a minimum of 40 h (*see* **Note 14**).

3.3. Charcoal Separation

1. Prepare DCC.
2. Set the centrifuge to pre-cool to 4°C.
3. Remove total tubes from rack.
4. Add 300 µL DCC suspension to all tubes except totals.
5. Mix 10 s and then centrifuge at 2700*g* for 30 min at 4°C.

6. Decant supernatants down a sink designated for disposal of radioactive waste and leave the tubes inverted over absorbent paper to drain for 5 min. Blot dry on fresh absorbent paper (*see* **Note 15**).
7. Count all tubes, including totals for 5 min in a γ-counter.
8. The results can be calculated manually by construction of a standard curve of counts bound (total–free) against AI standard values and interpolation of the unknowns. The correction required to give the result in pmol/mL/h for undiluted generates is as follows:

$$\text{Plasma Renin Activity} = \frac{\text{Angiotensin I (pg/tube)} \times 200}{1297\ \text{(mol/wt. AI)} \times 3}\ \text{pmol/mL/h}$$

4. Notes

1. The temperature of the water bath is critical. At temperatures above 37°C, renin is rapidly degraded, but at lower temperatures the amount of AI generated decreases.
2. The system of racks we use are made by Denley. These grip the top of the tubes so it is possible to mix the contents on a multivortex mixer in batches of 50 tubes, immerse the bottom of the tubes in an ice bath, centrifuge in batches of 100 tubes, and decant the supernatant from the charcoal pellet without removing the tubes from the racks at any point. This prevents the danger of transposition of tubes. If this or a similar system is not available, batch size should be limited to 100 tubes and much care must be taken throughout the assay.
3. The stock inhibitor solution must be made up at least the day before required because the constituents do not dissolve rapidly.
4. The pH of the buffer is critical because at a lower pH, less AI is generated.
5. NIBSAC research standard A,71/38 is supplied, freeze dried, in ampoules containing 9 µg and may be obtained from the Division of Endocrinology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts., EM6 3QG.
6. AI is very labile and must be handled rapidly at 4°C and stored frozen at –20°C. It must not be left in contact with unsilanised glassware and storage must be in plastic containers.
7. Antiserum in use is not commercially available, but there are several sources of antiserum to AI that should be safe to substitute. The titer,

sensitivity, and specificity of the antiserum chosen would have to be established.

8. The refrigerated centrifuge must be fitted with multi-tube carriers so that the complete batch of tubes can be processed together.
9. Too vigorous mixing of the DCC produces fines that are difficult to spin down and affect the integrity of the charcoal pellet. This can result in poor separation of the antibody bound from free fraction and to poor duplication.
10. Once the plasma samples have been pipetted for the generation step, the plasma should be refrozen as quickly as possible so that sample degradation is minimal and the estimation can be repeated if necessary.
11. Assays can be repeated from the generates which are stable for at least 1 mo if stored frozen. If dilution is required, the generates must be diluted with RIA diluent.
12. Charcoal blank tubes contain RIA diluent and iodinated AI but no antiserum. They give an indication of the efficiency with which the charcoal adsorbs unbound AI. Typically, values are above 99% of total counts.
13. RG1 and RG2 are generates from the previous batch reassayed in the RIA. If there is a change in control values between batches, these tubes will indicate whether the problem lies in the generation or RIA step.
14. Although the assay can be stopped at the end of the generation step, it is usual to continue immediately with the RIA. The sensitivity of the curve is improved if the incubation period is at least 40 h, but it can be extended to as long as 5 d. We typically incubate over the weekend.
15. Ensure that all radioactive waste is disposed of through correct channels.

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12

Measurement of Androgens

Michael J. Wheeler

Summary

Testosterone is the major androgen measured in clinical and research investigations of both men and women. Nevertheless, many other androgens have an important role in t he investigation of andrenal and gonadal physiology and pathology. Commercial assays are generally used in clinical laboratories but these have poor precision at low concentrations and poor sensitivity. Extraction assays, described in this chapter, can be much more sensitive and precise. There is interest in measuring free steriods and a steady-state gel filtration mehtod used in the author's laboratory is described. Methods are also provided for the measurement of steroids in saliva and hair.

Key Words: Androgrens; testosterone; free testosterone; saliva; hair; immunoassay.

1. Introduction

Androgens are secreted from the gonads and adrenal glands with some androgens produced by peripheral conversion of precursor androgens *(1)*. The most commonly measured androgen is testosterone and is useful in the investigation of gonadal function, hair growth, and aggression *(2)*. Concentrations of testosterone are also

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increased in abnormalities of the adrenal such as Cushing's syndrome and congenital adrenal hyperplasia (CAH) in human *(3)*. The measurement of weaker androgens, such as dehydroepiandrosterone (DHEA) and androstenedione (A), are of interest as precursors of the more potent androgens testosterone (T) and 5α-dihydrotestosterone (DHT), although there has been great interest in the role of DHEA in the immune system and in the ageing process of humans *(4,5)*. The measurement of androgens is useful clinically in monitoring the treatment of CAH *(6)*. The sulfated form of DHEA, dehydroandrostenedione sulphate (DHEAS), is unique in that it is secreted almost entirely by the adrenal glands and has been studied as a marker of adrenal androgen production. For example, it has been reported that between 5 and10% of patients with polycystic ovary syndrome have an increase in androgen concentration owing to increased androgen secretion by the adrenal glands. However, this finding is probably academic because it is unlikely to affect the treatment of such patients. DHEAS measurement may be helpful in the investigation of Cushing's syndrome when an adrenal adenoma is suspected *(7)*. In the majority of cases DHEAS is found to be below the reference range.

A simple solvent extraction method of steroids from serum, followed by immunoassay using specific antibodies, can be used to measure most of the individual androgens as total analyte concentration. Extraction is required to displace androgens from their binding proteins, sex hormone binding globulin (SHBG) and albumin, and remove water-soluble steroid conjugates that might crossreact with the antiserum. It is now generally accepted that the nonprotein bound fraction and some or all of the albumin-bound androgen is available to the tissues and, therefore, there is interest in measuring one of these two fractions *(8,9)*. Methods for the measurement of free testosterone are quite complex and therefore there has been a tendency in recent years to measure the nonSHBG bound fraction because the methodology is much simpler *(10,11)*. As testosterone is the most commonly measured androgen, simple methods will be described for the measurement of this androgen in different fluids with notes on the applicability

of each method to the other androgens. Exceptions are DHEAS and DHT. The concentration of DHEAS in serum is about 1000 times greater than the other androgens. In methods for this steroid, crossreaction of antibodies with other androgens, particularly DHEA, have no significant effect on the DHEAS result and can be ignored. Conversely, antibodies to DHT have a significant cross-reaction with testosterone that is present in serum at 3 to 30 times the concentration of DHT. Therefore testosterone must be eliminated before measurement of DHT. Testosterone is present in saliva ***(12)*** and, as the steroid diffuses readily across cell membranes, it is thought that it represents the free testosterone circulating in blood. Saliva is convenient to collect and is noninvasive, making it suitable for studies in children and where multiple samples are required. However, the concentration of the steroid in saliva is very low and necessitates the use of an extraction method. Therefore salivary testosterone is not measured routinely, but is of interest in some research studies. More recently there has been an interest, stimulated by the work of Baumgartnear et al. ***(13)***, in measuring androgens in hair in order to achieve a retrospective method for detecting androgen abuse ***(14,15)***. Hair has the attraction of providing an integrated value over an extended period of time because hair grows about 1 cm/mo. We chose the measurement of testosterone to investigate the procedure because, by analyzing male and female hair, large differences would be expected.

2. Materials

1. Phosphate buffer, 0.5 *M*, pH 7.2 (Stock Buffer): 68.1 g potassium dihydrogen phosphate (*see* **Note 1**), 89.1 g disodium hydrogen phosphate. $2H_2O$ dissolved in 1L distilled water.
2. Phosphate-buffered saline (PBS), 0.05 *M*, pH 7.2 : 100 mL stock phosphate buffer, 9 g sodium chloride, make up to 1 L in distilled water.
3. Bovine serum albumin (BSA) PBS (BPS), prepared by adding the appropriate amount of bovine albumin to 100 mL.
4. Testosterone standard (Sigma Chemical Co Ltd).
5. Antibody to testosterone (Guildhay Ltd, Surrey, UK).

6. Iodinated testosterone (Amersham Ltd, Bucks, UK).
7. Donkey anti-rabbit serum (Guildhay Ltd).
8. Nonimmune rabbit serum (Guildhay Ltd).

3. Methods

3.1. Total Testosterone in Serum

The method described is one that was developed by this laboratory for distribution to other centers *(16)* (*see* **Note 2**). At one time over 20 different hospital laboratories used the method. Agreement across sites was excellent and provided a roust standardized assay that was very sensitive and had a between-assay precision of 10% at about 0.2 nmol/L. Methods for other androgens, other than DHT and DHEAS, are similar to the one described here for testosterone.

3.2. Extraction Procedure

1. Add 300 µL female serum or 100 µL male serum with 200 µL assay buffer (*see* **Note 3**) or 300 µL standard to glass tubes (*see* **Note 4**), e.g., 16 × 100 mm).
2. Add 3 mL diethyl ether (Analar) (*see* **Note 5**).
3. Extract in a fume cupboard (*see* **Note 6**).
4. Allow the two phases to separate out (about 2 min).
5. Freeze the aqueous layer by placing the tubes in a tray with a mixture of cardice and industrial methanol or acetone (*see* **Note 7**).
6. Pour off the solvent phase into clean glass tubes large enough to take 3 mL solvent, e.g., 12 × 150 mm.
7. Evaporate to dryness under nitrogen or air.
8. Add 300 µL 1.0% BSA.PBS.

3.3. Radioimmunoassay

1. Add 100 µL extract into a polystyrene tube, e.g., 75 × 12 mm.
2. Add 100 µL anti-testosterone antiserum (*see* **Note 8**) in 1.0% BSA.PBS (*see* **Note 9**).
3. Add 100 µL iodinated testosterone (*see* **Note 10**).
4. Incubate for 2 h at room temperature.
5. Add 500 µL PEG solution (*see* **Note 11**).

6. Incubate for 30 min at 4ºC.
7. Centrifuge at 1500*g* for 30 minutes.
8. Decant the supernatant, blot the tubes.
9. Count the radioactivity.

3.4. Testosterone in Saliva

We collect saliva by asking subjects to place a No. 1 dental pad in the side of the mouth. After 3 min the pad was removed, placed into a plain polystyrene 5-mL blood tube, and placed into a freezer. Some subjects produced very little saliva owing to anxiety, which inhibited salivary flow. This problem was overcome by air-drying three drops of lemon juice, which stimulates salivary flow, onto the pad. Before assay, the tubes containing the pads were taken from the freezer and the saliva allowed to thaw. The pads were placed into a 5-mL plastic syringe and the saliva squeezed out into a clean tube. Saliva is viscid and when the pad containing the saliva is frozen, it breaks down the mucin and produces a liquid saliva. Any food particles also tend to remain in the syringe attached to the pad, providing a clear saliva sample. Radioimmunoassay (RIA) can be carried out as described previously, without prior extraction, by taking 100 µL saliva.

More recently Sarstedt have introduced the Salivette™. This device also uses a cotton cylindrical pad to collect the saliva. The pad is provided in a plain tube fitted into the top of a polystyrene conical centrifuge tube. The inner tube has holes in it and when the device is centrifuged, saliva in the pad is pulled out of the upper tube into the bottom of the conical centrifuge tube. After centrifugation, the inner tube that contains the pad, is discarded.

The concentration of testosterone in saliva is low so that the immunoassay must be very sensitive. The RIA described previously can measure down to at least 50 pmol/L by diluting the antibody.

3.5. Testosterone in Hair

1. Cut about 100 mg hair into 0.5-cm lengths and place in a small glass beaker.
2. Add 3 mL ethanol and briefly vortex mix (about 20 s).
3. Tip off solvent to waste and allow hair to dry.

