AN ACCURATE DETERMINATION OF HUMAN GROWTH HORMONE CONTENT IN DIFFERENT PITUITARY EXTRACTS, USING A RADIOIMMUNOASSAY WITH POLYACRYLAMIDE GEL ELECTROPHORESIS AS A BOUND-FREE SEPARATION SYSTEM

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Paolo Bartoline[2], Licio Marques de Assis, Irene Schwarz
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ABSTRACT

Human growth hormone was extracted and purified according to the method of Roos et al. (Roos, P., Fevold, H. R. and Gemzell, C. A. (1963) Biochim. Biophys. Acta 74, 526). A first control of its purification and integrity was performed through molecular weight determination by gel filtration on Sephadex G-100 and on polyacrylamide gel electrophoresis (PAGE). Its biological activity was confirmed by the growth promoted in non-hypophysectomized rats at plateau.

The main object, however, was the setting up of an accurate, reproducible method that could furnish the more absolute and comparable value of radioimmunoassayable HGH content in perfect agreement with the results obtained by other laboratories. This was accomplished through a radioimmunoassay system that uses HGH labelled with ^{125}I, where the separation of the bound from the free antigen is achieved on polyacrylamide gel electrophoresis, by a modification introduced in the original method of Davis.

The resulting values, extremely close to that stated by the KABI-Laboratories (Stockolm), though obtained in quite different conditions of incubation, antibody concentration and with no use of second antibody, represent a confident approach to a comparable measure of this hormone in extracts, which can also be applied to plasma determinations.

INTRODUCTION

The method used for the extraction and purification from frozen pituitaries[1] was chosen in preference to others because of its great simplicity and mildness, good yield and purification and lower levels of antigenicity produced by this extract during the treatment of pituitary dwarfs[2].

The goal being not only the preparation of HGH for clinical use but also that of kits for radioimmunoassay, an accurate and reproducible technique was needed that would ensure our having the hormone at a degree of purification suitable for both purposes and with a immunoactivity comparable to that presented by other available preparations.

For the labelling with ^{125}I, the method of Greenwood et al.[3] was used and for the separation of the labelled HGH from the reaction mixture the method of Cerasi et al.[4]. These authors stress the difficulty of making a comparison between HGH values obtained in different laboratories, when different standard preparations are used without prior evaluation of their immunoactivity. They question, moreover, the possibility of the absolute measure of this hormone, especially in view of factors of uncertainty introduced with the technique as, for example, the different conditions of incubation, of antibody concentration, presence and type of second antibody.

Holstrom et al[2] confirm the existence of great variations in the HGH dosage by laboratories employing different techniques, even when the same standard is used.

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(1) Centro de Aplicações Biomédicas de Radioisótopos e de Radiações — Área de Aplicações Médicas de Radioisótopos e de Radiações — Instituto de Energia Atômica. Cidade Universitária, São Paulo, SP.
(2) To whom correspondence should be addressed.

The present method, chosing as a reference standard the widely accepted WHO 1st IRP 66/217 for immunoassay[5], was able to provide results that were comparable with those obtained by other sources that had used the same standard preparation.

This system was selected after having experimented with different techniques of radioimmunoassay, especially as far as the various bound-free separation systems were concerned. Paper or cellulose acetate electrophoresis, chromatoelectrophoresis[6-9], precipitation through adsorption charcoal[10] and separation by gel filtration on Sephadex G-50 or G-75[6,11], did not give satisfactory results for our purpose, especially because of an incomplete bound-free separation, adsorption phenomena or interference of the "damaged" antigen with the formed complex.

Good results were obtained, however, applying the technique of polyacrylamide gel electrophoresis (PAGE), that was adapted to our particular needs starting from the method of Davis[1,2].

The use of PAGE, defined as the closest method to a general physical system in the separation of the bound from the free antigen, was mentioned by Hunter[6], even though not applied directly to a radioimmunoassay technique. Chalkley and Tanner[13] have applied a similar system to plasma antibodies determination only, where no real incubation takes place, and the reaction antigen-antibody occurs into two separate gel layers.

The present paper shows the application of a generally standardized electrophoretic technique to the obtaining of accurate and reproducible radioimmunological HGH determinations, and provides a useful tool for further studies on the electrophoretical behaviour of the complex antigen-antibody and of the "damaged" labelled antigen, showing the possibility of a complete separation between the two.

A publication presenting the application of this same technique to the detection and measurement of antibodies in patients treated with different HGH extracts has been already submitted.

