A liquid-phase two-site immunoradiometric assay for human prolactin

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The development of a 'two-site' immunoradiometric assay for human prolactin (hPrl) is described. The assay is based on the addition of radio-iodinated sheep anti-hPrl immunoglobulin G (IgG) and rabbit anti-hPrl serum to standards and unknowns followed by 3 h incubation. The use of solid phase reagents was avoided in order to minimize non-specific effects and the time required for reactants to reach equilibrium. Instead, the separation of hPrl-bound and free labelled antibody is achieved by the addition of sheep anti-(rabbit IgG) serum which precipitates bound labelled antibody by complex formation with rabbit anti-hPrl antibodies which are also hPrl-bound. Varying the order of addition of specific antibodies had a pronounced effect on the 'operating range' and sensitivity of resultant assays. This was attributed to competition between labelled and unlabelled antibodies for binding sites on the hPrl molecule. The immunoradiometric assay employing 'simultaneous addition' of specific antibodies was compared to a 'simultaneous addition' hPrl radioimmunoassay developed using the same sheep antiserum as that used to prepare the radio-iodinated sheep anti-hPrl IgG. This immunoradiometric assay is characterized by rapid equilibration of reactants, a wide 'operating range' (the precision of dose estimates was <10% over the range 8–10000 mU/l), and high sensitivity (2.6 mU/l, 13 pg). In contrast, the hPrl radioimmunoassay required an incubation of 18 h, demonstrated a much reduced 'operating range' (the precision of dose estimates was <10% only over the range 25–1500 mU/l) and reduced sensitivity (9.8 mU/l, 49 pg).

Immunoradiometric analysis (IRMA) is a method for the direct estimation of analytes in biological fluids based on the use of radioisotopically labelled specific antibodies (Miles & Hales, 1968). Such assays are in contrast with radioimmunoassays (RIA) (Yalow & Berson, 1960) which utilize radiolabelled antigen molecules as tracers. Whereas in RIA antibody must necessarily be limiting, in IRMA antibody may be added in excess, and this imparts several advantages to the technique (Ekins, 1976). Addition of antibody in excess has been reported to reduce the time required for reactants to reach equilibrium, remove the need for precise pipetting of antibody, and increase the 'operating range' and sensitivity of the assay (Hunter & Budd, 1981; Al-Shawi *et al.*, 1981). Despite these advantages, methodological limitations often prevent IRMA from achieving its

potential. One such limitation may result from the use of insolubilized (solid phase) reagents for the separation of antigen-bound from free labelled antibody. Despite their convenience many solid phases have undesirable physical properties which detract from the performance characteristics of the assay. For example, the sensitivity of IRMA, which is constrained by the signal-to-noise-ratio of end-point detection, may be adversely affected by non-inert solid phases which increase non-specific binding of the labelled antibody. Inconvenient washing of the solid phase is often employed to reduce non-specific binding (Addison & Hales, 1971) but this can reduce assay precision. Another example is the failure of solid phase to stay in suspension. This will protract incubation periods or necessitate continuous mixing of assay tubes. In addition, the coupling of antibody to solid phase may be associated with some loss of affinity (Gardner *et al.*, 1974). The advantages and disadvantages of solid phase reagents in immunoassay have been reviewed (Pourfarzane *et al.*, 1982). Problems in the development of IRMAs

Abbreviations used: hPrl, human prolactin; IRMA, immunoradiometric assay; RIA, radioimmunoassay; IgG, immunoglobulin G.

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also arise in the preparation of the purified immunoglobulin and a radio-iodinated derivative which retains high immunoreactivity. The harsh chaotropic conditions often utilized to recover antibody from antigen immunoabsorbent result in preparations of reduced immunointegrity (Miles *et al.*, 1974).

