Routine Ultramicro-Measurement of Human Growth Hormone in Plasma by Solid-Phase Radioimmunoassay

NORIO OGAWA
Third Department of Internal Medicine, Okayama University
School of Medicine, Okayama 700

Synopsis

A method for the ultramicro-measurement of human growth hormone (HGH) in plasma based on the solid-phase radioimmunoassay (RIA) using antibody-coated disposable microtiter trays was described. This method is simple, rapid and inexpensive, and particularly useful for ultramicro-measurement of plasma HGH levels in large numbers of plasma samples.

The lower limit of sensitivity for purified HGH was 25 pg/ml (P < 0.05). The precision of the measurement of HGH in plasma has been calculated, and the quantitative sensitivity of the method derived from the results given by its routine use was 56.8 pg/ml (P < 0.05). The HGH level of normal pooled plasma in 10 replicates was 1,515 pg/ml (± 126; 1 SD), and the coefficient of variation was 8.3%. The mean basal plasma HGH level after an overnight fasting in 23 normal males was 2,100 pg/ml (± 1,491; 1 SD) by this method and 2,036 pg/ml (1,643; 1 SD) by double-antibody RIA, and in 23 normal females, 2,589 pg/ml (± 1,189; 1 SD) and 3,300 pg/ml (± 1,690; 1 SD) respectively; there was no statistically significant difference between the basal HGH level obtained from this method and that from double-antibody RIA in normal subjects. On the other hand, the mean basal plasma HGH level after an overnight fasting in 21 hypopituitary patients was 484 pg/ml (± 282; 1 SD) by this method, and this value was significantly lower (P < 0.001) than 1,376 pg/ml (± 498; 1 SD) by double-antibody RIA. These data show that determination of basal plasma HGH levels by this method is of much value in the diagnosis of hypopituitarism.

The method of radioimmunoassay (RIA) developed by Yalow and Berson (1960) for the assay of human insulin had been soon applied to human growth hormone (HGH) (Utiger et al., 1962; Hunter and Greenwood, 1962; Glick et al., 1963). During the past 10 years various modifications of radioimmunological methods were applied to the detection and assay of HGH in plasma. All forms of RIA used at present include a procedure to separate antibody-bound and free tracer hormone when equilibrium is reached in the incubation mixture. The conventional procedures of RIA have utilized a wide variety of techniques for the separation of bound and free tracer hormone, such as a second antibody, chromatoelectrophoresis, solvent fractionation, salt precipitation, or adsorption to charcoal.

The technique of solid-phase RIA was originally developed by Catt et al. (1966) for the assay of HGH in plasma. The solid-phase RIA allows rapid removal of the free radioactive tracer antigen only by washing of the solid-phase with water on completion of the immune reaction.

The present paper describes the development and application of the method for the ultramicro-measurement of HGH in plasma,
based on the solid-phase RIA by using antibody-coated disposable microtiter trays. By further reduction in the amount of antibody with corresponding increase in the period of incubation, greater sensitivity has been achieved. This method has adequate sensitivity and precision for routine ultramicro-measurements of plasma HGH concentration, and is relatively simple to perform. By using this method, an attempt was made to differentiate patients with hypopituitarism from normal subjects on single basal plasma samples, although it had been hitherto generally accepted that determination of basal plasma HGH levels had not been of much value in the diagnosis of hypopituitarism (Glick et al., 1963; Hunter and Greenwood, 1964a).

**Materials and Methods**

*Disposable microtiter tray*

Disposable microtiter trays (S-MRC 96, Linbro Chemical, New Haven, Connecticut, U.S.A.) were immersed in 70% ethanol for 10 min and washed out thoroughly with distilled water, and then used for assay.

*Antiserum*

The rabbit antiserum to HGH were kindly supplied by Dainabot Radioisotope Lab., Ltd., Japan. In ordinary solid-phase or double-antibody RIA, the optimal dilution of this antiserum was 1: 20,000. In this assay, aliquots of antiserum were diluted 1: 80,000 in 0.05 M phosphate buffer, pH 7.5, without containing bovine serum albumin.