4. Add 50 mg hair to clean glass boiling tube.
5. Add 1 mL 1*M* NaOH and place in boiling water bath for 10 min.
6. Allow to cool before adding 5 mL diethyl ether.
7. Extract for 1 min on a rotamixer or 10 min on a multi-tube vortex mixer (*see* **Note 12**).
8. Allow two phases to separate.
9. Transfer 3 mL of the solvent mixture into clean glass tubes.
10. Evaporate to dryness.

In our original method, the dried extract was reconstituted in 5 µL acetonitrile:water (50:50) solvent mixture and purified further by HPLC. It is possible to carry out RIA on the dried extracts directly. In this case, 300 µL assay buffer is added and 100 µL taken for RIA, as described previously. However, the HPLC step is important when trying to identify an individual steroid specifically.

3.6. Free Testosterone

There are three common methods for the measurement of free testosterone; equilibrium dialysis, steady state gel filtration, and ultra centrifugation. The former is the most common method and is well described ***(17)***. This method and ultra-centrifugation significantly distort the equilibrium between the bound and free steroid and this distortion increases with time. The steady state gel filtration method maintains the equilibrium between free and bound moieties and is relatively simple to carry out. All methods are carried out at 37ºC, but maintaining this temperature in the centrifuge for ultra-centrifugation can be difficult because few centrifuges provide temperature control at 37ºC. The crude method of pre-warming the centrifuge bowl by prolonged spinning is unsatisfactory as the temperature is unknown and uncontrolled.

The method used in this laboratory is steady state filtration carried out on micro-columns ***(18)***. This method requires the construction of a water chamber with holes in the bottom to accommodate glass 15 × 0.5-cm columns. We use a perspex chamber. This has holes drilled in the bottom that are, in turn, fitted with small rubber grommets. The small glass columns fit through the grommets providing a water-tight fit. We have also modified a Quick-fit perspex

reservoir with a large rubber bung, with drilled holes in the bottom for the columns. A temperature-controlled circulating water pump is required to maintain the water in the chamber at 37ºC. The water level should be about 14 cm so that most of the column is immersed. This ensures the buffer in the column is at 37ºC when it enters the Sephadex.

At the bottom of each column is a small rubber bung pierced with a 1-cm metal tube. The end of the metal tube is initially sealed with knotted or sealed tygon tubing pushed over the end. The column is filled with buffer and a small disk of No. 1 Whatman filter paper is floated into the glass column. The disk is cut from No. 1 Whatman filter paper using no. 1-size cork borer. The disk is positioned over the end of the metal tube in the bung and Sephadex G50 (fine) is added to the column. The tygon tubing is removed and the buffer is allowed to flow through the column to pack the Sephadex. More Sephadex suspension is added until the column is packed to a depth of 10 cm. The tygon tubing is replaced and the column is ready for use. The apparatus should be able to accommodate several columns. Ten columns are ideal, as this is equivalent to a day's workload.

3.6.1. Serum Analysis in Sephadex Columns

1. Run excess buffer into the Sephadex and replace the tygon tubing.
2. Add 80 µL with titrated steroid pre-equilibrated serum.
3. Run the serum into the column, stopping the flow when all the serum has run into the column.
4. Carefully add assay buffer to fill the column causing minimal disturbance to the top of the column.
5. Remove the buffer with tygon tubing attached to a syringe.
6. Repeat twice more and then fill the column for a fourth time.
7. Remove the tygon tubing and collect three drops of buffer into scintillation vials. About 40 fractions are required to establish the profile and it will be necessary to replenish the buffer in the top of the column.
8. Add scintillation fluid and count each fraction for 10 min or 10,000 cts. Having this alternative option reduces counting time.
9. Plot out counts. A profile should result similar to the one in **Fig. 1**. It is only necessary to collect all the fractions from two or three

columns to ensure the technique is correct. To reduce the large amount of counting time, five fractions may be collected from each plateau, which reduces counting time to just over 1 h per sample. We use a coefficient of variation of < 5% as acceptable variation for the five aliquots.

10. The percentage free testosterone is calculated as:

$$\frac{\text{Mean cpm plateau 2} \times 100}{\text{Mean cpm plateau 1}}$$

11. Determination of the total testosterone concentration in the sample allows the concentration of the free testosterone to be calculated.

3.6.2. Other Methods for Free Testosterone

Commercial kits purported to measure free testosterone by direct measurement of serum are available from at least two companies. The kit manufactured by Diagnostics Products Corporation has been used in a number of studies. It gives much lower results for female serum and it has been questioned whether the kit actually measures free testosterone or simply a proportion of the total testosterone present in serum ***(11,19)***.

Others have used a variety of methods to calculate the free testosterone. The simplest method is to calculate a "free androgen index" based on the ratio of total testosterone concentration to the SHBG concentration ***(20)***. Although the ratio has been found to reflect free testosterone concentrations, it does occasionally give very high values that can be confusing. Other methods are based on the predetermined relationship between the SHBG concentration and free testosterone concentration while the more complex calculations are based on the binding affinities of albumin, SHBG and cortisol-binding globulin and the concentrations of these proteins and total testosterone in serum ***(21)***.

3.7. Dehydroepiandrosterone and Androstenedione

These steroids may be measured in serum, saliva, and hair using methods similar to those described for testosterone but with the requisite antibody and tracer. The immunoassay requires antisera produced specifically to the steroid of interest and an appropriately

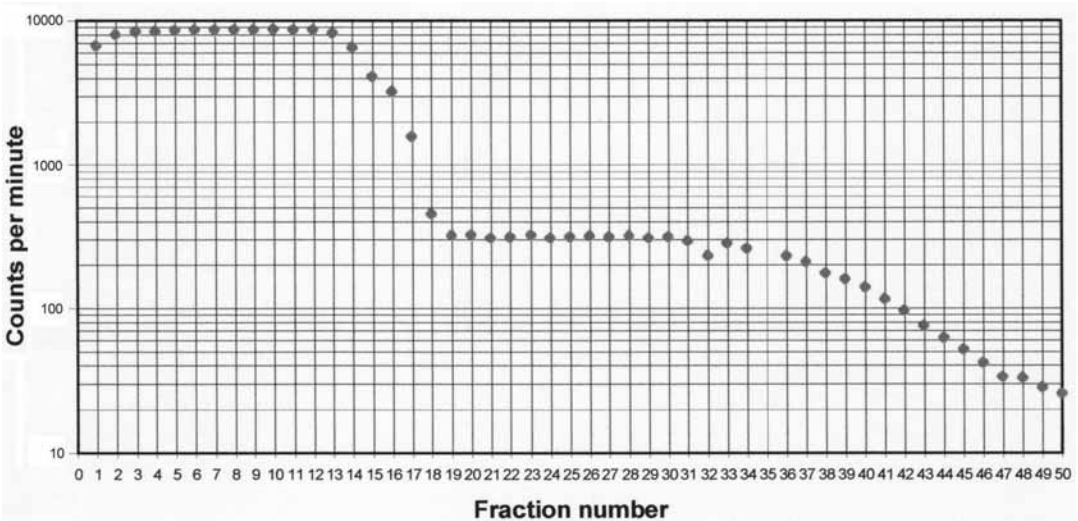

Fig. 1. Profile of radioactivity for free testosterone measurement by steady-state filtration.

labeled steroid. Commercial direct (nonextraction) assays for androstenedione exist and are sufficiently sensitive and specific for the majority of investigations. Direct assays for DHEA are not available commercially.

3.8. Dehydroepiandrosterone Sulfate

A simple direct immunoassay is readily developed in-house. Antisera to DHEA crossreact with the sulfated conjugate. Tritiated or iodinated DHEA may be purchased and used as tracer. DHEAS standards are prepared for quantitation. A typical standard curve has a concentration range of 0.2 µmol/L to 50 µmol/L. A simple methodology is as follows:

1. Dilute samples $^{1}/_{200}$ with assay buffer (1.0% BSA, 0.05 *M*, PBS, pH 7.4).
2. Add 100 µL standard or samples to appropriate tubes.
3. Add 100 µL DHEA antibody.
4. 100 µL DHEA tracer.
5. Incubate for 2 h or overnight at room temperature.
6. Add 500 µL PEG reagent.
7. Centrifuge for 30 min at 1500 rpm.
8. Decant off supernatant and allow inverted tubes to drain on absorbent tissue.
9. Count precipitate in tubes, plot standard curve, and calculate the concentration of DHEAS in the samples.

3.9. 5α-Dihydrotestosterone

Testosterone, that would be extracted with the DHT, must be removed first before extracting DHT because antisera to DHT have significant crossreaction with testosterone. Aqueous potassium permanganate is used to selectively oxidize and inactivate testosterone while leaving DHT essentially unchanged. The method described next is based on that of Puri et al. *(22)*.

3.9.1. Oxidation and Extraction

1. Add 500 µL patient serum to glass extraction tubes.
2. Add 5 mL diethyl ether.
3. Extract as described in method for testosterone (*see* **Note 13**).

4. Allow the two phases to separate.
5. Freeze aqueous layer in cardice/methanol.
6. Decant ether layer into clean glass extraction tubes and evaporate to dryness.
7. Reconstitute in 250 µL of fresh 50% w/v potassium permanganate (*see* **Note 14**).
8. Allow tubes to stand at room temperature for 3 h.
9. Extract with 3 mL diethyl ether.
10. Freeze aqueous layer and separate off ether layer as before.
11. Add 500 µL assay buffer and reconstitute for 1 h at room temperature.

3.9.2. Radioimmunoassay

1. Add 100 µL extract, 100 µL antiserum, and 100 µL tritiated DHT (*see* **Note 15**).
2. Incubate for 2 h at room temperature .
3. Add 500 µL 0.05% dextran/0.5% charcoal in assay buffer.
4. Incubate for 15 min in ice water bath.
5. Centrifuge for 20 min at 1500 rpm at 8ºC in refrigerated centrifuge.
6. Decant aqueous layer into scintillation mini-vials and count for 5 min.

3.10. Alternative Methods

All of the immunoassay methods described previously use radioactive tracers. The main advantage of RIA is the lack of interference effects. Most of the radioactive steroids are easily purchased. Enzyme, fluorescent, and chemiluminescent methods are available commercially or may be developed. Sensitivity is usually a problem with enzyme methods, which can also be affected by interferents. Fluorescent methods suffer similarly, but the use of time resolved fluorescence overcomes all or most of the problems and has improved sensitivity. Chemiluminescent labels are less available as single reagents and are more difficult to prepare and for most laboratories the preparation of in-house reagents is impractical.

4. Notes

1. Sodium salt may be used, but it is more difficult to dissolve.
2. Commercial kits, both for manual and automated analysis, are available for the direct, i.e., nonextraction) measurement of total testoster-

one from a number of diagnostic companies *(23–25)*. A number of procedures, such as adding large amounts of oestrogen, dihydrotestosterone, or urea, or adjusting the pH, are used to displace the steroid from the binding proteins. These methods are not as sensitive as the more laborious extraction method described. Extraction methods can precisely measure serum total testosterone down to at least 0.1 nmol/L, whereas most commercial direct assays have a functional sensitivity down to 1.0–1.5 nmol/L, inadequate for studies of prepubertal children, women, young animals and in saliva *(12,26–28)*.

3. Serum should be separated from red cells within 6 h of collection if kept at room temperature or within 24 h at 4ºC. Red cell enzymes convert androstenedione to testosterone and significantly increase testosterone concentration. The increase may be 50% after 24 h at room temperature.
4. A 1-min rinse of the glass tube with 1 mL ether is used in this laboratory to remove any traces of detergent or other contaminant that can interfere in the immunoassay.
5. Toluene may be used, but is not as efficient as ether although this does not matter if the standards are also extracted. Toluene is less flammable than ether but takes considerably longer to evaporate to dryness.
6. The length of the extraction procedure depends on how this is carried out. One minute is sufficient with either solvent with vigorous agitation, e.g., Rotamixer. We would recommend 10 min on a SMI shaker. Do not use bottles with cork/foil inserts in the caps as the solvent dissolves the glue and results in milky extracts and grossly elevated results.
7. The tube should not be left in the freezing mixture for more than about 1 min otherwise the ether begins to freeze. It is also important to ensure that the freezing mixture does not flow down the outside of the tube into the clean tube. This may be avoided by touching the bottom of the extraction tube on a pad of tissue before pouring.
8. There are a number of commercial companies that sell this reagent. Our source is Guildhay Antisera Ltd. This antiserum has a cross-reaction of about 16% with DHT and is used at a dilution of about 1/100,000.
9. This protein concentration reduces nonspecific binding (NSB) to about 2% and obviates the need to carry out a final wash step. A wash step will reduce the NSB to 1% or less.

