Development of a radioimmunoassay for measuring gonadotrophin releasing hormone in patients receiving treatment

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SUMMARY A radioimmunoassay for the measurement of gonadotrophin releasing hormone (GnRH) in plasma and urine using readily available reagents was developed. The GnRH assay showed good precision, recovery, and parallelism over a wide range of GnRH concentrations with a sensitivity of 15 pg/ml. The assay was compared with a commercially available kit (Buhlmann Laboratories). Although the Buhlmann kit showed acceptable precision, recovery, sensitivity, and correlation with the developed GnRH assay for plasma samples, lack of parallelism of serially diluted plasma and urine samples was consistently observed, together with a poor correlation with the developed GnRH assay for urine, suggesting a matrix effect with the Buhlmann kit.

The developed assay is suitable for measuring GnRH in samples obtained from patients receiving pulsatile infusions of GnRH. In contrast, the commercially available Buhlmann kit was unsuitable for measuring plasma GnRH as the kit had a top standard of only 160 pg/ml, well below the peak plasma concentration. It would not be possible to dilute samples for analysis because of the lack of parallelism of diluted samples compared with standards obtained with the Buhlmann assay.

Gonadotrophin releasing hormone (GnRH) is secreted by the hypothalamus in pulses and stimulates the synthesis and release of luteinising hormone and follicle stimulating hormone from the anterior pituitary gland.¹ The biological activity of GnRH depends on the pulse amplitude and frequency, changes in which change the relative proportion of luteinising hormone to follicle stimulating hormone secretion.²

Pulsatile administration of synthetic GnRH, which mimics the early follicular phase secretory pattern, has been used to induce ovulation in women with hypothalamic hypogonadism³ and in the management of polycystic ovarian syndrome.⁴ It has also been used successfully in the management of delayed puberty and with some success in men with hypospermia. Successful treatment of some of these disorders has become possible through the advent of the microcomputerised, programmable infusion pump which stimulates the physiological pattern of GnRH release. In such patients there is a requirement to assess pump

function and to evaluate patients with a suboptimal response of gonadotrophin release. Faster pulse frequencies reduce the plasma concentrations of both gonadotrophins. Long term administration of GnRH analogues, causing down-regulation of hormone secretion, is being assessed in the management of hormone sensitive disorders such as endometriosis⁵ and prostatic carcinoma.⁶

Although several workers have developed radioimmunoassays for GnRH in plasma and urine,⁷⁻¹⁰ there are few commercially available assays and no published comparisons of assay performance.

We describe the development of a radioimmunoassay to measure GnRH in plasma and urine using readily available reagents as part of a study in the use of pulsatile GnRH in the management of female infertility.

Material and methods

All reagents were analytical grade.

Phosphate buffer (0.05 mol/l, pH 7.4) containing 0.5% w/v bovine serum albumin (BSA) was used as

assay buffer. Sodium azide (0.1% w/v) was added as a preservative.

Sheep anti-GnRH antiserum (No 94) was kindly donated by Dr HM Fraser, MRC Reproductive Biology Unit, Edinburgh, Scotland. The immunological activity of the antiserum was directed against the carboxyl end of the molecule and reacted equally with the 1 to 4-10 GnRH peptides.¹¹ Cross reaction with other GnRH fragments and related peptides was negligible. The antiserum was supplied as a 1/10 dilution and stored in 5 µl aliquots at -20°C.

(3-[¹²⁵I]iodotyrosyl) GnRH with a specific activity of 78×10^6 kBq/µmol was supplied by Amersham International. The tracer was reconstituted in assay buffer to give about 30 000 cpm per assay tube.

Synthetic GnRH-HRF Ayerst No 1175 (0.5 mg) was dissolved in 2 ml sterile diluent (Ayerst Laboratories Ltd, Andover, England). This was made up to 1000 ml with distilled water to give a concentration of 0.5 µg/ml and was further diluted to give a stock standard of 1000 pg/ml which was stored in aliquots at -20°C. Before use the stock standard was diluted with assay buffer to give GnRH concentrations of 31.25 to 1000 pg/ml.

Polyethylene glycol 6000 (PEG, 20% w/v) in assay buffer, together with bovine gamma globulin (4% w/v) as carrier serum, was used to separate the bound from the free fractions.

