STANDARDIZATION OF RADIOIMMUNOASSAY TECHNIQUE FOR DETERMINATION OF PLASMA INSULIN AND GROWTH HORMONE

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Abstract

STANDARDIZATION OF RADIOIMMUNOASSAY TECHNIQUE FOR DETERMINATION OF PLASMA INSULIN AND GROWTH HORMONE.

The assays were performed with porcine insulin-^{125}I and human growth hormone (HGH)-^{125}I after labelling by the technique of Hunter and Greenwood and purifying by starch gel electrophoresis (for separation of the over-iodionated fractions) followed by further purification in gel Sephadex columns, to discard damaged components and free iodine. The final labelled fraction was more than 90% free from damaged hormone with excellent immunoreactivity. The assays were carried out with guinea-pig antisera specific to porcine insulin and to HGH. The separation of free from antibody-bound hormone was done with charcoal. These methods were standardized by evaluating the following parameters:

1. Accuracy. The accuracy was tested by the addition of progressively larger amounts of insulin (or plasma) to a known amount of plasma (or standard HGH). The recovery varied from 93 to 105% with a coefficient correlation r between the theoretical and found values of 0.997 for insulin and recoveries varying from 70 to 103% for HGH, with a coefficient correlation r of 0.985.

2. Specificity. Specificity was tested with progressively larger dilutions of a plasma sample, the correlation coefficient r with the apparent hormonal concentration being 0.998 for both insulin and HGH.

4. Precision. Precision was evaluated through the reproducibility of a pool of plasma studied within-assay and between-assay. The coefficient of variation for insulin was 7.4% and 7.6% for within-assay and between-assay, respectively, and 4.6% and 6% for HGH under the same conditions. The range of the standard curves studied was from 0.01 to 1 ng for insulin and 0.02 to 2.0 ng for HGH.

4. Sensitivity. Sensitivity was found to be 1.25 μU/ml and 1.5 ng/ml of plasma for insulin and GH, respectively.

INTRODUCTION

Insulin and human growth hormone (HGH), like other peptide hormones, are difficult to measure in biological material because of their low concentrations and the absence of radicals which could allow specific chemical reactions. Routine procedures for peptide hormones only became available when the specificity of immunological reactions was associated with the sensitivity and accuracy of radioactivity measurements, i.e. through the techniques known as radioimmunoassay [1].

The principles of radioimmunological assay, as applied to the measurement of peptide hormones, are illustrated in the set of competing reactions shown in Fig. 1 where Ag* represents the hormone labelled with an iodine isotope. This labelled hormone Ag* is the specific antigen for binding the antibody Ab. When they come in contact, a dynamic equilibrium is established between the antigen-antibody (Ag*-Ab) complex and its components Ab and Ag*, the latter constituting the free form (F) of the labelled hormone [2]. The addition to the system of the unlabelled hormone Ag will displace from the

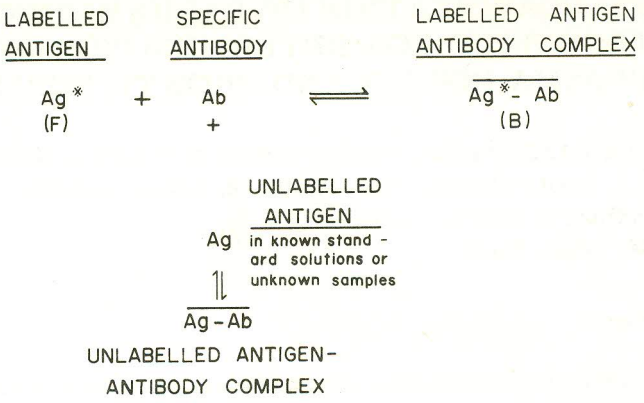

FIG. 1. Principles of radioimmunoassay: Competitive antigen-antibody reactions (reproduced from Yalow and Berson, AEC Symp. Series 13).

complex Ag*-Ab (B) a fraction of Ag* proportional to the relative quantities of Ag. Thus, the ratio of antibody-bound (B) to free (F) labelled hormone decreases progressively as the concentration of unlabelled hormone (standards and unknown samples) increases, the components Ag* and Ab being kept constant.

In our study the technique for obtaining highly purified labelled insulin and growth hormone has been standardized, a suitable specific activity being attained for both hormones. In this technique the separation of free labelled hormone from the antibody-bound fraction was carried out with the use of plasma-coated charcoal. The reliability of the assays has been carefully checked.