10. Iodinated testosterone can be bought commercially from Amersham International or ICN. Both sources provide material sufficient for several thousand tubes and much of the reagent is wasted because of the short shelf-life (8–12 wk). In-house production is possible, but is only worth the effort if several thousand samples are to be analyzed. ICN are willing to sell small amounts (about 37 kBq) of tracer taken from their commercial kit. This is sufficient for 200–500 tubes depending on the volume used.
11. PEG solution contains polyethylene glycol 6000 in 1.0% BSA.PBS with donkey or goat antiserum to immunoglobulin in the animal in which the testosterone was raised and normal serum from the same animal.
12. Occasionally emulsions form which are very difficult to break down. One way to break down the emulsions is to freeze the contents of the tube and allow them to thaw slowly.
13. Additional specimens of stripped serum as blanks, stripped serum containing 20 nmol/L testosterone and patient serum containing 20,000 dpm tritiated DHT, are also extracted. The latter two types of specimen confirm complete removal of testosterone and recovery of DHT, respectively.
14. Buy a small quantity of potassium permanganate every 6 mo, as its efficiency appears to decrease with age. Alternatively rely on the testosterone control sample that indicates efficiency of oxidization.
15. Iodinated DHT may be used in the RIA, but the reagent will have to be prepared in-house because no commercial source is available.

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13

Measurement of Gut Hormones in Plasma

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Summary

The gastrointestinal tract is the largest endocrine organ and holds a special place in endocrinology since the concept of blood-borne communication between cells was first established through experiments on the gut. Gut peptide hormones and neurotransmitters regulate the complex processes of digestion, motility, epithelial growth, and integrity. Investigation of this complex endocrine organ has depended on the development of sensitive and specific radioimmunoassay. Radioimmunoassays have also increased our understanding of pathophysiological processes affecting the gut, including rare gastroenteropancreatic neuroendocrine tumours. The object of this chapter is to describe the techniques used in the radioimmunoassay of common gastrointestinal hormones.

Key Words: Gut; gastroenteropancreatic neuroendocrine tumors; hormone; peptide; plasma; radioimmunoassay.

1. Introduction

The processes of digestion and absorption of nutrients from the gastrointestinal (GI) tract, epithelial cell proliferation, maintenance of mucosal integrity, and GI motility are regulated by gut peptide hormones and the enteric nervous system (*see* **Table 1**). A large

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Table 1
Gastrointestinal Peptides Measured for Diagnostic Purposes

Peptide hormone/ neurotransmitterr	Number of amino acids	GI site	Secreting cells	Released by	Main functions
A. Gastrin-CCK family					
Gastrin	17/34	Gastric antrum	G cells	Protein, high calcium	Gastric acid and pepsinogen secretion; mucosal growth
B. Secretin-glucagon family					
1. Glucagon-like peptides:					
Glucagon	29	Pancreas	A-cells	Hypoglycaemia, amino acids	Inhibits glycogen synthesis, stimulates glycogenolysis, gluconeogenesis, and ketogenesis
2. VIP and related peptides					
Vasoactive intestinal peptide	28	Small intestine	Neurons	Intraduodenal acid	Smooth muscle relaxation, stimulates intestinal and pancreatic secretion, inhibits gastric acid secretion
C. Pancreatic polypeptide family					
Pancreatic polypeptide	36	Pancreas	F cells	Hypoglycaemia	Gallbladder relaxation, reduces pancreatico-biliary secretion (not HCO_3)
D. Somatostatin	14/28	Gastric antrum, pancreas	D cells	Food, acid in duodenum	Generally inhibitory
E. Neurotensin	13	Ileum	N cells	Lipid	Inhibits gastric secretion, stimulates pancreatic HCO_3 secretion, gallbladder relaxation, glycogenolysis

number of gut peptides that act as hormones, autocrine and paracrine agents, and as neurotransmitters have been described. However, only a minority, when secreted in excess into the circulation by rare gastroenteropancreatic tumors, result in the development of well-defined clinical syndromes. Gastroenteropancreatic tumors are rare; they include carcinoid tumors, insulinomas (secreting excess insulin), gastrinomas (secreting excess gastrin), VIPomas (secreting excess vasoactive intestinal peptide [VIP]), glucagonomas (secreting excess glucagon and other proglucagon-derived peptides), and somatostatinomas (secreting excess somatostatin). Several GI pathologies are associated with alterations in circulating concentrations of gut peptides, which are likely to represent adaptation of the gut, for example, in response to loss of absorptive or secretory surface by altering secretion and motility in unaffected regions. Most GI peptides have short plasma half-lives (*see* **Table 2**).

For most practical purposes, GI peptide hormones are measured in plasma. Measurement of GI hormones by radioimmunoassay (RIA) has been the most effective technique for the diagnosis and follow-up of patients with gastroenteropancreatic tumors. This technique requires the production of polyclonal antibodies (usually in rabbits) against the peptide (ligand) of interest. It also requires the production of radiolabelled peptide (^{125}I radiolabeled peptide is usually used). The assay involves competition for antibody binding sites between a fixed amount of radiolabeled peptide and the amount of peptide in the sample or standard (*see* **Figs. 1** and **2**). A standard curve is established from which the concentrations of samples are calculated. RIA allows measurement of peptide levels (often as low as 10 pmol/L) in the circulation. Although different antisera are used by different laboratories, preventing standardization of results, there is consistency in the magnitude of hormone levels. The elevation in circulating peptide levels in patients with gastroenteropancreatic tumors is usually high, but a variety of conditions may be associated with more modest elevations (*see* **Table 3**). It is essential to be aware of other causes of elevated gut peptides in the plasma for accurate interpretation of assay results.

Table 2
Plasma Half-Life of Gastrointestinal Peptides, Sites of Tumors That May Overproduce Them, and the Normal Plasma Concentrations[a]

Peptide	Plasma half-life (minutes)	Site of tumor	Normal range in plasma (pmol/L)
Gastrin 17/34	5/42	Pancreas/antrum/duodenum	< 40
Glucagon	2.5	Pancreas	< 50
Vasoactive intestinal peptide (VIP)	< 1	Pancreas/sympathetic chain	< 30
Pancreatic polypeptide (PP)	7	Pancreas	< 200
Somatostatin	3	Pancreas/duodenum/lung	< 100
Neurotensin	1.5	Pancreas/lung	< 200

[a] Special Assay Service, The Hammersmith Hospital.

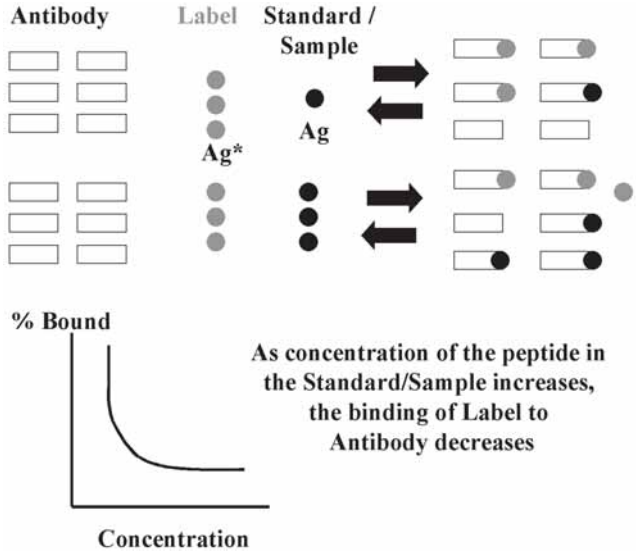

Fig. 1. The radioimmunoassay technique.

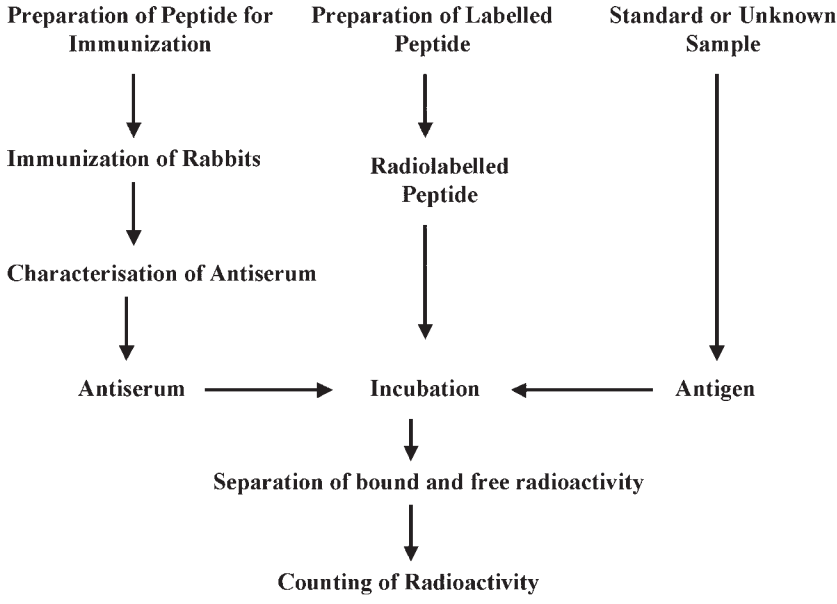

Fig. 2. The steps involved in development and running of a radio-immunoassay

Table 3
Causes of Increases in Plasma Concentrations of Commonly Measured Gut Peptides

General
Postabsorptive state
Renal failure
Hepatic failure
Stress (e.g., sepsis)
Hypergastrinaemia
(i) Associated with low gastric acid/achlorhydria
Atrophic gastritis-pernicious anaemia
Acid antisecretory drugs—proton pump inhibitors, histamine H2-blockers
Chronic renal failure
Helicobacter pylori infection
Post acid-reducing surgery
(ii) Associated with high gastric acid
H. pylori infection
Gastric outlet obstruction
Antral G cell hyperplasia
Retained gastric antrum post-surgery
Intestinal resection and short bowel syndrome
Gastrinoma (Zollinger-Ellison syndrome)
Hyperglucagonaemia
Prolonged fasting
Organ failure – liver, kidney
Drugs—oral contraceptive pill, danazol
Injury—trauma, burns, sepsis
Endocrine – Glucagonoma, Diabetic ketoacidosis, Cushing's syndrome
Familial—rare
Pancreatic polypeptide
Inflammatory bowel disease
Other neuroendocrine tumors
Elderly
Pernicious anaemia
Somatostatin
Somatostatinoma
VIP
VIPoma
Hepatic cirrhosis
Bowel ischemia

1.1. Collection of Blood for Radioimmunoassay of Gut Hormones

The correct collection of plasma for measurement of gut peptides is very important.

1. Collection is carried out in the morning after an overnight fast.
2. Collect blood (10 mL) in heparinized tube containing Trasylol (aprotinin, 200 μL, 2000 kallikein international units [KIU]). Aprotinin inhibits the action of plasma enzymes that may degrade gut hormones.
3. Mix by inversion.
4. Place on ice and transfer to laboratory or preferably spin in a centrifuge immediately. Separate plasma in a refrigerated centrifuge (~10,000*g* for 5 min) and remove plasma into suitable container (e.g., 5-mL Sterilin tube). Store at –20°C.
5. **Steps 1–4** should be carried out within 15 min.
6. Visible haemolysis makes sample unsuitable for measurement of gut hormones.
7. Complete patient details including medications should be stated.