Blood samples from patients and healthy laboratory volunteers were collected into cooled 10 ml edetic acid tubes containing 0.5 ml Trasylol (20 000 Kallibrein Inactivator Units/ml, Bayer Germany, Berkshire, England) and immediately centrifuged at 3000 rpm for five minutes. The plasma was stored at -20°C until assay. Random urine samples from similar subjects were frozen immediately and likewise stored at -20°C until assay. Samples were analysed within one week of collection.

Aliquots of GnRH standards, plasma, or urine (0.25 ml) were extracted with absolute ethanol (1.0 ml). The protein precipitate was removed by centrifugation (2500 × g for fifteen minutes at 4°C) and the supernatant decanted into clean glass tubes and evaporated under a stream of air at 70°C.

The procedure adopted for the final assay conditions is outlined in table 1.

The extraction procedure and analytical performance of the GnRH assay was evaluated and compared with a commercially available radioimmunoassay (Buhlmann Laboratories Ltd, Basel, Switzerland) which had a reported sensitivity of 2.25 pg/ml and a top standard of 160 pg/ml. Briefly, for the assay of plasma GnRH by the Buhlmann kit, sample (1.5 ml) was extracted with absolute ethanol (6.0 ml). After vortex mixing and centrifugation at 1000 × g for 20 minutes at 4°C the upper ethanolic extract was

Table 1 Procedure for radioimmunoassay of GnRH

	Total counts	NSB	Bo	Standards	Controls and samples
Buffer		0.1	0.05		
GnRH standard				0.05	
Control or sample					0.05
GnRH anti-serum			0.05	0.05	0.05
Vortex mix and incubate at 4°C for 5-6 hours					
¹²⁵ I GnRH	0.05	0.05	0.05	0.05	0.05
Vortex mix and incubate at 4°C for 18-20 hours					
Carrier serum		0.05	0.05	0.05	0.05
PEG solution		0.5	0.5	0.5	0.5
Vortex mix and incubate at 4°C for 15 minutes					
Centrifuge at 2500 g at 4°C for 15 minutes					
Aspirate supernatant					
Count for 300 seconds					
Results processed using the WHO Immunoassay Data Processing Program (PR Edwards, Version A5 for Apple II)					

evaporated to dryness and reconstituted in 1.5 ml assay buffer.

Reconstituted extract (0.5 ml) and ¹²⁵I GnRH (0.1 ml, 10000 cpm per tube) was incubated with rabbit anti GnRH antibody (0.05 ml) for 24 hours at 4°C. Goat anti-rabbit gammaglobulin (0.1 ml) was then added to separate the free fraction from the bound fraction. After incubation for a further 24 hours at 4°C tubes were centrifuged at 1000 × g for 20 minutes at 4°C. The supernatant was then aspirated and the precipitate counted. Results were processed using the WHO Immunoassay Data Processing Program (PR Edwards, Version A5).

Plasma and urine samples were obtained from healthy laboratory volunteers and from patients receiving pulsatile subcutaneous GnRH injections (Gonadorelin, Ayerst Laboratories Ltd, Andover, England) as treatment for infertility at the department of endocrinology, the Royal Free Hospital, London.

Results

OPTIMISATION OF ASSAY

Optimisation of extraction procedure

Bentonite and ethanol have been used to extract GnRH from plasma,⁹ and florisol and ethanol have been used to extract GnRH from urine.¹⁰ Because the previous studies used acidified methanol, pH 2.5, to elute the adsorbed GnRH from bentonite or florisol, the same procedure was used in this study.

Using ethanol extraction, the mean recovery of added GnRH (100 pg/ml) from normal male plasma and urine was 113% and 95%, respectively (n = 2). The recovery of extracted GnRH (100 pg/ml) from normal male plasma and urine using bentonite or florisol adsorption^{9,10} was only 6% and 34%, respec-

tively ($n = 2$). Thus ethanol extraction was used for all subsequent GnRH assays.

Experiments using normal human plasma and urine spiked with ^{125}I GnRH showed that the poor recovery using bentonite and florisil was due to the inability of the acidified methanol to elute GnRH from these adsorbents.