MATERIALS AND METHODS

Radioiodination

The hormones (porcine insulin and human growth hormone) were labelled with ^{125}I (Union Carbide) by the method of Hunter and Greenwood [3, 4] with several modifications as suggested by Yalow and Berson [5]. The yields of ^{125}I-labelled hormones were equal to or larger than 80%.

Porcine insulin (six times recrystallized) was supplied by Lilly Research Laboratories, and human insulin, for the standard curve, was a gift from Drs. A. Mirsky (Pittsburgh University) and M. Root (Lilly Lab.). Human growth hormone, prepared by Dr. A.E. Willhelmi, was obtained from the National Pituitary Agency.

Assessment of the radioiodinated hormone

To an aliquot of the preparation mixture contained in the iodination tube, 100 μl of human plasma, 2.4 ml of 0.02M veronal buffer at pH 8.6 and charcoal solution (1 mg/ml in veronal buffer) were added, 0.2 ml of the

charcoal solution being used for insulin and 1 ml for HGH. After centrifugation, the activities of the supernatant and precipitate were measured and expressed as a percentage of the total radioactivity. The charcoal adsorbs intact ^{125}I-hormones [6] and so separates them from damaged ^{125}I-hormones, ^{125}I and other reactants which remain in the supernatant.

Purification

After iodination, insulin-^{125}I and HGH-^{125}I were purified by starch gel electrophoresis [7, 8] as suggested by Yalow and Berson [9] followed by further purification in a Sephadex column. 40 μl of "blue plasma" (plasma stained with bromophenol blue) were added to the solution of labelled hormone after iodination with the purpose of binding damaged molecules and of preventing hormone losses by adsorption to glass, as well as to indicate the zone of migration of the plasma albumin in the starch gel electrophoresis. The various insulin components migrate in front of the albumin and the growth-hormone fractions just behind it (Fig. 2). The localization of the pure labelled hormone bands was done by autoradiography.

The suitable fraction of the labelled hormones (fraction 2) (Fig. 2) was eluted and centrifuged to remove gel particles. After adding 0.2 ml of "blue plasma", the eluate was transferred to a Sephadex column [3], G-50 being used for insulin and G-75 for HGH [3]. The elution from both columns was carried out with 0.02M veronal buffer, collecting 1-ml fractions.

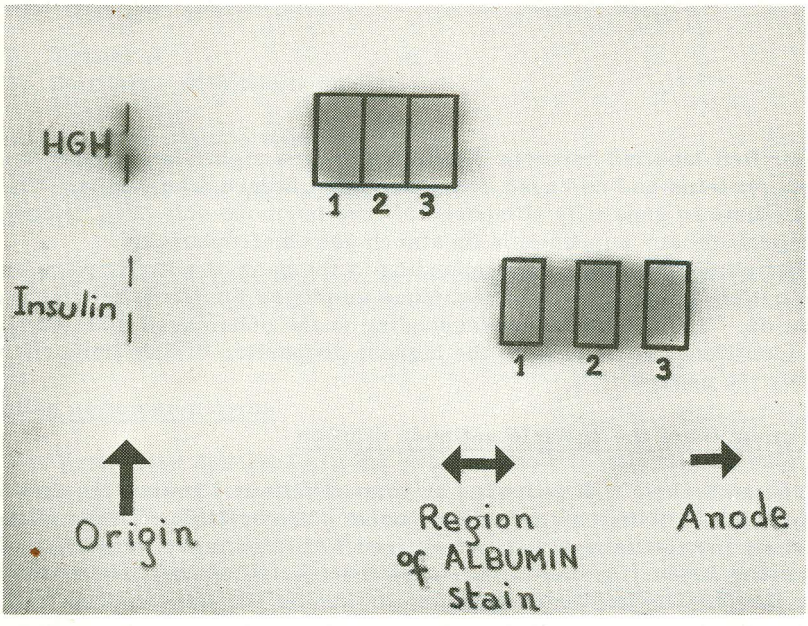

FIG. 2. Autoradiograph of starch gel electrophoretogram of HGH-^{125}I and insulin-^{125}I immediately after iodination.