For plasma gastrin measurement it is important for patients to be off of proton pump inhibitors for at least 2 wk and histamine H_2-blockers for 72 h; *Helicobacter pylori* infection has to be eradicated. Plasma should remain frozen during any transport.

1.2. Setting Up the Radioimmunoassay

Figure 2 shows the requirements for an accurate and reproducible RIA. These include:

1. A specific antibody that binds the peptide hormone with high avidity.
2. Stored peptide to be used as standard in the RIA.
3. Radiolabeled peptide hormone to compete for antibody binding sites with peptide in the standard or sample.
4. A method of separating antibody-bound and free fractions.

2. Materials

1. Chemicals to make buffers.
2. Balance for weighing reagents.

3. Large buffer flasks.
4. Distilled water and kettle.
5. Magnetic stirrer.
6. 30% Bovine serum albumin (BSA).
7. Access to cold room (temperature 4°C) and cold tray.
8. LP3 polypropylene tubes for assay.
9. Permanent markers (different color for each assay) for numbering tubes.
10. Eppendorf multipipet and tips.
11. Micropipets.
12. Vortex.
13. Minicounter and γ-counters.
14. Charcoal and dextran.
15. Sieve for charcoal slurry.
16. Syringe dispenser.
17. Centrifuge.
18. Glass Pasteur pipets (same pipet can be used for separating supernatant from pellet of all tubes without any need for washing in between).
19. Candle wax kept in oven with dispenser.
20. Curve drawing and calculating software.

Some antibodies and radiolabeled ligands are available either separately or as part of RIA kits. These commercial assays are however expensive and cannot therefore be used for routine measurement of plasma gut peptides. When an RIA kit is used, it is important to follow the instructions carefully. These are usually straightforward, but trouble-shooting may be difficult owing to small supply of reagents. Some companies carry out custom immunization; in this case, if regular use of the assay is going to be made, a regular supply of radiolabeled peptide is required.

3. Methods

3.1. Storage of Peptides

All peptides should be purchased from a reputable manufacturer. Peptides used for standard in the RIA should be weighed accurately and dissolved in a suitable solution for storage at concentrations

Table 4
Freeze-Drying Solution for Peptide Hormone Standards

Ingredient	Concentration	Purpose
Lactose	140 mmol/L (50 g/L)	Production of large soluble pellet
Bovine serum albumin	40 mmoL/L (2.5 g/L)	Prevents binding of peptide to surfaces
Citric acid	10 mmoL/L (2 g/L)	Peptide stability (low pH)
L-Cysteine hydrochloride	6 mmol/L (1 g/L)	Prevents oxidation damage
Aprotinin (Trasylol)	8×10^5 KIU/L	Inhibits proteolytic enzymes
Formic Acid	100 mmol/L (4.25 mL 90% formic acid)	Allows evaporation and aids freeze-drying

useful for the running the RIA. **Table 4** shows the composition of a suitable freeze-drying solution. Antibodies and radiolabeled peptide hormones are also stored in freeze-drying solution at a suitable concentration for running an average assay. Peptide standards, radiolabels, and antibody are aliquoted in glass vials and freeze-dried. Subsequently, they are stored at –20°C.

3.2. Antibody Production

For regular and routine measurement of gut peptides in plasma, a regular supply of antibody and radiolabeled ligand is needed. The production of sensitive and specific polyclonal antibodies is essential to RIA of gut peptides. Most gut peptides are small molecules (haptens) and are weakly immunogenic. Therefore, they require conjugation to a larger carrier molecule, which is usually BSA. The coupling of peptide antigen to BSA is usually carried out using gluteraldehyde or carbodiimide.

1. Usually six rabbits are immunized for each peptide.
2. The ratio of peptide to BSA is 4:1 for carbodiimide conjugation and 8:1 for gluteraldehyde conjugation.
3. 30–40 nmol of peptide is used for the primary immunization and 15–20 nmol for booster injections.
4. Primary immunization requires complete Freund's adjuvant (containing heat-killed mycobacteria 1mg/mL), incomplete Freund's adjuvant is used for booster injections. Freund's adjuvant is a mixture of 1 part of the detergent Arlacel A with 4 parts of *n*-hexadecane. It allows slow release of coupled hapten into the circulation and protects antigen against damage.
5. To prepare the emulsion for immunization:
   a. 4 mL of Freund's adjuvant (oily) is placed in a 20-mL Sterilin container.
   b. The conjugate is prepared by addition of gluteraldehyde or carbodiimide to peptide and hapten which have been dissolved in distilled water, pH 7.0 and incubated overnight at 4°C.
   c. Using a 1-mL syringe and small bore needle (23 gage), the peptide solution is withdrawn and forcefully injected into the oil with force. This is carried out up to four times until a water droplet placed on the surface of the emulsion stays as a single droplet.
6. Injections are carried out subcutaneously at four sites (0.25 mL/site), usually the flanks.
7. After 8–10 wk, the first booster injection is administered.
8. After 7–10 d, serum is collected. The ear is cleaned with Betadine and using aseptic technique, a nick is made in a peripheral vein. Up to 20 mL of blood is then collected in a 20-mL Sterilin tube.
9. The serum is removed and an antibody test is carried out.
10. Monthly boosts are given and blood collected 7–10 d after boosts.

3.3. Antibody Testing

To determine which antibody would be useful for a particular assay, the assay is set up with one set of tubes containing serial dilution of the antibody, while similar tubes will also contain standard at a concentration that is useful in the RIA. Radiolabel is added to all tubes. The antibody that produces the greatest drop in binding with the standard is further characterized and used in the RIA. An example is shown on **Table 5**. The tubes are set up as shown in **Fig. 3**.

Table 5
Antibody Testing

Tube no.		Antibody (% Bound)											
		A		B		C		D		E		F	
1	2	91.5	90.5	91.3	91.0	89.1	91.1	90.0	89.3	93.0	91.0	88.4	88.9
3	4	91.5	92.9	87.0	83.0	85.1	84.0	90.3	89.8	91.3	90.5	87.2	87.4
5	6	90.3	91.1	**53.1**	**23.0**	**62.3**	**37.0**	84.3	82.4	81.6	86.2	66.9	44.6
				57% Drop		**41% Drop**						**33% Drop**	
7	8	87.6	71.8	19.0	15.0	21.0	17.0	50.7	31.5	55.4	43.0	25.3	16.8
9	10	40.1	26.2	11.2	10.0	13.8	12.7	19.0	15.7	22.0	15.6	13.3	10.8
11	12	16.4	13.1	12.0	10.0	11.0	10.7	11.2	10.7	13.4	11.8	12.7	11.2

Serial dilutions of antibodies A–F; Even Tubes: 20 fmol of standard added.

Antibody B shows greatest drop in binding with the addition of standard; the dilution of the antibody B is then further investigated so that there is 50% binding (equilibrium) between the antibody and label in zero tubes (tubes without any standard or sample) and a useful full standard curve can be achieved. Antibodies C and F are also promising.

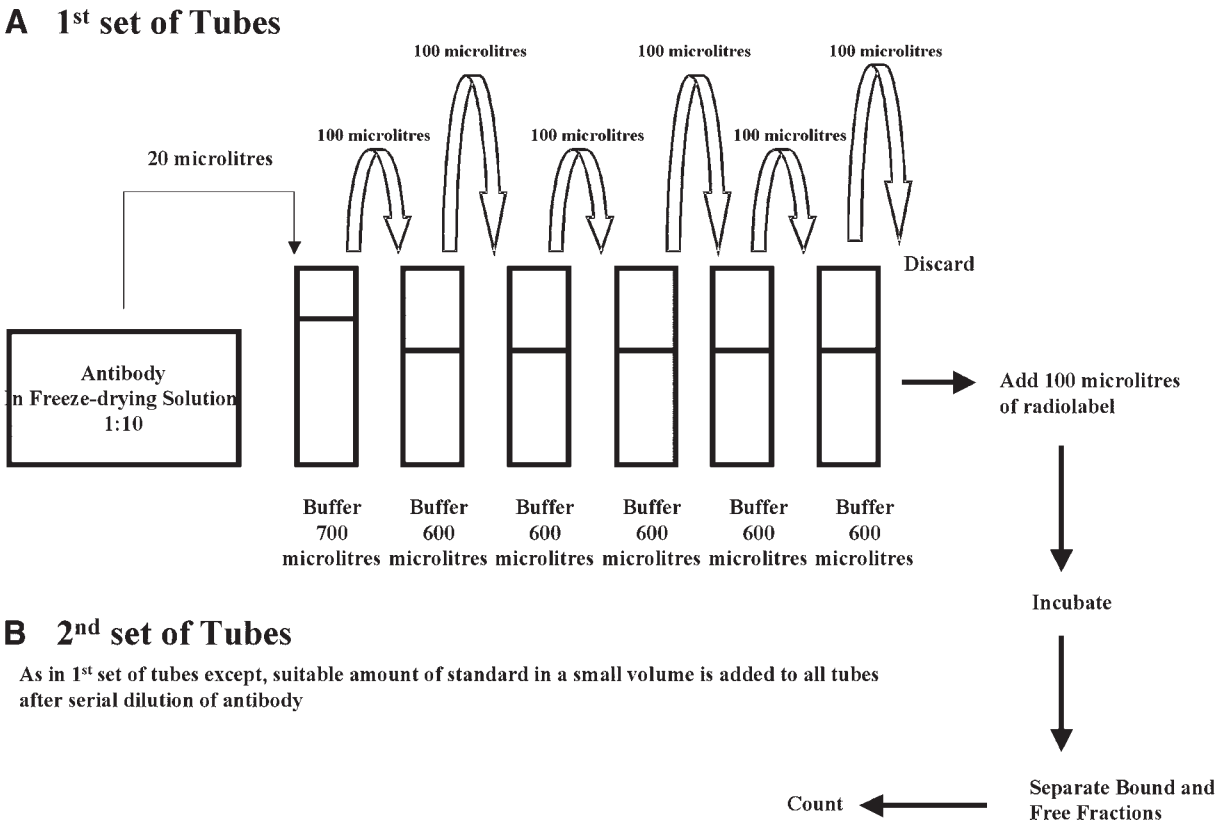

Fig. 3. Setting up tubes for antibody testing.

3.4 Radiolabel Production

Radio-iodination of peptides depends on whether the peptide has a suitable amino acid residue (e.g., tyrosine), the stability of the peptide, the harshness of the chemical reaction, and familiarity with a particular technique. The iodogen method has the mildest reaction and is used for iodination of VIP, glucagon, and Tyr11-somatostatin. The chloramine-T method is used for gastrin-17, peptide histidine methionine (PHM; derived from the same peptide precursor as VIP), and pancreatic polypeptide. High-performance liquid chromatography (HPLC) is the technique of choice for separating intact radioiodinated peptide from free radioiodine and damaged peptide. Usually one to three fractions are obtained, which are then tested in the assay. For label testing, the new label is always compared to the old label in the assay. Overnight incubation with double the concentration of antibody usually used in the assay will allow rapid identification of the best fraction for use in the RIA. Once identified, the fraction is dissolved in freeze-drying solution at sufficient counts for an average assay, and is freeze-dried and stored at –20°C.

3.5. Procedure for Radioimmunoassay

Assuming suitable antibody and label are available, setting up the RIA for gut peptides is straightforward.

1. Number assay tubes, everything is carried out in duplicate.
2. The designation of the tubes is shown in **Table 6**.
3. The content of the tubes is shown in **Table 7**.
4. Write an assay sheet to record the tube numbers and their corresponding contents.
5. Make up the buffers needed for the assays (5 L of buffers are usually made and kept for no longer than 14 d in a cold room at 4°C). The buffers used are:
   a. 0.06 *M* phosphate-EDTA buffer, pH 7.2. To make 5 L:
      Pre-boil 5 L of distilled water in an electric kettle (designated for buffers).
      Allow to cool.