MATERIALS

Human Pituitaries. These were obtained thanks to Professor Tales de Brito of the Department of Pathology, Faculty of Medicine (University of São Paulo).

Human growth hormone (HGH). NIH-GH-HS 2002 F, kindly furnished by Dr. A. E. Wilhelmi, was used for the labelling. HGH 1st IRP 66/127 for immunoassay was sent from the World Health Organization (WHO), and it was used for the standard curve in our radioimmunoassay. HGH-KABI, kindly provided by Astra Quimica do Brasil, was used to control the accuracy of our method and the agreement of our results with those obtained from a second laboratory. One ampoule of this preparation contains a declared activity of 4 I.U., determined by radioimmunoassay and using the same IRP 66/127 (WHO) as a standard.

Bovine growth hormone, for bioassay, was also sent by WHO. By definition 1 mg of this bovine pituitary extract is one international unit (1st Int. Std. established in 1955). The bioassay was performed according to Marx et al.[14], using normal female rats (whistar) at plateau of about six months of age, weighing between 230 and 250 g.

^{125}I, free of carriers and reductors, at high specific activity (up to 500 mCi/ml) was bought from New England Nuclear (Boston, Mass.).

Sephadex G-100 and G-75 (Pharmacia, Uppsala) were used respectivily in the purification of the pituitary extracts, in the molecular weight determination and in the separation of the labelled hormone from the labelling misture.

Guinea-pig antiserum against human growth hormone, already at a dilution of 1 : 2000 is distributed free by the National Institute of Health (NIH), Bethesda (Md.) with an antibody potenty as to allow its use up to 1 : 2 x 10^6; 1 : 3 x 10^6.

PAGE Technique for the Separation of the Bound from the Free Antigen

The method of Davis[12] was applied, introducing the following general modifications:

The separation gel was polymerized using Davis solution C (which produces a final concentration in acrylamide of 7%) with Riboflavin (sol. E) as a catalyst, instead of ammonium persulfate.

The sample from the incubation reaction was polymerized on this first gel at an acrylamide content of 2.5% using two new stock solutions, which, according to Davis nomenclature are composed as indicated in the table.

Stock solution G		Stock solution H		Sample gel polymerization mixture.	
Riboflavin	0.2 mg	Acrylamide	5.0 g	Stock solution G	75 µl
Saccharose	8.0 mg	Bis	1.25 g	Guinea-pig serum	5 µl
Davis Stock Solution B	5.0 ml	Water to	10.0 ml	Bromophenol blue (0.1 mg/ml)	5 µl
Water to	10.0 ml			Sample	200 µl
				Stock solution H	15 µl
				Total volume	300 µl

A layer of water is placed on top of this sample gel solution, as is usual, and the tube stand is placed directly under a daylight fluorescent bulb. The polymerization occurs in about 30 min.

No stacking gel is needed. After several experiments with the use of this third gel (2% in acrylamide) between the sample and the separation gel, we reached the conclusion that it affected neither the bound-free separation nor the band width.

A previous control of the ^{125}I activity enclosed in each sample gel can be performed before the electroforesis, counting in a well type γ-counter with the gel tube upside down. The run is performed in a Tris-glycine buffer at 2 mA per tube until the sample (blue band) has completely entered the separation gel. The amperage can be increased to 3 mA/tube after that. The voltage should never exceed 200 V because there is some indication that higher potential differences might interfere with the stability of the complex formed. Gels must therefore be used on the same day in which polymerization occurs. Finally they are cut into segments 0.5 or 0.7 cm. long and counted for their γ-activity in a well type counter.

Figure 1a shows the complete gel rod with its relative dimensions. In Figure 1b an alternative system is shown that can be of interest for more routine applications. The glass tube is made up of two separate halves, fastened together with a parafilm or any other type of transparent fixing band. After the run, the rod can be easily cut in the middle and the two separate parts counted without necessarily removing the gels from the glass tubes. This second method of counting proved to give satisfactory results, especially using a sample gel polimerized with 5% acrylamide. In fact under these conditions the antigen-antibody complex hardly migrated out of the sample gel, thereby allowing a practical count of the first band exactly geometrical to that of the free hormone, which migrates close behind the tracking dye.