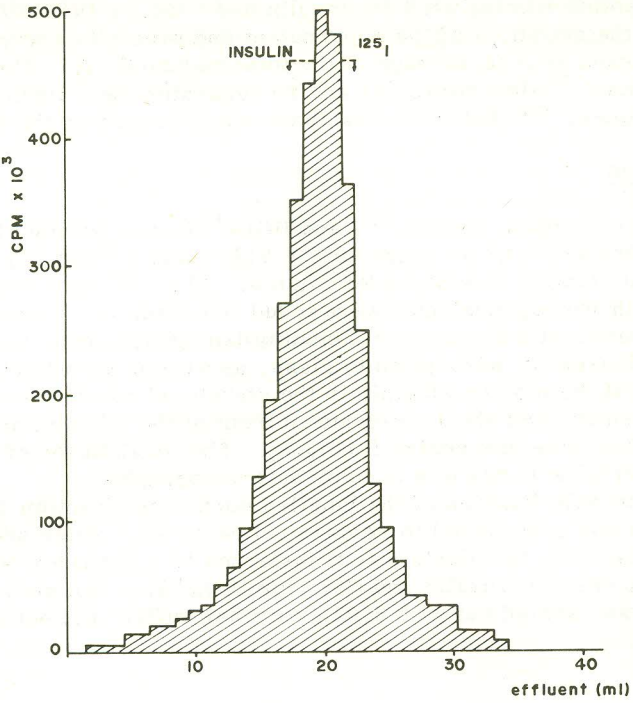

FIG. 3. Gel filtration on Sephadex G-50 of insulin-^{125}I after previous purification by starch gel electrophoresis.

The purified labelled hormone fraction emerges significantly later than plasma proteins and damaged components but before free iodine. The radioactive eluate is generally composed of free hormone only (Fig. 3) because of the preliminary purification on starch gel electrophoresis.

The eluted fractions corresponding to the peak of activity were submitted to the quality control with the technique employed for the assessment of the iodination. Thus, eluates corresponding to the undamaged labelled hormones were identified and those with the highest proportion of free hormone were kept for the assays.

Determination of the suitable antibody dilution

The antiserum was prepared by immunization of guinea-pig against porcine insulin or human growth hormone. The suitable dilution of the antiserum for the assays was determined by preliminary incubation of decreasing concentrations in the range from $1:1\times10^5$ to $1:4\times10^5$ for insulin and $1:1\times10^6$ to $1:3\times10^6$ for HGH.

The diluent used for all the solutions consisted of veronal buffer, 0.02M, pH 8.6, with 0.25% human albumin, 1% guinea-pig serum and ^{125}I-hormone (1000 counts $\cdot$ $min^{-1}\cdot ml^{-1}$).

The dilutions were tested daily during the incubation period according to the following protocol: to a sample of 2.4 ml of each dilution, 100 μl of blood bank human plasma and charcoal suspension (100 mg/ml) were added, 0.2 ml of the latter being used in the insulin assay and 1 ml for HGH, as previously indicated.

After centrifugation at 4°C the values for the supernatant (B) and precipitate (F) were measured in an automatic gamma well-counter (Nuclear Chicago) for a long enough time to accumulate about 10 000 counts and their ratio (B/F) was calculated. The eventual degradation of the labelled hormone during incubation was controlled by the use of standard diluent without antibody.

Incubation lasted from 3 to 4 days for insulin and from 5 to 6 days for HGH. The highest dilution of the antibody which presents a B/F ratio ranging from 0.8 to 1.2 [10] without a great increase in percentual damage to the hormone was selected.

Standard curve

The standards of human insulin were prepared from a dilution of 25 ng/ml according to the protocol shown in Table I. For HGH, the original dilution was 50 ng/ml.

"Tracer test tubes" (called tube A to D) and "controls" with 100 μl of diluent, instead of plasma or standard, were set up. To the controls, 2.4 ml of diluent were added and to the tracer, standards and unknown plasma samples, 2.4 ml of diluent with the antibody in the appropriate dilution (determined during the preliminary testing) were added in the following sequence:

1. Tracer A
2. Standards with odd number
3. Tracer B
4. Unknown plasma samples
5. Tracer C
6. Standards with pair number
7. Tracer D

Furthermore, tracer and control were prepared in excess being called "big tracer" and "big control", respectively. The incubation was carried out at 4°C.

Sampling of human plasma

The procedure described in the present work is compatible with the simultaneous assay of up to 500 samples. To 100 μl of each sample, 2.4 ml of diluent with the antibody in a suitable dilution were added. From each lot of 10 to 15 samples (usually in the same test), one is separated which will be its "control" for the damage in the hormone during the incubation period.

During incubation the amount of damage to the labelled hormone as well as its binding to the antibody was checked daily by the technique previously indicated (plasma-coated charcoal) with a sample from the "big control" and "big tracer", respectively.