*\textsuperscript{125}I-labelled HGH*

\textsuperscript{125}I-labelled HGH with a specific activity of 109 mCi/mg was obtained from Dainabot Radioisotope Lab., Ltd., Japan which was made up in 0.1 M borate buffer, pH 8.6, containing 0.5% bovine serum albumin.

*HGH reference preparation*

Human growth hormone for standards (NIH-GH-HS 1147) was prepered immediately before each assay as solutions containing 0, 10, 25, 50, 100, 250, 500, 1,000, 2,500, 5,000, 10,000 and 20,000 pg/ml in 95% horse serum.

*Assay procedure*

The cups of a disposable microtiter tray were coated with uniform volume (0.2 ml) of diluted antiserum for 24 to 48 hr at room temperature (about 18°C). After removal of the coating solution by suction, the cups of the microtiter tray were washed-out twice with tap water. It was demonstrated that the antiserum solution could be re-used at least twice (Furuno et al., 1973). 0.1 ml of HGH standards or unknown plasma samples, and 0.1 ml of \textsuperscript{125}I-labelled HGH containing approximately 10^4 cpm were added into the antibody-coated cups of the tray, and incubated for 48 hr at room temperature. After incubation, the contents were discarded and the cups washed-out twice with tap water. The cups were cut off each other by scissors, and then, they were placed in counting tubes and the radioactivity of the labelled antigen bound to the antibody-coated cups was counted in a well type scintillation detector. Without antibody, only 0.5% of the whole counts remained in the empty cups. The activity of the unknown plasma samples could be obtained from graph constructed by plotting the logarithm of the standard HGH solutions against their bound radioactivity counts (Fig. 1).

Duplicate determinations were usually made on each solution included in the assay.

**Investigation of plasma HGH levels**

For determination of basal HGH levels, 23 normal males, 23 normal females and 21 hypopituitary patients were studied. Blood samples were collected at 9 AM under basal conditions after an overnight fast.

Standard oral 50 g glucose tolerance tests were performed after an overnight fast in 13 normal male and 13 normal female subjects. Blood samples were drawn at times 0, 30, 60, 90, 120 min. The subjects were kept at rest in the horizontal position at least for 30 min before drawal of the first blood sample to minimize the effect of previous exercise.

All samples were taken in heparinized syringes, then centrifuged, and the plasmas were immediately separated and frozen till they were assayed.

**Results**

1. **Characterization of the method**

The standard assay curve for HGH in 5 replicates was shown in Figure 1. When logit 100 × B/B₀ was plotted against the logarithm of the dose of HGH, this assay was linear from 25 to 20,000 pg/ml of HGH (Fig. 2). For purified HGH, the smallest amount of HGH which was significantly different (P = 0.05) from zero was 25 pg/ml.
Fig. 1. Standard curve using a 1:80,000 final dilution of antiserum to coat the disposable microtiter tray, constructed by plotting the logarithms of the standard HGH solution against their 100 × B/B₀. Each point represents the mean of 5 replicates ± SD.

Fig. 2. Standard curve constructed by re-plotting on the logit-log paper.
Table 1. Estimate of standard deviation(s) from determination of duplicate pairs in various concentration range of plasma HGH, and the fiducial limits for each range.

<table>
<thead>
<tr>
<th>HGH concentration (pg/ml)</th>
<th>No. of duplicate pairs</th>
<th>s</th>
<th>Fiducial limits (P = 0.05) (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-99</td>
<td>25</td>
<td>20.1</td>
<td>Mean ± 28.4</td>
</tr>
<tr>
<td>100-499</td>
<td>59</td>
<td>71.4</td>
<td>Mean ± 99.9</td>
</tr>
<tr>
<td>500-999</td>
<td>60</td>
<td>94.4</td>
<td>Mean ± 132.1</td>
</tr>
<tr>
<td>1,000-4,999</td>
<td>173</td>
<td>160.0</td>
<td>Mean ± 221.7</td>
</tr>
</tbody>
</table>

The method was evaluated in the manner recommended by Brown et al., 1957. All calculations were based on duplicate determinations using 0.1 ml of plasma.