We have recently described a method for the purification of high-affinity antibodies to hPrI (Hodgkinson & Lowry, 1982) and this has prompted us to examine their use in an IRMA for the hormone. The assay is of the 'two-site' or 'sandwich' variety (Wide, 1971; Addison & Hales, 1971) but avoids the use of solid phase reagents and thereby the limitations imposed by their use. The current paper discusses the steps involved in establishing such an assay and compares its performance characteristics with an optimized hPrI RIA developed using the same sheep antiserum as that used to prepare the radio-iodinated antibody.

Materials and methods

Materials

hPrI for immunization, standards, radiolabelling and preparation of immunoabsorbent was prepared in this laboratory (Hodgkinson & Lowry, 1981). A sheep anti-(rabbit IgG) precipitating serum was kindly provided by Dr. R. Edwards of the North East Thames Regional Immunoassay Service. Horse serum 3 (unactivated) and normal rabbit serum were purchased from Wellcome Laboratories. Normal sheep serum and a horse anti-(sheep IgG) precipitating serum were obtained from ILS, 14-15 Newbury St., London E.C.1, U.K. Iodogen (1,3,4,6-tetra chloro-3 α ,6 α -diphenylglycoluril) was obtained from Pierce and Warriner (U.K.), Sephacryl S-300 from Pharmacia, bovine serum albumin from Sigma, Freund's complete and incomplete adjuvant from Difco Laboratories (Detroit, MI, U.S.A.) and all other reagents were AnalR grade and purchased from BDH.

Antiserum production

Antisera to hPrI were raised in sheep and rabbits by immunizations at 1 month intervals with antigen (250 μ g for sheep, 100 μ g for rabbits) emulsified in Freund's complete adjuvant for the primary injection [1 vol. of antigen in 0.9% (w/v) NaCl: 3 vol. of adjuvant] and subsequently with incomplete adjuvant. The animals were bled 10-14 days after each immunization and antisera were assessed by the preparation of dilution curves and Scatchard analysis of antibody affinity as previously described (Hodgkinson & Lowry, 1982).

Purification of sheep anti-hPrI IgG

The method used for the purification of sheep anti-(hPrI) IgG has previously been reported in

full (Hodgkinson & Lowry, 1982). Briefly, the specific immunoglobulin from a sheep anti-hPrI serum was isolated from a Na₂SO₄ precipitate of the immunoglobulin fraction by immunoabsorption to insolubilized hPrI and selective elution using a gradient of pH decreasing from pH 7 to pH 2 in the presence of acetonitrile (20%, v/v). The eluted anti-hPrI IgG was fractionated into two major peaks of affinity. The earlier eluting peak, of moderate affinity (affinity constant K_a 4.6×10^{10} l/mol) was pooled, concentrated to 1 mg/ml in an Amicon ultrafiltration cell fitted with a PM10 filter and stored at -20°C in 80 μ g (0.5 nmol) samples for iodination.

Preparation of radio-iodinated sheep anti-hPrI IgG

Radio-iodination was achieved by a modification of the Iodogen method (Salacinski *et al.*, 1981). Iodogen (100 μ l of a 92.5 μ M solution in dichloromethane) was added to a polypropylene vial [no. 690; Sarstedt (U.K.)] and the solvent was evaporated. To the vial were added in order 0.5 M-phosphate buffer, pH 7.4 (20 μ l), sheep anti-hPrI IgG (80 μ g, 0.5 nmol) and Na¹²⁵I (5 μ l, 0.5 mCi, 0.25 nmol; The Radiochemical Centre, Amersham). The iodination was allowed to continue for 10 min before transferring the vial contents to 0.05 M-phosphate buffer, pH 7.4 (250 μ l) for 5 min followed by addition of carrier sheep serum (250 μ l). The incorporation of iodine into IgG ranged from 75 to 90%, resulting in preparations with specific radioactivities between 4.7 and 5.6 μ Ci/ μ g. The mixture was applied to a column (1.0 cm $\times$ 90 cm) of Sephacryl S-300 previously equilibrated with 0.05 M-phosphate buffer, pH 7.4, containing bovine serum albumin (0.5%, w/v) and thiomersal (0.01%, w/v), and eluted at 3 ml/h. Fractions (1.5 ml) were collected. A single major peak of radioactivity was eluted in the position expected of sheep anti-hPrI IgG (K_{av} 0.26). To the pooled peak mannitol (1.0%, w/v) and normal sheep serum (10%, v/v) were added before lyophilization and storage at -20°C. The immunoreactivity of the radio-iodinated sheep anti-hPrI IgG (sheep anti-hPrI IgG-¹²⁵I) was assessed by its ability to reassociate with excess hPrI immunoabsorbent and was manifest by the high binding (>90%) of radioactivity achieved after incubating for 2 h. Non-specific binding of label to Sepharose 4B was <3%. Binding of sheep anti-hPrI IgG-¹²⁵I to hPrI immunoabsorbent had declined to 47% after 2 months storage.