TABLE I. SUMMARY OF ASSAY PROCEDURE

TUBES	Standard Curve			Veronal Albumin	Unknown Sample	Standard Diluent	Standard Diluent + Antibody
	*Standard Solution (µl)	Concentration mug/ml					
		Human Insulin	HGH	(µl)	(µl)	(µl)	(ml)
Tracer A	-	-	-	100	-	-	2.4
B	-	-	-	100	-	-	2.4
C	-	-	-	100	-	-	2.4
D	-	-	-	100	-	-	2.4
Control for Standard	-	-	-	100	-	2.4	-
Nº 1	1	0.01	0.02	99	-	-	2.4
2	2	0.02	0.04	98	-	-	2.4
3	3	0.03	0.06	97	-	-	2.4
4	4	0.04	0.08	96	-	-	2.4
5	5	0.05	0.10	95	-	-	2.4
6	10	0.10	0.20	90	-	-	2.4
7	15	0.15	0.30	85	-	-	2.4
8	20	0.20	0.40	80	-	-	2.4
9	30	0.30	0.60	70	-	-	2.4
10	40	-	0.80	60	-	-	2.4
11	50	0.50	1.00	50	-	-	2.4
12	60	-	1.2	40	-	-	2.4
13	70	0.70	1.4	30	-	-	2.4
14	100	1.00	2.00	-	-	-	2.4
Control for unknown sample	-	-	-	-	100	2.4	-
Unknown sample	-	-	-	-	100	-	2.4

* Human insulin : 25 ng/ml
HGH : 50 ng/ml

Incubation was stopped when the ratio of B/F was ≅ 0.8 - 1.2 and the damage was below 10% (for insulin) and 15% (for HGH). Charcoal was then added and after centrifugation at 4°C the radioactivity was measured in the precipitate (free hormone (F)) and in the supernatant (bound hormone (B), damaged hormone and free iodine).

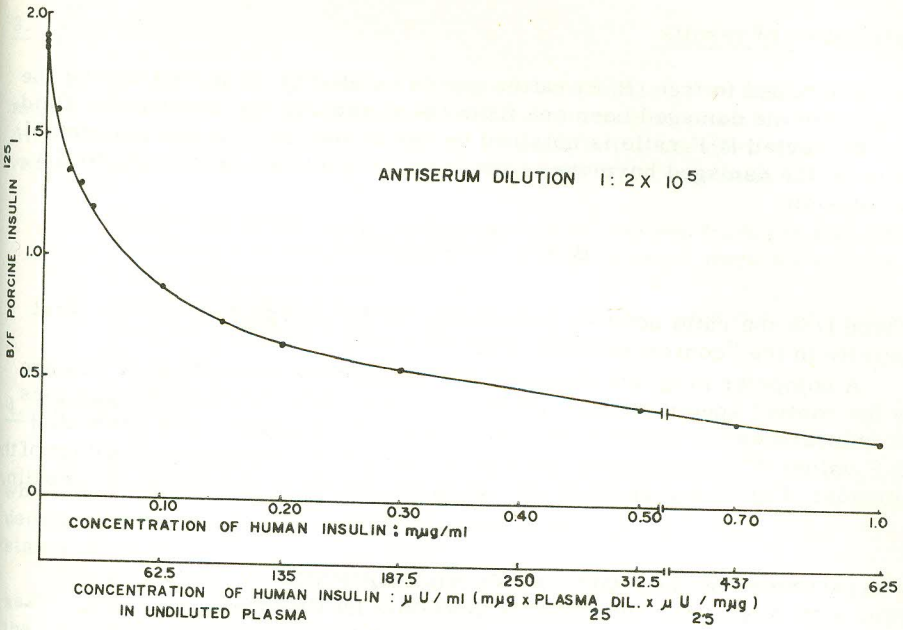

FIG. 4. Standard curve for assay of human insulin.

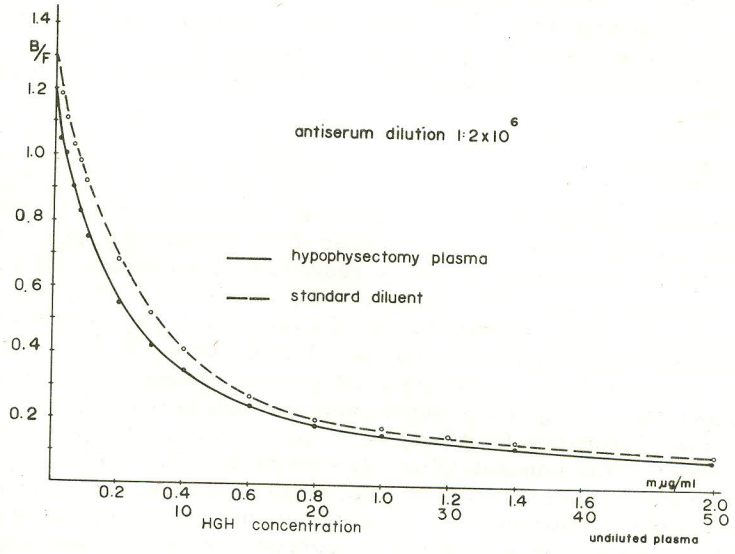

FIG. 5. HGH standard curves, with standard diluent and plasma from hypophysectomized patient.