Table 6
Designation of Assay Tubes

Tube number	Designation
1–2	Blank
3–4	1/2 X
5–6	2 X
7–10	Z
11–12	Standard 1
13–14	Standard 2
15–16	Standard 3
17–18	Standard 4
19–20	Standard 5
21–22	Standard 6
23–24	Standard 7
25–26	Standard 8
27–28	Standard 9
29–30	Standard 10
31–34	Zero
35–...	Samples
Zeros	2 Zeros after every 50 samples
Standard curve	
Other tubes	Quality controls
Final two tubes	Excess

Once cooled, to 4.5 L of the water add, while stirring:

48.00 g	$Na_2HPO_4.2H_2O$
4.13 g	KH_2PO_4
18.61 g	$Na_2H_2(EDTA)\cdot 2H_2O$ (ethylenediaminetetraacetic acid disodium salt)
2.50 g	NaN_3 (sodium azide).

Make the buffer up to 5 L.

Check pH with pH meter.

Label buffer with date and pH and store in cold room at –4°C.

For assay, 0.3% of 30% BSA is added to volume of buffer to be use and stirred well—1 mL 30% BSA for every 100 mL of buffer.

Table 7
Content on Assay Tubes

Tube Content	Blank	1/2 x	2 x	Zero	Standard	Samples	Excess
Buffer	550 µL	525 µL	450 µL	500 µL	Adjusted	500 µL	0
Antibody	0	50 µL	50 µL	50 µL	50 µL	50 µL	550 µL
Label	50 µL	25 µL	100 µL	50 µL	50 µL	50 µL	50 µL
Standard	0	0	0	0	1–100 µL	0	0
Sample	0	0	0	0	0	100 µL	0
Hormone Free Plasma	100 µL	100 µL	100 µL	100 µL	100 µL	0	100 µL
Total	700 µL	700 µL	700 µL	700 µL	700 µL	700 µL	700 µL

Blank: Non-specific binding; the lower the non-specific binding, the better. Assesses the integrity of label. Old labels give high blank binding.

1/2 x: This assesses whether greater sensitivity can be achieved by adding half the volume of label.

2 x: This allows assessment of the specific activity of the label.

Zero: Zero tubes are run at frequent intervals throughout the assay. The binding allows assessment of assay drift, which may be owing to syringe fatigue or reagent deterioration.

Excess antibody: This assesses the immunological integrity of the labeled peptide. The blank and excess tubes provide information about the quality of the label. The higher the excess binding, the better the assay.

Standards: A ten point standard curve is used.

Hormone free plasma (HFP): To all tubes except samples, 100 µL of HFP is added except glucagon for which Haemaccel is used instead. Since hormone free plasma is difficult to prepare, plasma from dated blood (available from the Transfusion Service) may be used.

Designated tubes for quality controls are also included containing "low," "medium," and "high" concentrations of the peptides.

For all peptide assays: Phosphate-EDTA buffer + 0.3% BSA is used except for the glucagon assay which requires barbitone buffer + 0.3% bovine serum albumin (BSA).

Incubation time: 3 d at 4°C.

b. 0.06 *M* phosphate-EDTA buffer with gel—used for charcoal/dextran separation:

   As in **item a**, except 12.50 g of gelatine is first dissolved in freshly boiled water and allowed to cool before other constituents are added and allowed to dissolve.

   Make up to 5 L.

   Label buffer with date and pH and store in cold room at –4°C.

For assay, 0.3% of 30% BSA is added to volume of buffer to be use and stirred well—1 mL 30% BSA for every 100 mL of buffer.

c. Barbitone buffer: to make 5 L:

Pre-boil 5 L of distilled water in an electric kettle (designated for buffers).

Allow to cool.

Then dissolve 51.55 g barbitone sodium and dissolve completely before adding 20 mL of 5 *M* HCl or 9.6 mL of concentrated HCl.

Allow acid to disperse well before adding 2.50 g NaN_3.

Leave to stir.

Check pH with pH meter.

Label buffer with date and pH and store in cold room at –4°C.

For assay, 0.3% of 30% BSA is added to volume of buffer to be use and stirred well—1 mL 30% BSA for every 100 mL of buffer.

6. Calculate volume of buffer needed for size of assay, add appropriate volume of 30% BSA (1 mL/100 mL to give 0.3% BSA). Stir on magnetic stirrer for 30 min.
7. Add buffer to the tubes with appropriate volumes using Eppendorf multipipet. Keep all buffered tubes, samples, and other reagents in a cold room at 4°C or on a cold tray until needed.
8. Make up standards from freeze-dried vials and appropriate buffers. Standards are weighed and dissolved in freeze-drying solution. They are then freeze-dried and stored at –20°C until use. Dissolve all standards in their respective assay buffers. Vortex to dissolve.

   Examples for standards are shown in **Table 8**.
9. Add standards to designated tubes as shown in **Table 9**.
10. Add hormone free plasma (HFP)/Haemaccel to all other tubes except samples.
11. Add samples using Eppendorf multipipet (100-L). Wash dispenser twice with water between samples and dry dispenser with tissue. Alternatively, samples can be added using a micropipet with a new tip for each sample. Always give the tubes a gentle shake throughout the assay after each addition of constituents.
12. Add quality controls.

Table 8
Examples for Standards

Peptide	Assay buffer	Standard vial (pmol)	Volume of buffer for standard to be dissolved in
Gastrin	Phosphate-EDTA + 0.3% BSA	1	2.5
VIP	Phosphate-EDTA + 0.3% BSA	1	2.5
PHM	Phosphate-EDTA + 0.3% BSA	2	2
Glucagon	Barbitone + 0.3% BSA	1	2
Pancreatic polypeptide	Phosphate-EDTA + 0.3% BSA	1	2
Neurotensin	Phosphate-EDTA + 0.3% BSA	1	1
Somatostatin	Phosphate-EDTA + 0.3% BSA	0.75	0.75

Table 9
Addition of Standards to Designated Tubes

Standard	Volume added from solution made above (µL)	Instrument used for adding standard	Buffer (µL)	HFP (µL)	Antibody (µL)	Label (µL)
1	1	Micropipette	500	100	50	50
2	2	Micropipette	500	100	50	50
3	3	Micropipette	500	100	50	50
4	5	Micropipette	500	100	50	50
5	10	Micropipette	500	100	50	50
6	15	Micropipette	500	100	50	50
7	20	Micropipette	480	100	50	50
8	30	Micropipette	470	100	50	50
9	50	Micropipette	450	100	50	50
10	100	Micropipette	400	100	50	50

Table 10
Examples of Titers Used

Peptide	Antibody Name	Working titer/final titer
VIP	V9 1:100	1:28000/1:448000
PP	HPP/CCK5 1:50	1:41667/1:666667
Gastrin	GAS 8 1:10	1:48125/1:770000
Glucagon	RCS5 1:50	1:24000/1:384000
Somatostatin	K2 1:10	1:6000/1:96000
Neurotensin	NT58	1:3947/1:63158
PHM	SY9 1:10	1:10,000/1:160000

13. Make up antibody to the appropriate titer in assay buffer. The titers have to be determined for every individual antibody after an antibody test (*see* **Subheading 3.2.**).
    Examples of titers used are given in **Table 10**.
14. Make up label in assay buffer to give a count of 300 (on minicounter) over 10 s/50 µL. Laboratory coats should be worn at all times. Hands should be covered with gloves during, and thoroughly washed after, the handling of radioactive material. Follow laboratory safety instructions for handling of radioactive material.
15. Add antibody and label to tubes using Eppendorf mulipipet (separate tips for antibody and label). The Eppendorf tips can be labeled with what they have been used for (e.g., buffer, label, or antibody); if these are washed thoroughly after every use, they can be reused. All tubes take 50 µL of label except 1/2X and 2X (25 µL and 100 µL, respectively). All tubes take 50 µL of antibody except Blank (no antibody) and Excess (550 µL).
16. Incubate at 4°C in a cold room for 3 d (2 d, i.e., over weekend to 5 d).
17. Charcoal separation after incubation period.

*3.6. Charcoal-Dextran Separation of Radioimmunoassay (*see ***Fig. 4)***

Charcoal has pores that allow anything that is not bound to antibody to collect in the charcoal. Dextran makes these pores of suffi-

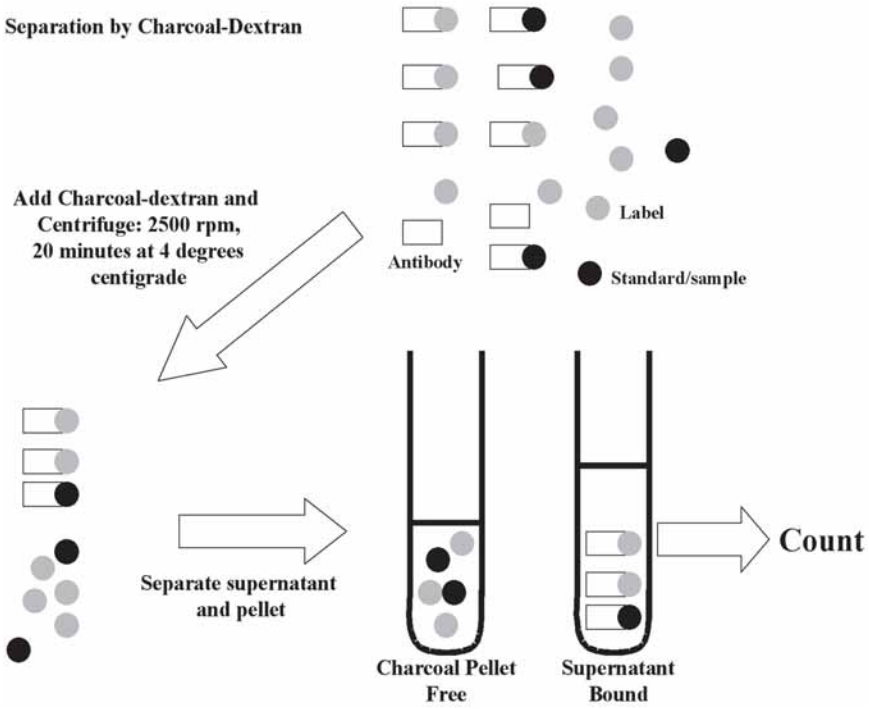

Fig. 4. Charcoal-dextran separation of free and bound fractions

cient size to exclude any bound antibody and allows better pellet formation.

Charcoal-dextran in phosphate-EDTA buffer (0.25 mL) with gel is added to each assay tube.

1. For gastrin, pancreatic polypeptide, neurotensin, and somatostatin, 8 mg of charcoal (with 10% dextran) is used per assay tube. Therefore, for a typically used volume of 400 mL of phosphate-EDTA with gel, 12.8 g of charcoal and 1.28 g of dextran is added.
2. For VIP, glucagon, and PHM, 6 mg of charcoal (with 10% dextran) is used for each assay tube. Therefore, for a typically used volume of 400 mL of phosphate-EDTA with gel, 9.6 g of charcoal and 0.96 g of dextran is added.
3. Weigh out appropriate amount of charcoal and dextran needed for size of assay.

4. Add the appropriate volume of buffer with gel needed for size of assay.
5. Set to stir in cold room at 4°C for 20 min or more and add BSA (0.3% BSA is used: 1 mL of 30% BSA is added for every 100 mL of buffer).
6. After 20 min, sieve the charcoal slurry into new beaker to remove large pieces and continue stirring in the cold room.
7. Rack assay tubes into centrifuge cassettes.
8. While still stirring, using Hamilton syringe dispenser, add one shot (0.25 mL) of the mixture to each assay tube.
9. Balance cassettes before putting in centrifuge.
10. Spin in centrifuge at 2500–3000 rpm (~1700*g*), 4°C for 20 min with centrifuge brake set at maximum.
11. When centrifugation is complete, separate bound (supernatant) from free (pellet) using Pasteur pipet.
12. Add molten wax (kept in oven at 200°C) using wax dispenser to supernatant tubes (stops spillage and contamination of counters).
13. Count the assay in γ-counter.
14. Calculate standard curve.
15. Calculate samples.