IRMA development

The buffer for all assay experiments was 0.064 M-phosphate, pH 7.4 (0.052 M-Na₂HPO₄/0.012 M-NaH₂PO₄), containing poly(ethylene glycol) 6000 (3%, w/v), bovine serum albumin (0.5%, w/v),

normal rabbit serum (1.0%, v/v), normal sheep serum (2.0%, v/v) and thiomersal (0.01%, w/v).

The separation of hPrl bound from free labelled antibody was facilitated by using specific antibodies raised in two different species. In our system the radiolabelled antibody was of sheep origin and the second specific antibody was raised in rabbits. Separation could then be achieved by addition of a sheep anti-(rabbit IgG) serum which precipitates hPrl-bound sheep anti-hPrl IgG-¹²⁵I by complex formation with rabbit anti-hPrl antibodies which are also hPrl-bound. The use of solid phase immunoadsorbents was thereby avoided. The amount of sheep anti-(rabbit IgG) serum [200 µl of a 1:20 (v/v) solution in 0.064 M-phosphate buffer, pH 7.4] required for the complete precipitation of rabbit anti-hPrl serum in the presence of normal rabbit serum (1.0%, v/v) as carrier had previously been established by a well-proven procedure (Buckler, 1971).

The amount of rabbit anti-hPrl serum required for the assay was established as follows. Sheep anti-hPrl IgG-¹²⁵I was added to assay buffer to give 50000 c.p.m./200 µl. Four pools of this reagent were prepared to which rabbit anti-hPrl serum was added to concentrations of 1:100, 1:350, 1:1000 and 1:10000 (v/v) respectively. Four standard curves were prepared by adding 200 µl of each of the above solutions to duplicate hPrl standards (100 µl) and vortex-mixing. The range of standards was 0–5400 mU/l (0–270 ng/ml, calibrated against WHO IRP 75/504) and were prepared in horse serum. Following an incubation period (3 h at room temperature), sheep anti-(rabbit IgG) precipitating serum was added, the tubes were vortex-mixed, incubated (0.5 h at room temperature) and centrifuged (1500 g, 4°C, 0.5 h). The soluble free fraction was aspirated to waste and the precipitated hPrl-bound labelled antibody was estimated in an Innotron Hydragamma-16 gamma counter.

The effect of changing the order of addition of specific antibodies was next assessed by the preparation of standard curves as follows.

(a) *Delayed addition of rabbit anti-hPrl serum.* To duplicate hPrl standards in horse serum (100 µl), sheep anti-hPrl IgG-¹²⁵I (50000 c.p.m., 100 µl) in assay buffer was added, the tubes were vortex-mixed, and incubated (1.5 h at room temperature) before the addition of rabbit anti-hPrl serum (100 µl, 1:250 dilution in assay buffer) and re-mixing. Following a further incubation (1.5 h at room temperature) hPrl-bound and free sheep anti-hPrl IgG-¹²⁵I were separated by the addition of sheep anti-(rabbit IgG) serum as described above.