Calculation of results

The bound to free (B/F) ratios are calculated by first subtracting the counts for the damaged hormone from the counts for the supernatant fluid. The corrected B/F ratio is obtained by taking into account the percentual value of the damaged hormone in the "control plasma", according to the expression:

$$B/F = \frac{B - [(B+F) \times D]}{F}$$

where D is the ratio between the activity in the supernatant and the total activity in the "control plasma" tube.

A computer program was used to calculate the percentage of damage in the control specimens and then to correct all B/F ratios of the samples or standard curve samples for their respective damage. The corrected B/F values of the standards are plotted against the corresponding dose of the hormone (Figs 4 and 5) expressed in ng/ml for HGH and μU/ml for insulin.

EVALUATION OF THE OPERATIONAL SEQUENCE FOR THE ASSAY OF PROTEIN HORMONES IN PLASMA

1. Accuracy

Accuracy was determined by measurement of the recovery of known quantities of purified hormones in buffer solution or plasma samples of known concentrations. To check the accuracy of the insulin assay, four solutions of standard insulin, with increasing concentrations: 0.02, 0.04, 0.05 and 0.10 ng/ml, were prepared in a plasma with a known insulin concentration. To check the accuracy of the GH assay, 10 determinations were made by adding increased quantities of GH from a pool of plasma with known HGH concentration to a solution of standard HGH in buffer. The correlation coefficient r between the theoretical and obtained values was determined.

2. Specificity

Specificity may be defined as the extent of freedom from interference by substances other than the one intended to be measured. This interference could be due to immunological cross-reactivity, the presence of unwanted labelled substances and differences in composition of the incubation medium.

A necessary requirement, but not the only one to be met for checking complete specificity, is provided by the dilution test which requires that the hormone concentrations measured at different dilutions of plasma by reference to the standard curve give the same value for undiluted plasma [1].

For the dilution test four samples of each dilution from a pool of plasma with a known insulin concentration were prepared: 1:25, 1:50, 1:100 and 1:125 for insulin and 1:25, 1:27, 1:35, 1:50, 1:83 and 1:250 for HGH. The hormonal concentration for each dilution determined by reference to a standard curve was plotted against the corresponding dilutions (Figs 6 and 7) and the correlation coefficient was determined for both hormones.

3. Precision

Precision can be defined as the extent to which a given set of measurements of the same sample can give results as close as possible to one another. An estimate of precision was obtained by calculating the coefficient of variation of measurements done under two different conditions:

(a) Within the same assay: 20 aliquots were taken from a plasma pool of known concentration and their concentrations were measured within the same assay.
(b) Between different assays: 20 aliquots of the same plasma pool were assayed 20 times in the course of 1 year.

4. Sensitivity

Sensitivity may be defined as the smallest amount of unlabelled hormone which can be distinguished from no hormone. According to this, sensitivity depends on the error in the determination of B/F in the initial slope of the standard curve [11].

The antiserum giving the steepest slope will provide the most sensitive assay, since small variations in unlabelled hormone concentration within the early part of the standard curve (low hormone concentrations) will correspond to greater changes in the B/F ratio. In order to have maximal sensitivity, it is required that the specific activity in the tracer be high. This means that a small concentration of the tracer and a high antibody dilution must be used.

RESULTS

Assessment of the radioiodinated hormones

In 1 year labelling of the hormones was carried out 16 times with an average efficiency of 73% (ranging from 60 to 83%) for insulin and 71% (ranging from 62 to 83%) for HGH.

Purification of the labelled hormone

The preliminary purification by starch gel electrophoresis, to select the labelled hormone fraction with the highest immunoreactivity (devoid of excessive iodination and damaged molecules), i.e. fraction 2 (Fig. 2), gave a mean recovery of 73% of the radioactivity in the corresponding gel section. This eluate gave a mean value of 88% (ranging from 85% to 91%) of its total radioactivity as free labelled hormone for both hormones.