Accuracy

The only practical way to evaluate accuracy is by recovery experiments in which known quantities of pure HGH are added to the plasma samples before analysis and the results compared with those from a control plasma sample not containing added HGH. The recoveries of HGH added to plasma samples were ranged 95.5 to 107.8%.

Precision and sensitivity of the assay when applied to plasma

The results of duplicate determinations in different concentration ranges were analysed by estimating the standard deviation (s) by the following formula: \( s = \left( \frac{\sum d^2}{2n} \right)^{1/2} \), where \( d \) is the difference between the two results in a duplicate determination and \( n \) is the number of duplicate pairs. For each concentration range of duplicates, the fiducial limits were calculated from the relationship: \( M \pm ts/\sqrt{N} \), where \( M \) is the mean of each concentration range, \( t = 1.960 \) (P = 0.05) and \( N = 2 \) (duplicate determinations). From 317 duplicate determinations, the precision of the method obtained in practice was calculated as described above. Table 1 summarized the values of \( s \) and the fiducial limits.

The qualitative sensitivity of the method, that is the smallest amount of HGH that can be distinguished from zero, is given by the formula: \( ts/\sqrt{N} \). For example, the sensitivity of the method (P = 0.05) is 28.4 pg/ml with a maximum error of ±100%.

The quantitative sensitivity of the method is defined by the lowest concentration of HGH which can be measured within a specific maximum error. This is calculated from the formula: \( 100 ts/x/\sqrt{N} \), where \( x \) is the maximum percentage error. For example, if it is specified that the percentage error should not be more than ±50% (P = 0.05), then the smallest amount of HGH which can be measured with this accuracy is 56.8 pg/ml.

Replication experiments

Pooled plasma of normal subjects in 10 replicates gave a mean ± SD of 1,515 ± 126 pg/ml. The coefficient of variation at this level was 8.3%.

Effect of concentration of protein on this method

The concentration of plasma protein in physiological range had no effect on bound radioactivity.

II. The clinical application of this solid-phase method to the ultramicro-measurement of HGH in the plasma

Comparison was made between this solid-phase method and the double-antibody method by direct analysis of the same basal plasma samples obtained from 23 normal males, 23 normal females and 21 hypopituitary patients (Fig. 3). Basal plasma HGH levels after an overnight fasting in normal men were 2,100 pg/ml (±1,491; 1 SD) by this method and 2,036 pg/ml (±1,643; 1 SD) by the double-antibody method, and in women, 2,589 pg/ml (±1,189; 1 SD) and 3,300 pg/ml (±1,690; 1 SD), respectively; there was no statistically significant difference between the HGH levels obtained from this method and those from
the double-antibody method. On the other hand, basal plasma HGH levels after an overnight fasting in patients with hypopituitarism were 484 pg/ml (±282; 1 SD) by this method, and this was significantly lower (P < 0.001) than 1,376 pg/ml (±498; 1 SD) by the double-antibody method. By using this method, in only 6 out of 46 normal subjects, the basal HGH levels were below 1,047 pg/ml which was Mean + 2 SD obtained from the basal HGH levels of 21 patients with hypopituitarism.

Standard oral 50 g glucose tolerance tests (OGTT) were carried out in normal subjects. In only 1 out of 13 normal male subjects and only 2 out of 13 normal female subjects, plasma HGH levels during OGTT decrease less than 1,047 pg/ml (Fig. 4). Thus high concentrations of blood glucose were unable to suppress completely HGH secretion in normal subjects.

**Discussion**

The technique of solid-phase radioimmunoassay (RIA) was originally developed for the measurement of HGH in plasma (Catt *et al.*, 1966, 1967). This simple and sensitive procedure was soon applied to the measurement of other peptide hormones—namely human placental lactogen (Catt *et al.*, 1967), luteinizing hormone (Catt *et al.*, 1968), insulin (Cott *et al.*, 1971) and prolactin (Fell *et al.*, 1972). The solid-phase materials had been prepared in the form of powder (Catt *et al.*, 1966), small discs (Catt *et al.*, 1968; Cott, 1969) and tubes (Catt *et al.*, 1967; Cott *et al.*, 1971;
Fig. 4. Plasma HGH concentrations during a standard oral glucose tolerance test (50 gm) in normal subjects.