Suggested Reading

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14

Measurement of Melatonin and 6-Sulphatoxymelatonin

Benita Middleton

Summary

Melatonin and its major metabolite, 6-sulphatoxymelatonin, can routinely be measured by RIA or ELISA. Plasma, serum or saliva samples maybe used for melatonin measurement and urine for the metabolite. Melatonin is a hormone produced by the pineal gland witha clear circadian rhythm giving low levels by day and a peak during the night in normally entrained individuals. The timing of sample collection for measurement is therefore crucial. Melatonin levles are primarily assessed to determine the timing of an individual's internal body clock and therefore indicate any misalignment to the 24 h day.

Key Words: Melatonin; 6-sulphatoxymeltonin; ciracadian rhythm; internal body clock; misalignment; jet lag; shift work; rhythm disorders.

1. Melatonin

Melatonin (5-methoxy-*N*-acetyltryptamine) is an indole hormone secreted by the pineal gland. Pineal activity modulates the hypothalamic pituitary axis and the serotonergic system and may therefore be implicated in clinical illness and psychiatric disorders. Melato-

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nin is involved in the transduction of time of day and time of year information to the organism. In all mammals (including humans) it acts as a signaling system indicating the change from day to night *(1)*. There is some evidence to suggest that endogenous melatonin may influence sleep propensity and core body temperature. It has also been linked to a number of other physiological functions including, retinal function *(2)*, enhanced immunity *(3)*, oncostatic effects *(4)*, and antioxidant mechanisms *(5)*.

In humans, melatonin is secreted rhythmically with low levels during the hours of daylight and a peak during darkness. This melatonin rhythm is circadian being generated by the biological clock, situated in the hypothalamic suprachiasmatic nuclei (SCN). In normally entrained individuals (i.e., synchronized to the 24-h light/dark cycle) levels begin to rise during the evening (20:00–23:00 h) reaching maximum values between 02:00 and 04:00 h and returning to baseline levels during the late morning (08:00–10:00 h). This clearly defined circadian rhythm in melatonin production is a useful marker of the phase position of the endogenous SCN clock *(1)*.

The primary reason for the measurement of melatonin is to assess the timing (phase position) of the biological clock and to identify any misalignment of the circadian system to the 24-h d, for example as seen following travel across time zones, during night shift work *(6)*, and in some categories of blindness *(7)*. Melatonin is a very robust marker rhythm as levels are only affected by light exposure *(8)* and, to a lesser extent, by changes in posture *(9)*. Exposure to light at night suppresses melatonin levels, which return to normal after lights off. In ideal circumstances it is very important to collect regularly timed samples across a complete 24-h day; analysis of single time point samples gives little or no indication of the melatonin profile. In individuals who have been maintaining a regular sleep-wake cycle for a minimum period of 7 d, samples may be collected in dim lighting during the evening hours only (18:00–24:00 h) to determine the dim light melatonin onset (DLMO) *(10)*.

Secondary reasons for the measurement of melatonin are to determine pineal gland function and in the diagnosis of pineal tumors.

Finally, the light-induced melatonin suppression test can be utilized to investigate the integrity of the retina-retino-hypothalamic tract-SCN-pineal pathway.

Melatonin concentrations can be measured in both plasma and saliva, while the major metabolite, 6-sulphatoxymelatonin (aMT6s), can be assessed in urine. The principle technique currently in use is quantitative radioimmunoassay (RIA) ***(11–18),*** there are also commercially available enzyme-linked immunosorbent assays (ELISA). For accurate assessment of circadian phase plasma is the matrix of choice as values are high and samples can be collected at carefully controlled time points (preferably a minimum of hourly for 24 h). Saliva melatonin levels are approx 30% of plasma levels, but an advantage is that saliva sample collection is noninvasive. Care must be taken to ensure that plasma and saliva samples are collected under dim light conditions (preferably <10 lux at eye level) with controlled posture. Urine samples should be collected over regular time intervals (routinely every 4 h and 8 h overnight). The timing of the collection period and volume of urine passed must all be recorded. The major benefit of urine collections for aMT6s is that these can be done in subjects' homes and over long periods of time. A drawback is that the samples are integrated over several hours and so phase assessment is less accurate, however, as an initial investigation urinary aMT6s is ideal allowing calculation of the 24-h output and time of peak values.

Sample size and preparation is also an important consideration when choosing whether to collect plasma, saliva, or urine. A minimum of 1.5 mL plasma or saliva is needed if measurements are to be made in duplicate whereas 100 µL of urine is more than sufficient. Urine samples do not require treatment before measurement and are stable for up to 5 d at room temperature. Saliva samples are best harvested by using Salivettes (Sarstedt) with cotton plugs that are capable of holding up to 3 mL saliva. Samples should be frozen as soon after collection as possible and then centrifuged prior to assay. The saliva ELISA melatonin assay requires saliva samples to be pre-treated but saliva samples to be assayed by RIA do not require pretreatment. Blood samples should be collected in lithium/

heparin tubes and centrifuged immediately after collection with the plasma being separated and frozen (–20°C) as soon as possible. The ^{3}H melatonin assay allows plasma samples to be measured directly but both ^{125}I assays require plasma or serum samples to be extracted, one with chloroform and the other by passage through a C_{18} reverse phase column. Plasma and saliva samples must be kept frozen, even during transport.

All RIA and ELISA assays described here involve production of a standard curve and it is essential that each curve is constructed in the correct matrix and nonspecific binding (NSB) is determined. It is very important for consistency and reproducibility of assay performance that suitable quality control samples (low, medium, and high values) are included in each assay.

2. Materials

2.1. Plasma ^{3}H-Melatonin Assay (Assay Has Been GCMS Validated)

1. Double glass-distilled water (DGDW) make fresh.
2. 0.1 *M* tricine buffer pH 5.5 containing 0.9% NaCl and 0.1% gelatin. Store at 4°C.
3. Affinity- or charcoal-stripped serum (12.5-mL lyophilized aliquots). Store at –20°C.
4. Melatonin standard (1 mg/mL) in absolute ethanol. Store at –20°C.
5. Melatonin antiserum batch G/S/704/8483 (Stockgrand Ltd, University of Surrey, Guildford, UK). Store at –20°C.
6. ^{3}H-Melatonin (Amersham International, Amersham, Bucks, UK). Store at –20°C.
7. Dextran-coated charcoal (2% charcoal, 0.2% dextran). Store at 4°C.
8. Scintillation fluid (2.5 L Toluene (low sulfur grade) + 12.5 g 2,5-diphenyoxazole (PPO) + 0.75g 1,4-*bis*(5-phenyloxazol-2-yl)benzene (POPOP).
9. Disposable polysterene LP3 tubes.
10. Single-channel adjustable pipets 5–1000-µL, pipet tips.
11. Repeating pipet for 100, 200, 500 µL vol.
12. 500 or 1000 mL graduated cylinder.

13. Vortex mixer.
14. Shaker.
15. Weighing balance.
16. Metal spoon or spatula.
17. Magnetic stirring bar.
18. Magnetic stirrer.
19. Refrigerated centrifuge.
20. Absorbent paper towels.
21. Automatic dispenser for 4 mL vol.
22. Polypropylene minivials (7 mL).
23. Scintillation counter.

2.2. Saliva and Plasma ^{125}I-Melatonin Assay (Assay Has Been GCMS Validated)

1. DGDW, make fresh.
2. 0.1 *M* tricine buffer, pH 8.0, containing 0.9% NaCl and 0.2% gelatin. Store at 4°C.
3. Melatonin standard 1 mg/mL in absolute ethanol. Store at –20°C.
4. Melatonin antiserum rabbit or sheep. Store at –20°C.
5. ^{125}I-Melatonin (Amersham International). Store at 4°C.
6. Anti-sheep or anti-rabbit second antibody suspension (SacCell, IDS Ltd. Tyne and Wear, UK). Store at 4°C.
7. Brij-Saline wash solution: 9 g NaCl + 2 mL Brij 35 in 1 L DGDW. Store at 4°C.
8. Chloroform.
9. 10-mL glass screw-capped vials.
10. Disposable polysterene LP3 tubes.
11. Single-channel adjustable pipets 5 µL–1000 µL, pipet tips.
12. Repeating pipet for 100, 200, 500 µL vol.
13. 500 or 1000 mL graduated cylinder.
14. Vortex mixer.
15. Shaker.
16. Weighing balance.
17. Metal spoon or spatula.
18. Magnetic stirring bar.
19. Magnetic stirrer.

20. Refrigerated centrifuge.
21. Absorbent paper towels.
22. γ-counter.

2.3. Saliva and Plasma ^{125}I-Melatonin Assay: Bühlmann Test Kit

These kits are supplied by Bühlmann Laboratories AG, Switzerland. (Buhlmann Melatonin RIA test kit code: RK-MEL or Bühlmann Direct saliva melatonin test kit code: RK-DSM.) Store at 4°C.

1. Single channel 100 µL, 1000 µL, 5-mL adjustable pipets, pipet tips.
2. Disposable polysterene LP3 tubes.
3. Disposable borosilicate glass tubes.
4. Methanol (HPLC grade).
5. Hexane (HPLC grade).
6. DGDW.
7. Refrigerated centrifuge.
8. Vortex mixer.
9. Magnetic stirring bar.
10. Magnetic stirrer.
11. Aspiration device.
12. γ-counter.

2.4. Saliva and Plasma Melatonin ELISA: Bühlmann Test Kit

This kit is supplied by Bühlmann Laboratories AG, Switzerland. (Bühlmann direct melatonin saliva ELISA kit code: EK-DSM.) Store at 4°C.

1. Single channel 50–1000 µL, adjustable pipets, pipet tips.
2. Multichannel pipet, pipet tips, and troughs.
3. Deionized water.
4. 500 or 1000-mL graduated cylinder.
5. Wash bottle.
6. Disposable borosilicate glass tubes.
7. Methanol (HPLC grade).
8. Hexane (HPLC grade).
9. DGDW.

10. Polypropylene microfuge tubes.
11. Refrigerated centrifuge.
12. Vortex mixer.
13. Plate mixer.
14. Absorbent paper towels.
15. Microtiter plate reader able to read at 450 nm.

2.5. Urine ^{125}I–6-Sulphatoxymelatonin Assay (Assay Has Been GCMS Validated)

1. 6-sulphatoxymelatonin kit from Stockgrand Ltd. Code: aMT6s-HU-K100. Store at 4°C.
2. DGDW, make fresh.
3. Disposable polysterene LP3 tubes.
4. Single-channel adjustable pipets 5–1000-µL, pipet tips.
5. Repeating pipet for 100, 200, 500 µL vol.
6. 500 or 1000 mL graduated cylinder.
7. Vortex mixer.
8. Shaker.
9. Magnetic stirring bar.
10. Magnetic stirrer.
11. Refrigerated centrifuge.
12. Absorbent paper towels.
13. γ-counter.

3. Methods

3.1. Plasma ^{3}H-Melatonin Assay

1. Blood samples for analysis are collected into lithium heparin tubes and centrifuged immediately at 1500*g* for 15 min at 4°C. Plasma may then be stored at –20°C until assayed. No further preparation is required but samples may require centrifugation if large fibrin clots form (*see* **Note 1**).
2. Affinity-stripped plasma and charcoal-stripped plasma are prepared from blood harvested during the afternoon, when endogenous levels of melatonin are low. Affinity stripped plasma is produced by use of melatonin antisera coupled to a solid matrix (Stockgrand

Ltd). Charcoal stripped plasma is made using 10% activated defined charcoal. Lyophilized aliquots are reconstituted with 12 mL DGDW (*see* **Note 2**).