Further purification in Sephadex yielded the three eluates (1, 2, 3 in Fig. 2) with the highest activity, with a mean for free labelled hormone of 97% (94-98%) of the total radioactivity present in the fraction for insulin and 94% (90-97%) for HGH.

TABLE II. DETERMINATION OF SUITABLE ANTISERUM DILUTION AND DAMAGE CONTROL FOR INSULIN ASSAY

Antiserum dilution	1st day		2nd day		3rd day		4th day	
	% damage	B/F	% damage	B/F	% damage	B/F	% damage	B/F
$1:1\times10^5$	4	1.30	4	1.87	4	1.88	5	1.95
$1:2\times10^5$	4	0.83	4	1.16	4	1.26	5	1.44
$1:3\times10^5$	4	0.57	4	0.97	4	0.99	5	1.16*
$1:4\times10^5$	4	0.41	4	0.57	4	0.70	5	0.83

* See text.

TABLE III. DETERMINATION OF SUITABLE ANTISERUM DILUTION AND DAMAGE CONTROL FOR HUMAN GROWTH HORMONE ASSAY

Antiserum dilution	1st day		2nd day		3rd day		4th day		5th day		6th day	
	% damage	B/F	% damage	B/F	% damage	B/F	% damage	B/F	% damage	B/F	% damage	B/F
$1:1\times10^6$	7	0.63	9	1.24	9	1.40	10	1.57	11	1.57	11	1.42
$1:2\times10^6$	7	0.37	9	0.69	9	0.85	10	1.00	11	1.17	11	1.33*
$1:3\times10^6$	7	0.18	9	0.26	9	0.43	10	0.60	11	0.70	11	0.78

* See text.

Determination of the suitable dilution of the antibody

Table II presents the results of a typical study for insulin antiserum in which the percentage of damage and the B/F ratio corresponding to every dilution titre during the 4 days of incubation are indicated. It can be seen that the highest acceptable dilution of the antiserum with a low level of damage (5%) was $1:3\times10^5$ with a B/F ratio of 1.16 (indicated by an asterisk).

The results of a typical study for the HGH antiserum is shown in Table III. It can be seen that the highest antibody dilution after 6 days of incubation, with an acceptable damage (11%), was $1:2\times10^6$ with a B/F of 1.33 (indicated by an asterisk).

EVALUATION OF THE CHARACTERISTICS OF OPERATIONAL SEQUENCE FOR THE ASSAY OF PROTEIN HORMONES IN PLASMA

1. Accuracy

(a) Insulin: The percentage of recovery varied between 93 and 105%. The correlation coefficient r between theoretical and obtained insulin values was 0.997 which can be considered excellent in relation to the protocol used (Table IV), being significantly greater than zero, at the level of 1%.

TABLE IV. RECOVERY EXPERIMENT – ADDITION OF HUMAN INSULIN STANDARD TO A KNOWN PLASMA

TUBES Nº	Amount present (plasma)	Amount Added (standard)	Theoretical Value	Found Value	Recovery
	ng/ml	ng/ml	ng/ml	ng/ml	%
1 A	0.02	-	0.02	0.019	99.5
1 B	0.02	-	0.02	0.021	105
2 A	0.02	0.02	0.04	0.037	93
2 B	0.02	0.02	0.04	0.042	105
3 A	0.02	0.03	0.05	0.051	102
3 B	0.02	0.03	0.05	0.047	94
4 A	0.02	0.08	0.10	0.101	101
4 B	0.02	0.08	0.10	0.097	97

r=0.997

TABLE V. RECOVERY EXPERIMENT – ADDITION OF INCREASING QUANTITIES OF PLASMA TO HGH STANDARD (CONSTANT AND KNOWN)

TUBES	Concentration of HGH Standard (C)	Results			
		Theoretical Value	Found Value	Calculated Found Value (Found-C)	Recovery
	ng/ml	ng/ml	ng/ml	ng/ml	%
1	6.25	4.95	9.75	3.50	70.7
2	6.25	4.95	10.25	4.00	80.8
3	6.25	9.90	16.10	9.85	99.5
4	6.25	9.90	16.50	10.25	103.5
5	6.25	14.85	20.00	13.75	92.6
6	6.25	14.85	19.00	12.75	85.8
7	6.25	18.80	25.25	19.00	95.9
8	6.25	18.80	22.80	16.55	83.6
9	6.25	24.75	28.20	22.00	88.9
10	6.25	24.75	30.00	23.75	95.9

(b) HGH: The percentage of recovery varied between 70.7 and 103.5%. The correlation coefficient r between the theoretical and obtained HGH values was 0.989 which can also be considered excellent for the corresponding number of samples in the protocol (Table V), being significantly greater than zero, at the level of 1%.