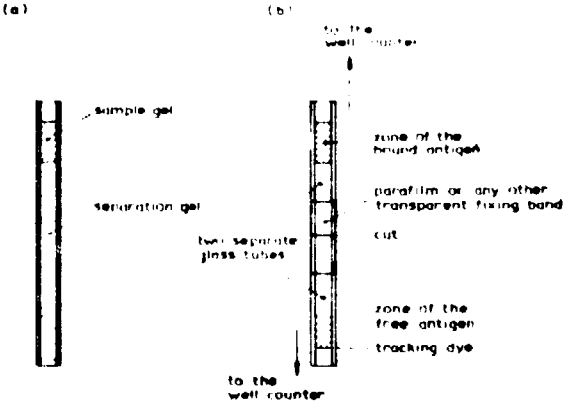

Figure 1 – The gel rod (a). For more routine applications the system (b) can be used.

RESULTS

a) Extraction and Purification of HGH from Pituitaries

According to the method of Roos et al.[1] a Sephadex G-100 refrigerated column, size 5 x 90 cm, at a flow rate of 43 ml per hour, was used for the purification of 1.59 g of a crude extract obtained from about 100 g of starting material (200 hypophysis).

In Figure 2 the protein profile after passage on Sephadex is plotted together with the total immunoactivity of each of the ten collected fractions. In peak III, corresponding to the HGH fraction, a yield of 386 mg is obtained, which is very close to that presented by Roos. In Table I the specific activities are shown in international units per milligram, determined by radioimmunoassay on the lyophilized poweder. As one can see, fraction IIIc has a level of purification good enough to enable its use for the labelling and as standard of reference. Peak IV, not mentioned by Roos, must represent very small fragments of molecules, since before the dialysis its absorbance at 280 nm was about three times higher. Peak I presented an atypical hormonal activity that might be due to polymeric forms.

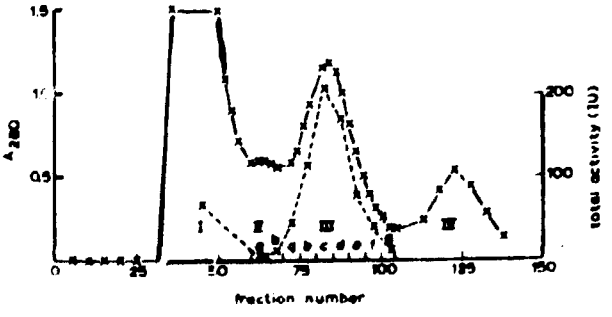

Figure 2 - Gel filtration of human pituitary extract on Sephadex G-100, 5 x 90 cm; flow rate 43 ml/h; fraction size, 15 ml; 1.6 g sample applied in 21 ml. ——, absorbance at 280 nm; - - - - - -, HGH activity determined by RIA using WHO 1st IRP 66/217 as a standard.

Table I

Immunoactivity of the Fractions Separated on Sephadex G-100

Peak	Fraction	Weight (mg of lyoph.)	Spec. activity (I.U./mg)	Total activity (I.U.)
I		292.0	0.14	40.88
II	a	36.8	0.08	2.94
	b	30.0	0.28	8.40
III	a	36.2	1.20	43.44
	b	62.3	1.84	114.63
	c	93.2	2.24	208.77
	d	75.3	2.24	168.67
	e	55.2	1.36	75.07
	f	32.7	1.16	37.93
	g	19.1	0.68	12.99
Peak II + Peak III		440.8	1.53*	672.84

* This value is obtained from the total activity of the pool, calculated on this same table The corresponding experimental value (1.59 I.U./mg) is reported in Table III.

b) Molecular Weight Determination

To confirm that peak III corresponded to the purest HGH fraction and that the hormone was present in a monomeric non-degraded form, two different estimations of molecular weight were performed.

The first, by means of gel filtration on Sephadex G-100[15], produced a value of 22 000 and the second, on polyacrylamide gel electrophoresis[16,17] about 21 500. These values are in agreement with those presented by different authors for HGH[17-19].

The standard proteins used in both cases to mabe the reference curve were: bovine serum albumin, ovalbumin, myoglobin, cytochrome, insulin and glucagon. Bovine growth hormone (WHO) was also used as a reference.

c) Labelling

In the labelling procedure the method of Greenwood et al.[3] was followed, using 5 μg of HGH-NIH and about 1 mCi of ^{125}I free of carriers and reductors, in a total volume of 75 μl, sodium metabisulfite included. The Chloramine-T reaction was conducted under continuous magnetic stirring.