3. A working melatonin standard is prepared from the stock 1 mg/mL melatonin solution.
   100 µL (1 mg/mL) to 100 mL in DGDW = 1 µg/mL.
   500 µL (1 g/mL) to 50 mL in DGDW = 10 ng/mL.
   300 µL aliquots of this standard can be stored at –20°C for up to 2 mo.
4. The lyophilized antisera is reconstituted as directed to give a working dilution.
5. ^{3}H Melatonin from Amersham is stored at –20°C. An intermediate stock is prepared by diluting 20 µL to 2 mL with absolute ethanol. A working solution is prepared fresh for each assay by making 50 µL intermediate stock up to 10 mL with assay buffer.
6. Label polysterene LP3 tubes in duplicate for total, NSB, and standards *(7)* with maximum binding (MB) (zero melatonin) tubes in quadruplicate (*see* **Note 3**). Label additional tubes in duplicate for samples and quality controls.
7. Pipet 1.2 mL assay buffer into the Total tubes; 200 µL assay buffer and 500 µL melatonin free plasma into the NSB tubes.
8. Prepare top standard of 500 pg/mL by diluting 125 µL (10 ng/mL melatonin) to 2.5 mL in melatonin free plasma.
9. A standard curve is constructed by adding varying amounts of 500 pg/mL standard and melatonin free plasma as shown in **Table 1** to give a standard curve range of 0–500 pg/mL (*see* **Note 4**).
10. Add 500 µL of each quality control or sample in duplicate to the correctly numbered tubes (*see* **Notes 5** and **6**).
11. Add 200 µL working antiserum dilution to all tubes except total count and NSB tubes. Cover tubes with cling film.
12. Vortex and incubate at room temperature for 30 min.
13. Add 100 µL working ^{3}H-melatonin to all tubes, cover with cling film.
14. Vortex and incubate at 4°C for 18 h (overnight).
15. Add 500 µL dextran-coated charcoal to all tubes except totals. Incubate at 4°C for 10 min (*see* **Note 7**).
16. Centrifuge at 1500*g* for 15 min at 4°C.
17. Place 700 µL aliquots of all supernatants into minivials containing 4.0 mL scintillation fluid (*see* **Note 8**).
18. Shake vials at room temperature for 1 h (*see* **Note 9**).

Table 1
Volumes for ^{3}H Direct Plasma Melatonin Standard Curve

MT standard 500 pg/mL (µL)	MT free plasma (µL)	MT concentration (pg/tube)
0	500	0
5	495	2.5
10	490	5
25	475	12.5
50	450	25
100	400	50
200	300	100
500	0	250

19. Count the radioactivity in all vials for 4 min (or 4000 cpm) in a scintillation counter.
20. Melatonin concentrations in the samples and quality controls are determined by reference to the standard curve (*see* **Note 10**).

3.2. Saliva and Plasma ^{125}I–Melatonin Assay

1. Saliva samples (*see* **Note 11**) collected into Salivettes (*see* **Note 12**) should be centrifuged at 1500*g* for 15 min at 4°C prior to assay. This extracts saliva from the cotton plug, saliva collected directly into tubes must also be centrifuged to remove particulate matter.
2. Plasma samples are extracted using chloroform. Place 1.2 mL plasma in a 10-mL screw-capped borosilicate glass tube. Add 5.0 mL chloroform using a glass pipet, vortex for 10 s and centrifuge for 10 min at 1500*g*, 4°C. Remove aqueous layer and evaporate chloroform to dryness under nitrogen in a 40°C water bath. Resuspend in 1.2 mL assay buffer (*see* **Note 13**).
3. A working melatonin standard is prepared from the stock 1 mg/mL melatonin solution.
   100 µL (1 mg/mL) to 100 mL in DGDW = 1 g/mL.
   500 µL (1 g/mL) to 50 mL in DGDW = 10 ng/mL.
   300 µL aliquots of this standard can be stored at –20°C for up to 2 mo.
4. The lyophilized antisera is reconstituted as directed to give a working dilution.

Table 2
Volumes for ^{125}I Saliva and Plasma Melatonin Standard Curve

MT standard 200 pg/mL (µL)	Assay buffer (µL)	MT concentration (pg/tube)
0	500	0
5	495	1
10	490	2
25	475	5
50	450	10
100	400	25
200	300	50
500	0	100

5. ^{125}I Melatonin from Amersham International is stored at 4°C. A working solution is prepared fresh for each assay by diluting the stock solution to give 10,000 cpm. The dilution will vary depending on the activity date of the tracer and the specific activity.
6. Label polysterene LP3 tubes in duplicate for total, NSB, and standards *(7)* with MB (zero melatonin) tubes in quadruplicate (*see* **Note 3**). Label additional tubes in duplicate for samples and quality controls.
7. Leave Total tubes empty. Pipet 700 L assay buffer into the NSB tubes.
8. Prepare top standard of 200 pg/mL by diluting 50 µL (10 ng/mL melatonin) to 2.5 mL in assay buffer.
9. A standard curve is constructed by adding varying amounts of 200 pg/mL standard and assay buffer as shown in **Table 2** to give a standard curve range of 0–200 pg/mL (*see* **Notes 4** and **14**).
10. Add 500 µL of each quality control or sample in duplicate to the correctly numbered tubes (*see* **Notes 5** and **6**).
11. Add 100 µL working antiserum dilution to all tubes except total count and NSB tubes. Cover tubes with cling film.
12. Vortex and incubate at room temperature for 30 min.
13. Add 100 µL working ^{125}I-melatonin to all tubes, cover with cling film.
14. Vortex and incubate at 4°C for 18 h (overnight).
15. Add 100 µL anti-sheep or anti-rabbit second antibody suspension (SacCell IDS Ltd.) to all tubes except totals and mix. Incubate at

room temperature for 1 h mixing every 15 min (*see* **Note 15**).
16. Add 1 mL Brij/saline wash solution to all tubes except totals (*see* **Note 16**).
17. Centrifuge at 1500*g* for 15 min at 4°C.
18. Decant supernatants from all tubes except totals. Blot tubes dry on absorbent paper.
19. Count the radioactivity in all tubes for 1 min in a γ-counter.
20. Melatonin concentrations in the samples and quality controls are determined by reference to the standard curve (*see* **Note 10**).

3.3. Saliva and Plasma ^{125}I–Melatonin Assay: Bühlmann Test Kit

Instruction leaflets accompany each kit. The steps for each assay are outlined next.

1. Plasma or serum samples should be filtered or centrifuged at 1500*g* for 10 min at 4°C prior to extraction to remove fibrin, red cells, or other material likely to clog the column.
2. Label an extraction column for each sample to be extracted and place in a polypropylene or glass tube.
3. Add 1 mL methanol to each column and centrifuge for 1 min at 200*g* (*see* **Note 17**).
4. Add 1 mL methanol to each column and centrifuge for 1 min at 200*g*.
5. Add 1 mL DGDW to each column and centrifuge for 1 min at 200*g* (*see* **Note 18**).
6. Add 1 mL DGDW to each column and centrifuge for 1 min at 200*g*.
7. Add 1 mL sample to the correctly labeled column and centrifuge for 1 min at 200*g*.
8. Add 1 mL 10% methanol in water to each column and centrifuge for 1 min at 500*g*.
9. Add 1 mL 10% methanol in water to each column and centrifuge for 1 min at 500*g*.
10. Add 1 mL hexane to each column and centrifuge for 1 min at 500*g*.
11. To elute the extract, place each column in a clean correctly labeled borosilicate glass tube. Add 1 mL methanol and centrifuge for 1 min at 200*g*. The column can then be used to extract the next sample (*see* **Notes 19** and **20**).
12. Evaporate the methanol to dryness by placing in a 37°C water bath under nitrogen.

13. Reconstitute each sample with 1 mL of incubation buffer and mix well using a vortex mixer (*see* **Note 21**).
14. Equilibrate the extracts for 30 min at room temperature.
15. Label polysterene LP3 tubes in duplicate for total, NSB, MB, and standards ***(5)***. Label additional tubes in duplicate for samples and quality controls.
16. Leave Total tubes empty. Pipet 500 µL incubation buffer into the NSB tubes and 400 µL incubation buffer into MB tubes.
17. Reconstitute lyophilized standards with 5 mL incubation buffer (*see* **Note 22**).
18. Add 400 µL standards (0.5, 1.5, 5, 15, and 50 pg/mL melatonin) to correctly labeled tubes (*see* **Note 23**).
19. Add 400 µL of the extracted samples and controls to the correspondingly labeled tubes.
20. Add 100 µL melatonin antibody to all tubes except totals and NSB tubes.
21. Add 100 µL ^{125}I melatonin to all tubes and vortex.
22. Incubate for 18 h (overnight) at 4°C.
23. Mix solid phase second antibody thoroughly and add 100 µL to all assay tubes except totals. Vortex mix all tubes (*see* **Note 15**).
24. Incubate for 15 min at 4°C.
25. Add 1 mL cold DGDW to all assay tubes except Totals.
26. Centrifuge for 2 min at 2000*g* 4°C. Aspirate all supernatants except totals (*see* **Note 25**).
27. Count pellets for 2 min in a γ-counter.
28. Melatonin concentrations in the samples and quality controls are determined by reference to the standard curve (*see* **Note 10**).

3.4. Saliva and Plasma Melatonin ELISA: Bühlmann Test Kit

Instruction leaflets accompany each kit. The method is an indirect "sandwich" ELISA assay with capture antibody preadsorbed to 12×8 well strips making up one 96-well microtiter plate. The steps for each assay are outlined next.

1. Plasma or serum samples should be extracted using C_{18} columns as for the Bühlmann ^{125}I melatonin assay except that 200 µL of sample is extracted and the extract reconstituted in 250 µL reconstitution buffer (*see* **Note 13**).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	HighQC	HighQC								
B	Zero	Zero	UNK 1	UNK 1								
C	Std A	Std A	UNK 2	UNK 2								
D	Std B	Std B	UNK 3	UNK 3								
E	Std C	Std C	UNK 4	UNK 4								
F	Std D	Std D	UNK 5	UNK 5								
G	Std E	Std E	UNK 6	UNK 6								
H	LowQC	LowQC	UNK 7	UNK 7								

Fig. 1. Diagram of suggested microtiter plate layout.

2. Saliva samples must be pretreated. Pipet 200 µL controls and saliva samples into correspondingly labeled polypropylene microfuge tubes (*see* **Note 26**).
3. Add 25 µL pre-treatment solution to each tube.
4. Vortex for 10 s and leave to stand for 10 min.
5. Add 25 µL neutralizing solution to each tube and vortex for 5 s.
6. Prepare wash buffer by making up 100 mL stock solution to 1 L with deionized water.
7. Select sufficient 8 well strips to test the number of blanks, standards, controls, and samples required (*see* **Note 27**).
8. Wash coated strips twice with wash buffer. Empty wells and blot dry (*see* **Note 28**).
9. Add 100 µL blanking reagent to wells A1 + A2 (for plate layout *see* **Fig. 1**).
10. Add 100 µL zero calibrator to wells B1 + B2.
11. Add 100 µL 5 standards (1, 3, 9, 27, and 81 pg/mL) in duplicate to wells C1 + C2; D1 + D2, and so on (*see* **Note 29**).
12. Add 100 µL pretreated low and high controls to wells H1 + H2 and A3 + A4.
13. Add 100 µL pretreated sample in duplicate to subsequent wells B3 + B4; C3 + C4, and so on.
14. Add 50 µL melatonin biotin conjugate to each well. Cover with a plate sealer and mix on a plate mixer at 800–1000 rpm for 60 s (*see* **Notes 30** and **31**).
15. Incubate at 4°C for 3 h.
16. Remove and discard plate sealer, empty wells, and wash four times with wash buffer. Empty wells and blot dry on absorbent paper (*see* **Note 32**).

17. Add 100 µL enzyme label to each well.
18. Cover plate with a new plate sealer, place on plate mixer, and mix at 800–1000 rpm at room temperature for 1 h.
19. Remove and discard plate sealer, empty wells, and wash four times with wash buffer. Empty wells and blot dry on absorbent paper.
20. Add 100 µL TMB substrate to each well (*see* **Note 33**).
21. Cover plate with a new plate sealer, place on plate mixer, and mix at 800–1000 rpm at room temperature for 30 min out of direct light.
22. Remove and discard plate sealer. Add 100 µL stop solution to all wells (*see* **Note 34**).
23. Read the absorbance at 450 nm in a microtiter plate reader (*see* **Note 35**).
24. Melatonin concentrations in the samples and quality controls are determined by reference to the standard curve (*see* **Note 10**).