(b) *Simultaneous addition of sheep anti-hPrl IgG-¹²⁵I and rabbit anti-hPrl serum.* To duplicate hPrl standards (100 µl) a reagent mixture (200 µl) containing sheep anti-hPrl IgG-¹²⁵I (50000 c.p.m.,

200 µl) and rabbit anti-hPrl serum (1:500) in assay buffer was added; the tubes were vortex-mixed, incubated (3 h at room temperature) and hPrl-bound and free labelled antibody was separated as above.

(c) *Delayed addition of sheep anti-hPrl IgG-¹²⁵I.* To duplicate hPrl standards (100 µl), rabbit anti-hPrl serum (100 µl, 1:250 dilution in assay buffer) was added, the tubes were vortex-mixed, and incubated (1.5 h at room temperature) before the addition of sheep anti-hPrl IgG-¹²⁵I (50000 c.p.m. in 100 µl of assay buffer) and re-mixing. Following a further incubation (1.5 h at room temperature) bound and free labelled antibody were separated as described above.

The rate of equilibration of reactants in the 'simultaneous addition' IRMA was assessed. Standard pools (10, 25 and 1000 mU of hPrl/l) in horse serum were prepared to which a reagent mixture, containing sheep anti-hPrl IgG-¹²⁵I (50000 c.p.m. in 200 µl) and rabbit anti-hPrl serum (1:500) in assay buffer, was added in the ratio 1 vol. of standard:2 vol. of reagent mixture. The pools were mixed and incubated at room temperature. Aliquots (0.3 ml) were removed at 0.5, 1, 2 and 4 h and hPrl-bound and free sheep anti-hPrl IgG-¹²⁵I were separated as described above.

Development of a comparative hPrl RIA

Radio-iodinated human prolactin (¹²⁵I-hPrl) was prepared as described previously (Hodgkinson & Lowry, 1981). Buffer for the RIA was 0.064 M-phosphate (pH 7.4) containing bovine serum albumin (0.5%, w/v) and thiomersal (0.01%, w/v). An antiserum dilution curve, of another aliquot of the same sheep anti-hPrl serum (K_a 1.5×10^{10} l/mol) that was used to prepare the sheep anti-hPrl IgG-¹²⁵I, demonstrated that at a final dilution of 1:660000 42% of added ¹²⁵I-hPrl (20000 c.p.m., 75 pg) was bound in the absence of unlabelled hPrl. This dilution was used for assay. Standards (100 µl) were as described for the IRMA. Previous studies had shown that although the assay required an incubation of 18 h, there was no advantage in incubating for longer periods, at temperatures other than ambient, or in delaying the addition of ¹²⁵I-hPrl. The assay procedure was as follows. To duplicate hPrl standards (100 µl), ¹²⁵I-labelled hPrl (100 µl) and sheep anti-hPrl serum (100 µl, initial dilution 1:220000) were added, vortex mixed and incubated for 18 h at room temperature. Separation of antibody-bound and free ¹²⁵I-labelled hPrl was achieved by precipitation of the bound fraction using horse anti-(sheep IgG) serum. Addition of the second antibody was followed by mixing, incubation (0.5 h at room temperature), centrifugation (1500 g, 4°C) and aspiration of the soluble free fraction to waste.

Results and discussion

IRMA development

The 'operating range' and the efficiency of precipitation of hPrl-bound sheep anti-hPrl IgG-¹²⁵I was shown to be critically dependent on the dilution of rabbit anti-hPrl serum (Fig. 1). A progressive increase in the precipitation of bound sheep anti-hPrl IgG-¹²⁵I was observed at a hPrl concentration of 5400 mU/l, with decreasing dilution of rabbit anti-hPrl serum. At 1:10000 rabbit anti-hPrl, precipitation was <30% of that at either 1:100 or 1:350. Furthermore, at 1:10000 rabbit anti-hPrl the standard curve demonstrated the high dose 'hooking' which is a major source of negative bias in IRMA (Miles, 1977). Alternatively, the response of the standard curve to lower doses of hPrl (0–600 mU/l) was reduced at the lower dilution of rabbit anti-hPrl serum (1:100) compared with greater dilutions (1:350, 1:1000). This is suggestive of competition between the two anti-hPrl antibodies for binding sites on the hPrl molecule despite their different species of origin, but the extent of competition was reduced com-