After purification on Sephadex G-75[4], the radioactivity distribution was that shown in Figure 3, which relates to one of our best labelling reactions. The yield of labelled protein was calculated at a value of 86%. The damaged hormone corresponded to only 12.3% of the total ^{125}I eluted from the column, and the free iodide represented only 13.5%.

After more than a month of using the labelled HGH, the amount of damaged antigen reached proportions of about 15 – 20%. This started to affect the results of the radioimmunoassay technique. In view of this, a second purification was done on the same type of column, under the same conditions,

which produced a newly purified [^{125}I]HGH with no detectable loss in binding capacity in comparison with that immediately following the first purification. It had, of course, a lower specific radioactivity, due to the decay time, and a higher dilution in terms of cpm/μl. This last factor did not influence our incubation technique in any way whatsoever, since the initial dilution range needed was very high (about 1 : 100 on the central fraction eluted from Sephadex), and the only modification one had to introduce to maintain the same counts per incubate was to use a lower dilution.

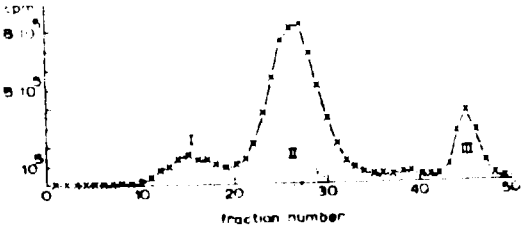

Figure 3 – Separation of [^{125}I]HGH from the labelling mixture on Sephadex G-75. Column size, 2 x 32 cm; flow rate, 12 ml/h: fraction volume, 2 ml. I, damaged HGH; II, undamaged HGH; III, free iodide.

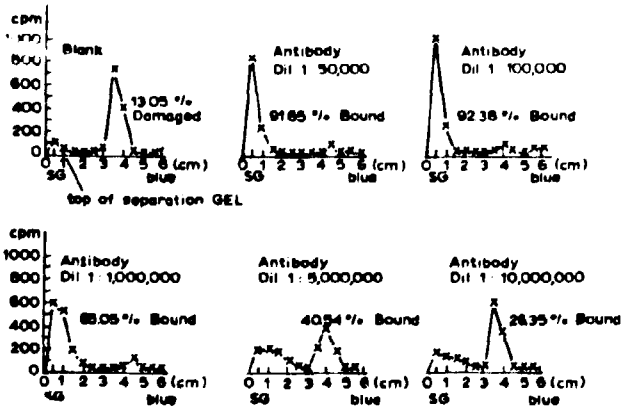

Figure 4 – Separation of bound from free antigen on polyacrylamide gel electrophoresis. Sample gel (SG), 5% acrylamide; separation gel, 5% acrylamide; gel slices, 0.5 cm long.

It is very interesting to note that, after the second purification, practically no more "damaged" antigen originated, remaining constant aroud the value of 5 – 8%, which allows very reproducible assays.

The conclusion we reached was that after a good labelling reaction the same [^{125}I]HGH can be used for a period of at least three or four months by simply modifying the amount of labelled antigen and consequently the optimal antibody dilution.

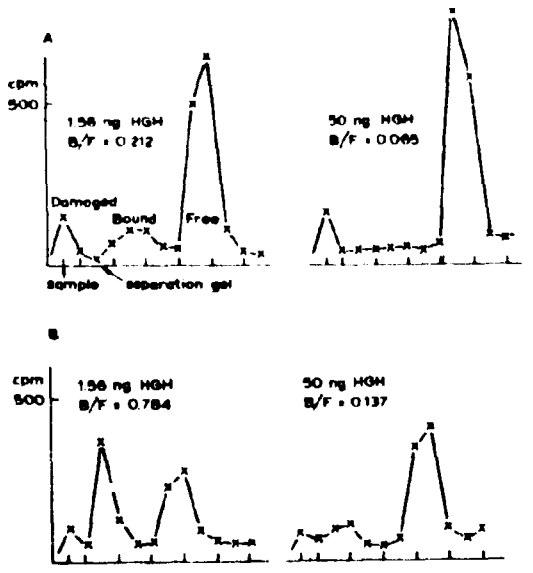

Figure 4A – Complete separation on the damaged from the bound antigen on a RIA with a very low, antibody-antigen ratio. Figure 4B. Incomplete separation of the damaged antigen in a regular radioimmunoassay.

d) Bound-free Separation on PAGE and Choise of the Optimal Antibody Dilution

For this separation initially a separation gel at 5% acrylamide was used. As may be seen from Figure 4, under such conditions the damaged antigen remains in the sample gel, under the antigen-antibody complex. This complex, however, progressively enters the gel according to the decreasing antibody concentration, possibly showing the formation of different types of aggregates. Using a 2% sample gel and a 7% separation gel, as was subsequently our practice, it is possible to separate almost completely the damaged antigen from the complex which, due to the increased concentration of the separation gel, remains mainly in the first segment (Figure 4A and B).