3.5. Urine ^{125}I–6-Sulphatoxymelatonin Assay

1. Make up each bottle of concentrated buffer to 200 mL with DGDW (*see* **Note 36**).
2. Reconsitute each vial of lyophilized antibody with 30 mL assay buffer.
3. Reconstitute each vial of lyophilized charcoal-stripped urine with 25 mL assay buffer to give a 1:250 dilution.
4. Urine samples may be assayed directly at a 1:250 dilution.
5. Label polysterene LP3 tubes in duplicate for total, NSB, and standards *(8)* with MB (zero melatonin) tubes in quadruplicate (*see* **Note 3**). Label additional tubes in duplicate for samples and quality controls.
6. Leave Total tubes empty. Pipet 500 µL 1:250 charcoal stripped urine and 200 µL assay buffer into the NSB tubes.
7. A standard curve is constructed by adding varying amounts of 200 pg/mL standard and 1:250 charcoal-stripped urine as shown in **Table 3** to give a standard curve range of 0–50 ng/mL (*see* **Note 37**).
8. Dilute each quality control or sample in duplicate 1:250 and add 500 µL to the correctly numbered tubes (*see* **Note 38**).
9. Add 200 µL working antiserum dilution to all tubes except total count and NSB tubes. Cover tubes with cling film.
10. Vortex and incubate at room temperature for 30 min.
11. Add 100 µL working ^{125}I-aMT6s to all tubes, cover with cling film (*see* **Note 39**).

Table 3
Volumes for aMT6s Standard Curve

aMT6s standard 200 pg/mL (µL)	aMT6s-free urine 1:250 (µL)	aMT6s pg/tube	aMT6s ng/mL urine
0	500	0	0
5	495	1	0.5
10	490	2	1
20	480	4	2
40	460	8	4
70	430	14	7
100	400	20	10
200	300	40	20
500	0	100	50

12. Vortex and incubate at 4°C for 18 h (overnight).
13. Add 100 µL dextran-coated charcoal to all tubes except totals and mix. Incubate at 4°C for 15 min (*see* **Note 7**).
14. Centrifuge at 1500*g* for 15 min at 4°C
15. Decant supernatants from all tubes except totals. Blot tubes dry on absorbent paper.
16. Count the radioactivity in all tubes for 1 min in a γ-counter (*see* **Note 39**).
17. aMT6s concentrations in the samples and quality controls are determined by reference to the standard curve (*see* **Note 10**).

4. Notes

1. Plasma samples that have a high lipid content or are haemolysed may give false high melatonin concentrations owing to color quenching during counting.
2. It is essential that fresh DGDW be used for preparing all reagents. Lack of binding is usually the result of poor water quality.
3. By having four tubes to measure MB it is possible to calculate the limit of detection for each individual assay. The limit of detection is usually calculated by subtracting two standard deviations of the averaged MB duplicates from the counts at MB and intersecting this

value with the standard curve produced in the assay. The assay sensitivity for the plasma ^{3}H direct melatonin assay is 5 pg/mL.

4. Extra precision can be achieved in the standard curve by mixing the standard curve tubes individually on a vortex mixer.
5. It is essential that a standard curve plus low, medium, and high quality control samples be included in each assay run.
6. It is important to limit the number of samples run in each assay to prevent assay drift. If more than 30 samples are run, it is advisable to place a set of quality control samples before, in the middle, and at the end of the samples. With less than 30 samples, quality controls should be included before and after the unknown samples. If possible, all samples collected from one individual should be run in the same assay.
7. The preparation, storage, and use of the dextran-coated charcoal is important to achieve the highest specific binding. Suspend activated charcoal at 2% w/v in assay buffer. Stir for 5 min, centrifuge at 500*g* for 5 min at 4°C. Discard supernatant and any fines. Resuspend in the original volume of assay buffer and add 0.02% dextran T70. Stir overnight at 4°C and store at 4°C. When using charcoal to separate the bound from the free ensure that it is used cold and is fully mixed before adding to the assay tubes and keep stirring during addition.
8. It is possible to decant the whole supernatant from each tube into the minivials in place of measuring 700 -µL aliquots.
9. The toluene-based scintillation fluid gives a two-phase counting system. The shaking is necessary to extract all the ^{3}H melatonin into the organic phase. It provides a greater efficiency of counting than a detergent-based system and is less sensitive to quenching by colored constituents of the plasma.
10. Melatonin concentrations can be calculated automatically if the scintillation or γ-counter has a suitable computer program to fit a spline-smoothed curve. Concentrations can also be determined manually by using log-lin graph paper. The percent bound is plotted on the vertical (linear) axis against the known melatonin concentrations (pg/mL) from the standards on the horizontal (log) axis. The best fitting curve can then be drawn and the melatonin concentrations of the unknown samples read from the curve.
11. The Salivettes used to collect the saliva samples must have the untreated cotton swabs.

12. During the saliva collection period, subjects should ideally refrain from eating, smoking, taking caffeine containing beverages, or cleaning their teeth. If possible they should also maintain constant posture in dim light (<10 lux) conditions. This will prevent contamination of the saliva samples with any compounds likely to interfere with the assay and minimize light suppression of melatonin.
13. If samples are being extracted by any method, quality control, samples must always be extracted at the same time using the same methodology.
14. If running plasma samples in the ^{125}I melatonin assay the standard curve can be adjusted to use the same standard concentrations as for the ^{3}H direct plasma assay. This should ensure that all samples tested fall on the standard curve. The sensitivity of this assay is 2 pg/mL.
15. The second antibody suspension should be well shaken prior to use and must be mixed throughout the time taken to add to the assay tubes.
16. Mix the tubes for the final time just prior to the addition of the Brij/saline wash solution to ensure the suspension is well washed and the NSB is low. The tubes will be too full to mix after the wash solution is added.
17. It is very important to use HPLC grade methanol and hexane to avoid contaminants and ensure full melatonin extraction.
18. Deionized double distilled water of ultrapure quality free of organic residues must be used.
19. If an extraction column is to be reused to process another plasma sample it is important to repeat the wash steps comprising 2×1 mL methanol and $2 \times$ 1mL DGDW. This is to ensure the column performs equally well each time it is used.
20. Used columns can be stored at room temperature in a light and dust tight container.
21. Reconstituted samples may be assayed immediately after 30 min equilibration at room temperature or can be stored frozen at –20°C in capped tubes.
22. When reconstituting lyophilized reagents take care when opening the vials. Some powder tends to attach to the rubber bungs and may be lost. The vials should be mixed gently to avoid excessive foaming. When reconstituting the standards to avoid contamination, make sure that the vial caps are not switched. If reusing frozen standards and others, ensure that they are fully thawed and well mixed before use.

23. The standard curve range for this assay is 0–50 pg/mL. In our experience this is insufficient for the measurement of samples collected during the night. Plasma melatonin values vary greatly between individuals, but on average the maximum levels reached are 70–80 pg/mL. Indeed, some individuals may have levels as high as 200 pg/mL, although others may struggle to reach 20 pg/mL. High samples need to be diluted and extracted again. The extraction procedure and limited range of the standard curve means that the assay is time consuming when running large numbers of samples.
24. Saliva melatonin levels are on average 30% of the plasma levels, so more saliva samples will fall on the standard curve, however, there are individuals who can reach levels of 100 pg/mL and these would need to be diluted and repeated. The limit of detection for the assay in unextracted samples is 0.3 pg/mL, but the average daytime levels are 2.6 pg/mL in plasma so that the extra sensitivity does not improve assessment of the melatonin profile.
25. It is possible to decant the tubes rather than aspirate the supernatant. In both cases duplication improves with the number of assays performed.
26. Pretreated saliva samples may be assayed immediately or stored at 4°C for up to 24 h or at –20°C for longer term periods of time.
27. Unused microtiter strips must be resealed in the aluminum bag with the desiccant sachets as they must be kept moisture and dust free.
28. Use a plastic wash bottle to easily wash the microtiter strips. This allows the wash buffer to be squirted directly into the wells and, in many cases, appears to give better reproducibility than an automatic plate washer.
29. The standard range of 0–81 pg/mL will allow measurement of the majority of saliva samples however, plasma samples may be too concentrated and need to be repeated diluted to ensure that they fall on the standard curve.
30. Using a plate mixer is important because it enhances the assay performance by improving duplicates and reproducibility.
31. When using a multichannel pipet, it is a good idea to check the following to ensure accuracy: make sure the tips are attached securely and aspirated volumes look identical. Also check expelled volumes are equal by looking at the side of the microtiter strips.
32. The kit is only supplied with three plate sealers, if all the microtiter strips are not used in one assay run, there will be no plate sealers left for the second assay run.

33. It is important to allow the TMB substrate to warm up to room temperature before adding it to the wells.
34. After adding the stop solution check the wells and remove any air bubbles with a pipet tip before reading the absorbance.
35. The absorbances must be read within 30 min of stopping the reaction.
36. The buffer concentrate will need to be warmed to 40°C to dissolve the gelatin completely. This can be done by standing the bottles in a water bath.
37. The standard curve range of 0.5–50 ng/mL should allow for measurement of aMT6s in the majority of urine samples collected. However, overnight samples may be too concentrated and require diluting 1:10 prior to assay. Values obtained should then be multiplied by 10. In a similar fashion daytime samples may have a very low concentration and need to be run more concentrated (i.e., 50 µL urine sample:450 µL assay buffer). Values obtained should then be divided by 25.
38. The urine samples must all be diluted 1:250 before assay. This can be done directly into the assay tube using an automatic diluter (2 µL urine:498 µL assay buffer). If done manually an intermediate dilution is recommended of 1:10, i.e., 50 µL urine + 450 µL assay buffer with a further 1:25 dilution into the assay tube (20 µL 1:10 dilution + 480 µL assay buffer).
39. The ^{125}I-aMT6s is diluted for use in the assay to give 10,000 cpm in 100 µL aliquot.

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Hormone Assays in Biological Fluids

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Although automated systems can measure all common hormones in human blood, they are expensive and often incapable of measuring hormones in urine, saliva, and animals. In *Hormone Assays in Biological Fluids*, expert researchers who have developed and applied significant new assays describe in step-by-step detail readily reproducible methods for measuring a broad variety of hormones, related peptides, and synthetic steroids in various biological fluids. The hormones measured range from glucocorticoids in biological fluids, urinary steroids, aldosterone in blood, and plasma renin activity, to gut hormones in plasma, melatonin, prolactin, 6-sulfatoxymelatonin, and androgens in blood, saliva, and hair. The emphasis is on noncommercial assays so that investigators can develop novel methods suited to their special needs. Commercial assays are also described for comparative purposes. Tutorials on radioimmunoassay, gas chromatography–mass spectrometry, high-performance liquid chromatography, and polymerase chain reaction techniques help readers choose the best methods for their purpose. The protocols follow the successful Methods in Molecular Medicine™ series format, each offering step-by-step laboratory instructions, an introduction outlining the principles behind the technique, lists of the necessary equipment and reagents, and tips on troubleshooting and avoiding known pitfalls.

Wide-ranging and highly practical, *Hormone Assays in Biological Fluids* illuminates the many different methods available for a broad range of hormone assays, enabling researchers to choose the optimal existing methods or develop novel assays for their specialized purposes.

FEATURES

- **Cutting-edge hormone assays that facilitate choosing the best analytical method**
- **Numerous examples of assays for a wide variety of substances**
- **Transferable examples of assays for a wide variety of substances**
- **Help to choose the best method or, if necessary, to develop a new method**
- **Step-by-step instructions to ensure rapid implementation and successful results**
- **Tricks of the trade and notes on troubleshooting and avoiding known pitfalls**

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