Optimisation of radioimmunoassay

Experiments were performed in which the incubation time, incubation temperature, first antibody dilution and tracer quantity were varied. The optimal antibody dilution was 1/30 000, which gave a Bo of 45%, and the most sensitive (steep) standard curve at low GnRH concentrations. Although tracer quantity was varied from 5000 to 50 000 cpm/tube, no significant improvement in sensitivity was noted in reducing the tracer quantity below 30 000 pm. Table 1 shows the conditions which were found to be optimal with regard to incubation time and temperature, quantities of label, carrier serum, and PEG.

Stability of GnRH in plasma

Three blood samples collected in trasylol with GnRH concentrations of 100, 250, and 500 pg/ml were each split into eight aliquots. For each sample, one aliquot was extracted immediately and another was extracted two and half hours later after standing at room temperature. Both were then analysed. The remaining six aliquots were stored at -20°C and -70°C and then assayed in duplicate after extraction for GnRH at one, two, and four weeks. GnRH immunoreactivity decreased by 20% at room temperature over a two and a half hour period and after two weeks' storage at -20°C and -70°C . Samples stored at -20°C or -70°C for up to one week before extraction showed less than 5% reduction in GnRH immunoreactivity. Samples were therefore stored at -20°C and extracted and analysed within one week of collection.

EVALUATION OF THE GNRH ASSAY AND BUHLMANN KIT

Sensitivity

The sensitivity of each assay was calculated as two and a half times the standard deviation of cpm zero standard ($n = 20$). For the GnRH assay the sensitivity was 15 pg/ml compared with 2.1 pg/ml for the Buhlmann kit.

Recovery

The recovery of GnRH through the ethanol extraction procedure was evaluated for both assays by adding ^{125}I GnRH to plasma and counting the radioactivity in the final extract. For both assays 77–79% of the added ^{125}I was recovered. To allow for this incomplete recovery

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standards were always extracted in an identical manner with that of controls and samples.

The recovery of added GnRH to plasma and urine was assessed by adding 0.05 ml of standard GnRH to 1.0 ml plasma or urine to give final concentrations of GnRH, ranging from 8–500 pg/ml. The top standard for the GnRH assay was 1000 pg/ml so recovery was assessed between 50–500 pg/ml. As the top standard for the Buhlmann Kit was 160 pg/ml, however, recovery was assessed between the lower values of 8–40 pg/ml. Added GnRH could be recovered quantitatively by either assay with the exception of low concentrations of GnRH in urine using the Buhlmann Kit (table 2).

Precision studies

For the determination of within assay precision of the GnRH assay, three pools of plasma at different GnRH concentrations were analysed 20 times in one analytical run. Similarly, three pools of plasma were analysed 20 times to give within assay precision of the Buhlmann kit.

For the determination of between assay precision, three pools of plasma at different GnRH concentrations were analysed in nine consecutive assays. To minimise the effect of the poor stability of GnRH in plasma on the results, the assays were carried out over one week (table 3).

Comparison of standards used in the two assays

Appropriate dilutions of the GnRH standard supplied with the Buhlmann kit were analysed in duplicate, using the GnRH assay. Similarly, appropriate dilutions of the Ayerst Gonadorelin standard used in the GnRH assay were analysed in duplicate using the Buhlmann kit. Both sets of standards gave identical results with either assay.

Parallelism

Plasma and urine samples, containing high GnRH concentrations, were diluted with assay buffer and compared with the standard curve for parallelism.

Table 2 Mean recovery of GnRH added to a pool of plasma and urine ($n = 2$)

	GnRH assay		Buhlmann kit	
	GnRH added (pg/ml)	Per cent recovery	GnRH added (pg/ml)	Per cent recovery
Plasma	100	99	8	92
	250	86	16	107
	500	95	40	116
Urine	50	137	8	56
	250	102	16	90
	500	89	40	118

Table 3 Within assay and between assay precision

	GnRH concentration pg/ml (Mean (SD))	CV (%)
<i>Within assay precision:</i>		
GnRH assay (n = 20)		
Plasma 1	84 (6.7)	8.0
Plasma 2	184 (11.8)	6.4
Plasma 3	353 (10.2)	2.9
Buhlmann kit (n = 20)		
Plasma 4	20 (1.6)	8.1
Plasma 5	93 (12.2)	13.1
Plasma 6	122 (20.7)	17.0
<i>Between assay precision:</i>		
GnRH assay (n = 9)		
Plasma 1	88 (15.0)	17.0
Plasma 2	207 (28.0)	13.5
Plasma 3	397 (61.1)	15.4
Buhlmann kit (n = 9)		
Plasma 4	18 (4.5)	25.1
Plasma 5	94 (21.1)	22.4
Plasma 6	122 (17.0)	14.0

There was a high degree of parallelism using the GnRH assay but not when using the Buhlmann kit (Figs 1a and b, respectively).