In either case, however, the method allows a good resolution between bound and free antigen and consequently permits the calculation of the percentage of bound to total, at different antibody concentrations, so that in Figure 5 one can easily determine the dilution which is the most suitable for the incubation in radioimmunoassay. These incubations, for the purpose described above, and for the radioimmunoassay itself, have always been carried out according to the following procedure:

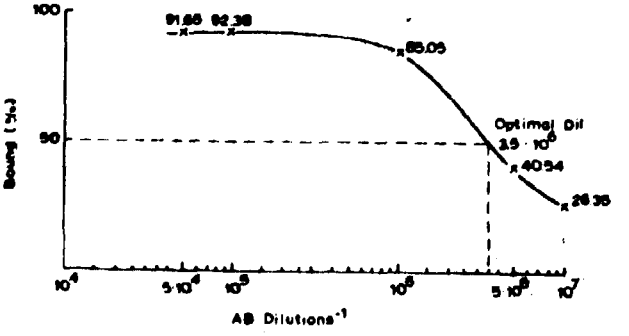

Figure 5 – Determination of the optimal antibody dilution for incubation in RIA.

Antibody, diluted up to . x 10^6	900 µl
[^{125}I] HGH, diluted to obtain approx. 10^4 cpm	50 µl
HGH std. or unknown, in radioimmunoassay	
or	
buffer veronal, in antibody titration	50 µl
Total volume	1000 µl

Only 200 µl of this incubate were used in each determination on PAGE. The buffer used in all dilutions is Veronal 0.025 M, pH 8.4, with 0.25% bovine serum albumin and 1% guinea-pig serum.

Incubations were conducted at 4°C for 3 or 5 days. Even though a considerable increase in binding was observed between the 3rd and the 5th day, both periods of time gave good results in our assays.

This type of incubation, as can be seen, allows five different electrophoretic tests that can be performed at various incubation times. Of course the corresponding sensitivity, calculated around 0.5 ng/ml (25 absolute pg.), can be increased four times using a total incubation volume of 250 µl and a more concentrated antibody solution.

e) Determination of the Resulting Assay Sensitivity and Detection Range

Once the optimal antibody dilution for a certain detectable amount of labelled antigen has been established it is advisable to keep this relationship constant, before actually running the assay, to determine the minimum amount of unlabelled antigen necessary to produce a good fall in B/F. This is accomplished by incubating different concentrations of HGH standard (WHO) with the previously calculated fixed proportions of antibody and [^{125}I] HGH. The amount that gives a fall in B/F of about 10%[6] is chosen as the first detectable dose (sensitivity), and the upper limit for the detection range covered by the curve will be at a value of approximately 15 – 20 times.

It must be remembered however that, while a very high sensitivity is not required for analyzing purified extracts, in the case of serum HGH determinations this sensitivity must be at least 1 – 2 ng/ml. Hence the curves shown in Figures 6 and 7, though used for analysis of extracts, demonstrate a sensitivity and cover a range useful for serum determinations too.

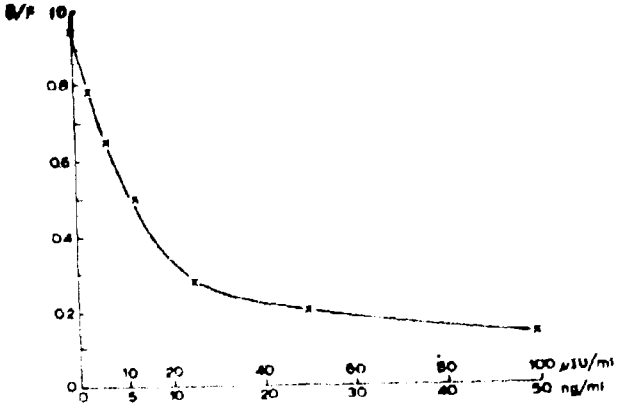

Figure 6 – Standard curve for RIA on PAGE, using HGH-WHO 1st IRP 66/217.