2. Specificity

As can be seen in Figs 6 and 7 the measured concentration of hormone obtained by reference to a standard curve decreases proportionately with the dilution factor, the correlation coefficient r between plasma dilution and hormonal concentration being 0.998 which is significant for both hormones, at the level of 1%.

3. Precision

The mean and standard deviation and the coefficient of variation (CV) of 20 aliquots from the same pool of plasma, in the same assay, were: 164.25 ±12.19 and 7.4% respectively for insulin and 12.85 ± 1.25 and 4.6% respectively for HGH.

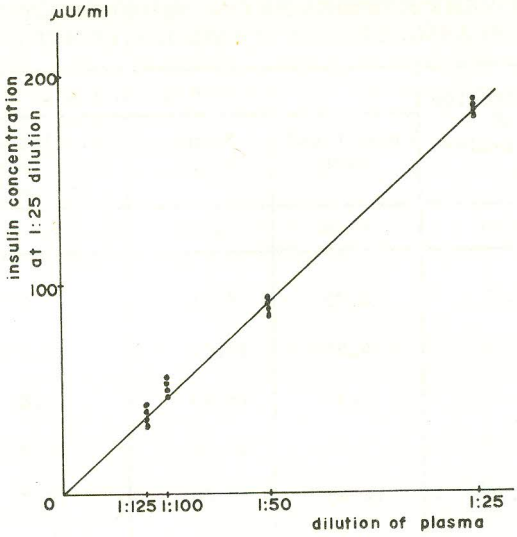

FIG. 6. Specificity: effect of dilution of plasma on apparent insulin concentration.

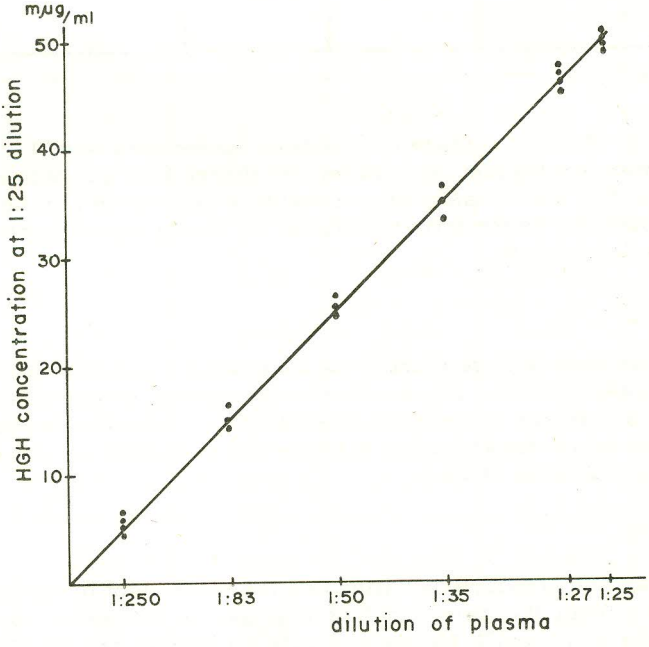

FIG. 7. Specificity: serial dilutions of a pool of plasma with high HGH concentration.

For 20 aliquots (from the same plasma pool as above) measured in different assays, the results were as follows: 160.0 ± 12.2 and 7.6% respectively for insulin and 12.82 ± 1.61 and 6% respectively for GH. Therefore, the precision for insulin assay can be considered as good (CV = 5-10%) both within- and between-assays. On the other hand, HGH assay precision can be accepted as excellent (CV < 5%) for within-assays and good for between-assays.

4. Sensitivity

The sensitivity was calculated to be 0.002 ng for the insulin standard curve (corresponding to 1.25 μU/ml of undiluted plasma) and 0.06 ng for the HGH standard curve (equivalent to 1.5 ng/ml of undiluted plasma).

DISCUSSION

The method of Hunter and Greenwood [3], as modified by Yalow and Berson [2], was used in the iodination procedure. Human plasma was added to minimize damage to the hormone and to minimize adsorption losses. The reagents were added in sequence as quickly as possible.

An important aspect in radioimmunoassay is the labelling of peptide hormones with radioactivity levels which would permit statistically suitable counting yet maintain, at the same time, a low concentration of the tracer hormone.