Fig. 1. Influence of rabbit anti-hPrl serum dilution on the hPrl IRMA

Standard curves were prepared by the simultaneous addition of sheep anti-hPrl IgG-¹²⁵I (50000 c.p.m. in 200 μ l) and rabbit anti-hPrl serum at a range of dilutions ($\bullet$, 1:100; $\blacksquare$, 1:350; $\blacktriangle$, 1:1000; $\circ$, 1:10000) to hPrl standards (100 μ l, 0–5400 mU/l) in horse serum followed by 3 h incubation at room temperature. Separation of bound and free labelled antibody was achieved by addition of a sheep anti-(rabbit IgG) precipitating serum followed by centrifugation and aspiration of the soluble free fraction to waste.

pared with an assay developed using the same antibody preparation for radio-iodination and coupling to a solid phase (S. C. Hodgkinson, unpublished work). The choice of dilution (1:500) of rabbit anti-hPrl serum for continued development of the IRMA was made from consideration of the requirements of hPrl assays as diagnostic tools and economy.

The 'operating range' and sensitivities of standard curves were also dependent on the order of addition of specific antibodies (Fig. 2). Delaying the addition of rabbit anti-hPrl serum resulted in a standard curve (Fig. 2, curve A) with increased response to low doses of hPrl when compared with curves prepared by either simultaneous addition of labelled and unlabelled antibodies (Fig. 2, curve B) or by delaying the addition of sheep anti-hPrl IgG-

Fig. 2. Prolactin IRMA assay procedure
Standard curves were prepared by: addition of a reagent mixture (200 μ l) containing sheep anti-hPrl IgG-¹²⁵I (50000 c.p.m.) and rabbit anti-hPrl serum (1:500) to hPrl standards (100 μ l) followed by 3 h incubation and separation of bound and free labelled antibody ($\blacksquare$, 'simultaneous addition', curve B); addition of sheep anti-hPrl IgG-¹²⁵I (50000 c.p.m. in 100 μ l) to hPrl standards followed by 1.5 h incubation, addition of rabbit anti-hPrl serum (100 μ l, 1:250), a further incubation (1.5 h at room temperature) and separation of bound and free labelled antibody ($\bullet$, delayed addition of rabbit anti-hPrl serum, curve A); addition of rabbit anti-hPrl serum (100 μ l, 1:250) to standards followed by 1.5 h incubation, addition of sheep anti-hPrl IgG-¹²⁵I (50000 c.p.m. in 100 μ l), a further incubation (1.5 h at room temperature) and separation of bound and free labelled antibody ($\blacktriangle$, delayed addition of sheep anti-hPrl IgG-¹²⁵I, curve C).

Fig. 3. Rate of equilibration of the 'simultaneous addition' hPrI IRMA

Pools of hPrI standard (10, 25 and 1000 mU/l) were prepared in horse serum to which a reagent mixture containing sheep anti-hPrI IgG- ^{125}I (50000 c.p.m. in 200 μl) and rabbit anti-hPrI serum were added in the ratio of 1 vol. of standard : 2 vol. of reagent mixture. The pools were incubated at room temperature and aliquots (0.3 ml) were removed after 0.5, 1, 2, 3 and 4 h. Separation of bound and free labelled antibody was achieved by the addition of sheep anti-(rabbit IgG) precipitating serum as described in the text.