Fell et al., 1972). The antibody-coated disposable microtiter tray method (Takeda et al., 1972; Nagayama et al., 1972; Ogawa et al., 1973; Furuno et al., 1973) is more simple and more inexpensive than the tube method.

Although various customary RIA methods can be used as a routine on provocative tests, it would seem that further increase in sensitivity is desirable. Without the statistical evaluation, Catt et al. (1966) reported that the sensitivity of the powder form solid-phase method for plasma HGH estimation was always 0.4 ng/ml or less, frequently under 0.1 ng/ml. The results of present study demonstrate that greater sensitivity has been achieved in modification of the microtiter tray method by further reduction in the amount of antibody with corresponding increase in the period of incubation. The lower limit of quantitative sensitivity calculated from the precision of this solid-phase ultramicrodetermination method ('this method') during routine use has been taken as 56.8 pg/ml rather than that calculated from the sensitivity of the standard curve, i.e. 25 pg/ml. The discrepancy between the lower limit of quantitative sensitivity calculated from the routine use and that from the standard curve had been also reported by Hunter and Greenwood (1964a), and they described that this differentiation suggested that measurement of HGH in plasma had been subject to a greater degree of imprecision than its measurement in pure solution or that there had been some degree of nonspecificity detectable at the lower limit of the method, or both. Anyway, 'this method' is thus extremely convenient for the ultramicro-measurement of HGH.

Up to this time, it had been generally accepted that determination of basal plasma HGH levels had not been of much value in the diagnosis of hypothalamic or pituitary hypofunction, because in many normal subjects these levels had been below the sensitivity of available assays (Glick et al., 1963; Hunter and Greenwood, 1964a; Okada, 1970). Further,
Okada (1970) reported that, because of the tracer damage, the basal HGH levels in hypopituitary subjects obtained from the double-antibody method were higher than those from the chromatoelectrophoretic method. So, by using double-antibody RIA, it was difficult to differentiate patients with hypopituitarism from normal subjects on single basal plasma samples, and it had been also accepted that the differentiation between normal and hypopituitary subjects required serial estimations throughout provocative tests (Hunter and Greenwood, 1964a). But using this method, avoiding the problem of the tracer damage and achieving greater sensitivity, it was possible to detect the differentiation between normal subjects and patients with hypopituitarism on single basal plasma samples.

On the other hand, it was interesting that basal plasma HGH levels of hypopituitary subjects did not show zero but 484 ± 282 pg/ml (ranged 97 to 960 pg/ml). Kenny et al. (1973) reported that patients who had undergone surgery for craniopharyngiomas, showed 'catch-up' growth without HGH responsiveness to provocative tests and with normal prolactin levels. On the basis of the above data, it might be conceivable that the remaining basal levels of HGH present induced the phenomenon of 'catch-up' growth in some of these patients.

Although several studies (Roth et al., 1963; Hunter and Greenwood, 1964b) have reported that glucose suppresses normal resting HGH secretion, this point is difficult to establish unequivocally because initial fasting levels may already be only barely detectable by customary methods. Plasma HGH concentrations were determined by this method during OGTT in normal subjects, and there was a distinct decline but not reaching the sub-nanogram range. Thus high concentrations of blood glucose could not completely suppress HGH secretion in normal subjects. This suggests that the mechanism of HGH secretion in normal subjects is thoroughly different from that in hypopituitary subjects.

Acknowledgement

I wish to express my particular thanks and appreciation to my respected teachers, Professor Tadashi Ofuji and Dr. Jiro Takahara for their cordial guidance and encouragement. I am also grateful to Dainabot Radioisotope Lab., Ltd., for supplying the anti-HGH serum, and National Instituts of Health, U.S.A. for supplying HGH reference preparation.

References

December 1973

Igaku-no-Ayumi (Tokyo) 85, 540. (in Japanese)