Correlation

Correlation of the GnRH assay with the Buhlmann kit was assessed by measuring GnRH in plasma and urine samples from healthy laboratory staff and from patients receiving subcutaneous pulsatile GnRH treatment. As parallelism for the Buhlmann kit was poor, it was considered inappropriate to dilute samples with GnRH concentrations above 160 pg/ml.

Results were analysed by least squares linear regression. Deming's correction for slope was not applied because S_{sex}/S_x was less than 0.2, where S_{sex} represents the standard deviation of a single measurement of

GnRH by the Buhlmann kit method at a GnRH concentration of 60 pg/ml, and S_x is the standard deviation of the data set for the GnRH values obtained using the Buhlmann kit.¹² A value of S_{sex}/S_x of less than 0.2 means that the precision of the individual data points of the x data set relative to the total spread of the x data set is good. Hence there is minimal error in using the least squares linear regression model for calculating the slope of the regression line.

There was a good correlation between both assays for plasma samples within the range of the Buhlmann kit standards. Correlation for urine samples was poor (Fig 2).

Profile

Fig 3 shows a typical plasma GnRH profile from a patient who received a single subcutaneous injection of 15 μg GnRH (Gonadorelin, Ayerst Laboratories). Samples were analysed using the GnRH assay.

Discussion

Although several workers have developed radioimmunoassays for GnRH in plasma and urine,⁷⁻¹⁰ there are few commercially available assays and no published comparisons of assay performance. The developed GnRH assay showed good precision and recovery over a wide range of GnRH concentrations and parallelism. In contrast, the commercially available Buhlmann kit was unsuitable for measuring plasma GnRH in patients receiving treatment as the kit had a top standard of only 160 pg/ml, well below the peak plasma concentration observed in such patients. Although precision and recovery using the Buhlmann kit were acceptable, the poor parallelism observed made it unacceptable to dilute plasma samples with high GnRH concentrations for reanalysis.

Fig 1 Parallelism of plasma and urine samples using (a) the GnRH assay and (b) the Buhlmann kit.

Fig 2 Correlation between the GnRH assay and the Buhlmann kit for (a) plasma samples and (b) urine samples. Results below the limit of detection for the GnRH assay (15 pg/ml) were not included in the calculation of the regression coefficients.

The Buhlmann kit was considerably more sensitive than the developed GnRH assay and might, therefore, be the method of choice to detect low concentrations of GnRH. This was not in the remit of our assay development, however, and we have not addressed the potential of the assay as a diagnostic test for hypothalamic-pituitary disorders.¹³

Although there was a good correlation between both assays for plasma samples within the range of the

Buhlmann kit standards, there was a constant positive bias which was not due to differences in the standards as both sets of standards gave identical results with either assay. The poor correlation on the urine samples and the lack of parallelism with the Buhlmann kit suggests a matrix effect with this assay.

Despite the acceptable recovery of added GnRH to plasma and urine for unextracted samples, we have occasionally observed spuriously raised GnRH concentrations, using unextracted plasma. Therefore, extraction into ethanol was chosen as the procedure to use. Extraction into ethanol gave a recovery of about 80% and evaporation under a stream of air at 70°C significantly reduced the time of extraction without any loss of immunoreactivity. We were unable to confirm the use of bentonite⁹ or florisil¹⁰ adsorption followed by acid methanol elution as a suitable method for extracting GnRH from plasma and urine. This was because we were unable to elute the GnRH from the adsorbents. Our results have shown that samples must be stored at -20°C or -70°C and assayed within one week of collection. The assay described is suitable for measuring GnRH in samples obtained from patients receiving treatment with GnRH.

Fig 3 Plasma GnRH concentrations following a single subcutaneous injection of 15 µg GnRH at time 0.

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