^{125}I (Fig. 2, curve C). The sensitivities (lowest detectable dose of hPrI as judged by a $2.5 \times \text{s.d.}$ increase of duplicate counts from counts at zero hPrI) of curves A, B and C were 0.8, 2.6 and 15 mU/l respectively. Alternatively, the standard curve reached a plateau at a lower concentration of hPrI when delayed addition of rabbit anti-hPrI serum was employed (curve A) compared with curves B and C. These findings further illustrate the competition between the labelled and unlabelled hPrI antibodies. Delayed addition of the labelled antibody (Curve C) is not a protocol that would be used in a 'liquid phase' IRMA since it offers no advantage. However, it is a protocol which is often employed in IRMA's utilizing solid phase antibody immunoabsorbents, ostensibly to extract and concentrate antigen and thereby increase assay sensitivity. In assays where there is competition between labelled and unlabelled antibodies this may be deleterious and explain why many investigators have not been able to demonstrate improved sensitivity in IRMA compared with RIA despite the theoretical advantages of the technique (Woodhead *et al.*, 1974). Simultaneous addition of sheep anti-hPrI IgG- ^{125}I and rabbit anti-hPrI serum (Fig. 2, curve B) was chosen for subsequent experimentation since the requirements of a clinical hPrI assay are achieved using this protocol and because it simplifies the assay by eliminating the extra pipetting step.

The reactants in the 'simultaneous addition'

Fig. 4. Comparative 'simultaneous addition' hPrI IRMA and RIA standard curves

The hPrI IRMA (●, curve A) standard curve was prepared by the addition of a reagent mixture (200 μl) containing sheep anti-hPrI IgG- ^{125}I (50000 c.p.m.) and rabbit anti-hPrI (1:500) to duplicate hPrI standards (100 μl) in horse serum followed by 3 h incubation at room temperature. Separation of hPrI-bound and free labelled antibody was achieved by addition of sheep anti-(rabbit IgG) precipitating serum as described in the text. The hPrI RIA (○, curve B) standard curve was prepared by the addition of ^{125}I -labelled hPrI (20000 c.p.m. in 100 μl) to duplicate standards (100 μl , 0–5000 mU/l) in horse serum followed by the addition of sheep anti-hPrI serum (1:220000 initial dilution, 100 μl) and incubation for 18 h at room temperature. The separation of bound and free ^{125}I -labelled hPrI was achieved by precipitation of the bound fraction by addition of a horse anti-(sheep IgG) precipitating serum and centrifugation as described in the text. The sheep anti-hPrI IgG- ^{125}I was derived from a different portion of the same sheep anti-hPrI serum that was used in the RIA. A dilution curve of a hyperprolactinaemic can also be seen (■, curve C).

IRMA were shown to attain equilibrium rapidly (Fig. 3). Equilibrium was >90% established after an incubation of 1 h. This is in part due to adding specific antibodies in excess and because the immunological reaction is carried out in the liquid phase. An incubation time of 3 h was chosen for subsequent work.

IRMA versus RIA

Comparative hPrI standard curves prepared using the 'simultaneous addition' IRMA and the RIA can be seen in Fig. 4. Characteristic of the