By increasing the number of substitutions of radioiodine atoms in the hormone molecule we could increase the specific activity of the labelled hormone. However, over-iodination tends to decrease the immunoreactivity of the preparation [12]. Thus, the hormone must be labelled at a specific activity consistent with the maintenance of the immunochemical integrity and stability of the labelled molecule.

A compromise between a counting time required to obtain statistical accuracy and a maximal specific activity without significant alterations (other than the iodine substitution) of the preparation can be attained by labelling insulin at a specific activity between 100 and 300 mCi/mg and HGH between 50 and 100 mCi/mg.

Purification of the labelled hormone is always necessary because of the appearance of certain labelled components distinct from those of the main hormonal fractions, i.e. contaminating proteins, altered hormonal peptides in the unlabelled (hormonal heterogeneity) and labelled (damaged component) material.

The procedures used in our laboratory (starch gel electrophoresis followed by Sephadex chromatography) give the assurance that the assay is started with an almost pure labelled peptide preparation without over-iodination and with practically no damaged components or free iodine contamination. In effect, the final yield from Sephadex columns has always been above 94% for insulin and 90% for HGH for the total radioactivity in the corresponding effluents. With these techniques for purification of the labelled peptides, only a small degree of damage occurs even after a few weeks of storage.

To separate the free from the antibody-bound hormone and damaged components, plasma-coated charcoal was chosen as the specific adsorbent

of the free hormone [13], the damaged components and the antibody-bound hormone having less affinity for the adsorption sites.

As suggested by Palmieri et al. [13] the optimal quantities of adsorbent for a plasma concentration of 1:25 and an incubation volume of 2.5 ml were as follows: 20 mg and 100 mg for insulin and HGH, respectively, suspended in 0.02M veronal buffer. As reported by these authors, this method is as efficient as other separation techniques (dextran-coated charcoal and talc), the other conditions of assay not being affected.

We have checked and found that standard curves obtained by separation of bound and free labelled hormone by means of talc (100 mg for insulin and 200 mg for HGH) or charcoal coated with blood bank plasma gave entirely reproducible curves with either peptide (unpublished data). The reliability of the radioimmunoassay techniques for insulin and HGH in human plasma, as standardized in our laboratory, was evaluated by their accuracy, specificity, precision and sensitivity.

Accuracy, as determined by means of recovery experiments, was examined and shown to be excellent, the correlation coefficient r between the theoretical and found values being 0.98, which is significant at the 1% level both for insulin and for HGH.

The specificity of our assay, as defined previously, satisfied the following criteria, proposed by Yalow and Berson [14]:

(a) The non-specific effect of the incubation medium needs to be controlled. This may be achieved by setting up standards in plasma devoid of hormone (e.g. from hypophysectomized subjects for HGH assay at the same dilution as is used for the unknowns). As can be seen in Fig. 5 there were no significant differences in the results obtained in prepared buffers with those in plasma devoid of hormone. The same can be said for insulin when one uses plasma from fasting subjects which is submitted to charcoal extraction.

Actually, in plasma assayed at a dilution of 1:25 there is no interference from the buffer and salt concentration and these are the main non-specific plasma factors that influence the immunochemical reaction.

(b) The hormone added "in vitro" to unknown plasma should be recovered quantitatively. This criterion was fully satisfied by our recovery experiments.

(c) On dilution of an unknown plasma, the apparent hormone concentration should decrease proportionately with the dilution factor as determined by reference to a standard curve. This criterion was satisfied as the correlation coefficient between plasma dilution and hormonal concentration was 0.998 for both insulin and HGH.

(d) Added hormone and measured hormone should behave in a similar way in a variety of physical and chemical systems. However, this criterion is not completely satisfied because of the presence, in small proportion, of "big insulin" and "big growth hormone" in plasma.

The reproducibility analysis allowed us to evaluate the precision of the methods used in our work and it was shown that for within-assays the precision was good for insulin and excellent for growth hormone. For between-assays the reproducibility of the methods could be considered as good.

Finally, sensitivity, in the way it was evaluated, was found to be 1.25 μU/ml and 1.5 ng/ml of plasma for insulin and growth hormone, respectively, which is adequate for clinical work. However, it should be

pointed out that these assays were carried out with the routine test schedules. If necessary, the sensitivity of the reaction may be considerably increased by reducing the tracer concentration and/or increasing the antiserum dilution.

In conclusion, the statistical evaluation of the characteristics of operational sequence for the assay of insulin and growth hormone established in our laboratory indicated that the methods employed are accurate and specific and have good precision and sensitivity, being adequate for routine and investigational work.

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