IRMA (Fig. 4, curve A) is the large change in bound radioactivity (21-fold) across the range of standards. This is due to the low non-specific binding (sheep anti-hPrI IgG- 125 I precipitated in the absence of hPrI was 2.1%) and high specific binding (46.2% at 10000 mU of hPrI/l) of sheep anti-hPrI IgG- 125 I which was added in excess. There was no need to wash the precipitate or to dilute assay tube contents before centrifugation to reduce non-specific binding, as is often the case in IRMAs that employ a solid phase (Hunter & Budd, 1981). In contrast the change in bound radioactivity across the range of standards obtained with the RIA (Fig. 4, curve B) was much reduced (7.3-fold) and could only be increased at the expense of assay sensitivity. The non-specific binding was 3.8% (125 I-labelled hPrI precipitated in the absence of sheep anti-hPrI serum). Fig. 4, curve C is a dilution curve of a hyperprolactinaemic serum prepared using the IRMA. The serum demonstrated complete parallelism with the IRMA standard curve. The concentration of hPrI in the serum was 26300 mU/l (1.31 μ g/ml) and the dilution curve shows no sign of 'hooking' at the higher concentrations of hPrI. The sensitivity (lowest detectable dose of hPrI) of the IRMA was 2.6 mU/l (0.13 ng/ml, 13 pg). This represents a moderate improvement (3.8-fold) over the 9.8 mU/l (0.49 ng/ml, 49 pg) calculated for the RIA and yet was achieved with a labelled antibody preparation of specific radioactivity equivalent to less than 1 molecule of 125 I to 2 molecules of IgG. The greater sensitivity of the IRMA could be explained by the higher affinity of the purified sheep anti-hPrI IgG (K_a 4.6×10^{10} l/mol) than of the unfractionated antiserum (K_a 1.5×10^{10} l/mol) used for the RIA. But a much greater increase in sensitivity than could be explained on the basis of affinity (12-fold; 0.8 mU/l, 4 pg, by IRMA; 9.8 mU/l by RIA) was observed on comparison of the RIA with the IRMA employing delayed addition of rabbit anti-hPrI serum. These results compare very favourably with the detection limits of other published immunoassays. Sinha *et al.* (1973) reported the sensitivity of their homologous hPrI RIA as 100 pg, which is in agreement with a collaborative study carried out on the performance characteristics of hPrI RIAs (Aubert *et al.*, 1974). There are also two 'two-site' immunoradiometric assays commercially available for the estimation of the hormone, one of which employs monoclonal antibodies. The limits of detection of these assays are 15 pg for the monoclonal method and 25 pg for the assay utilizing polyclonal antibodies. The sensitivity of the 'simultaneous addition' hPrI IRMA described here is therefore equivalent to, or greater than, that of these assays. But the 'delayed addition' method demonstrates an additional improvement in sensi-

Fig. 5. Comparative precision profiles of the 'simultaneous addition' hPrI IRMA and the hPrI RIA. $\circ$, IRMA; $\bullet$, RIA. Data for the calculation of precision profiles were obtained from the standard curves seen in Fig. 4 and the assay of 20 unknown sera by each method.

tivity (4 pg). This greater increase in sensitivity must be due to the labelled antibody methodology employed here.

Comparative precision profiles (Ekins, 1981) of the IRMA and of the RIA can be seen in Fig. 5. Characteristic of the IRMA is the wide operating range of the standard curve. This is a consequence of adding antibody in excess and is particularly apparent in the high dose region (500–10000 mU of hPrI/l) which is of importance in the management of hyperprolactinaemia. The precision potential of dose estimated is $< 5\%$ over the range 20–5000 mU/l and $< 2.5\%$ between 80 and 1700 mU/l. Furthermore, it is still $< 10\%$ at 10000 mU/l, reducing the need to dilute high samples. In contrast, a precision profile calculated using the hPrI RIA data can also be seen in Fig. 5. The RIA demonstrates a much reduced operating range. The precision potential of dose estimates is $< 5\%$ only over the range 65–920 mU of hPrI/l. Furthermore, at no stage does the precision equal or better that obtainable with the IRMA. This is in contrast to the findings of other investigators (Hunter & Budd, 1981). The between-assay precision of the IRMA was 6.3% as calculated from 10 estimates of the hPrI content of a serum pool (actual hPrI, 400 mU/l) carried out on successive days. A satisfactory correlation was obtained when sera ranging in hPrI content from 100 to 1000 mU/l were assayed by the IRMA and the RIA. The correlation coefficient was 0.92; slope 1.13; and the y intercept -2 mU/l.

The use of a second antibody precipitation system has proven an ideal method for the separation of bound and free labelled antibody in the 'two-site' IRMA for hPrI. The assay is simple to per-

form, requiring only the simultaneous addition of specific antibodies to initiate and the addition of a precipitating second antibody to complete. The IRMA can be further simplified by replacing the sheep anti-(rabbit IgG) precipitating serum with an antiserum directed against the Fc region of rabbit IgG. The precipitating antiserum may then be added simultaneously with the two specific antibodies, thereby eliminating the second pipetting step. The assay is now undergoing clinical validation.

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