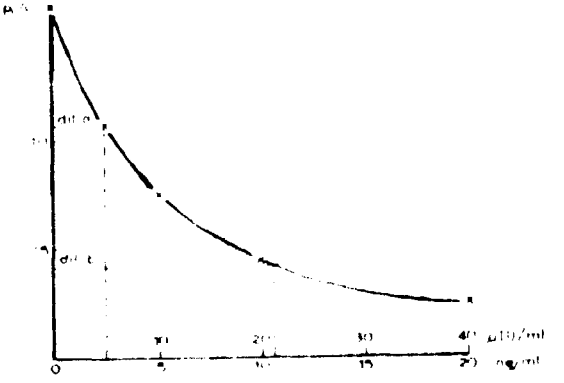

Figure 7 Example of radioimmunological determination on two different dilutions of KABI-CRESCORMON. For the standard curve, WHO-HGH 1st IRP 66/217 for immunoassay has been used. Dilution a: stated content, 5 μ I.U./ml (2.5 ng/ml); found content, 5 μ I.U./ml. Dilution b: stated content 20 μ I.U./ml (10 ng/ml); found content, 21.2 μ I.U./ml.

f) Radioimmunoassay

Using HGH-WHO 1st IRP for immunoassay in a range between 1.5 and 50 ng/ml, the curves in Figures 6 and 7 were obtained. These show the applicability of the method and the most useful zone of the curve for our determinations. Later we used only the zone between 2 and 20 ng/ml, which presented the best accuracy.

Table II sets out the various determinations, carried out with the present method on KABI-Crescormon at different dilutions. An ampoule of this hormone, prepared for clinical use, has a stated activity of 4 international units and a declared content of about 2 mg of purified extract. The total content of the ampoule was dissolved completely, frozen in separate fractions, and each new dilution was prepared starting from one of these fractions. In the various determinations performed, the incubation time was not always the same, antibody dilution also varied according to the different concentrations of labelled antigen used, and the latter was derived from the same labelling reaction, lasting up to four months and more. Experiments 1, 2 and 3 were done with the original [^{125}I] HGH. Experiment 4 with the same labelled antigen after re-purification. In experiment 4 was also used a second ampoule of HGH-WHO 62/217 for the standard curve.

In Table III are presented the value obtained applying the same method to the evaluation of the immunoactivity of HGH-IEA (*Instituto de Energia Atômica*) and of HGH-NIH that was used for the labelling. As far as the first one is concerned, it can be seen that the value is considerably lower than that presented in Table I and corresponds to the central fraction of peak III (Figure 2). This is due to the fact that these last determinations were conducted on the pool of the whole peak III and II, including some fractions relatively poor in HGH. A good agreement, though, is found between the calculated and the experimental immunactivity of the pool, the first being 1.53 I.U./mg and the second 1.59 I.U./mg (Tables I and III).

HGH-NIH seems to have a definite higher activity than HGH-WHO 1st IRP 66/127 prepared in 1968 by the same laboratory (Dr. A. E. Wilhelmi). This explains why curves of reference, that were prepared here and in other laboratories using HGH-NIH as a standard, gave constantly lower values in serum HGH determinations.

Table II

Determination on KABI-CRESCORMON

Experiment Nº	Stated content* (ng)	Found* content (ng)	I.U./mg
1	2.5	2.50	2.00
	10.0	10.60	2.12
2	5.0	5.30	2.12
3	5.0	5.60	2.24
4	2.0	2.40	2.40
	4.0	4.35	2.17
	8.0	8.40	2.10
			Average 2.164
			S.D. ± 0.1267
			S.E. ± 0.0479
		Fiducial Limits (p = 0.95)	2.047 – 2.281

* These values were calculated according to the various dilutions and considering the content of 4 I.U./ampoule (about 2 I.U./mg HGH) declared by KABI.

Table III

Immunoactivity of IEA (Pool) and NIH-HGH

HGH preparation	Specific activity (I.U./mg)	Average (I.U./mg)
IEA		
Pool Peak II + Peak III	1.76	
	1.30	
	1.84	1.59
	1.40	
	1.66	
NIH		
GH-HS 2002 F	2.99	
	2.49	2.70
	2.62	

g) Bioassay

The result obtained on the pooled peak III by immunoassay, was also confirmed by the biological assay. It should be noted that this assay was not performed for purpose of quantification but to confirm that our extract had a biological activity as well as an immunoactivity. In fact we undertook our assay only once, using six groups of rats, each including five individuals. Two control groups were injected with saline, one group was treated with 0.5 mg/day of bovine growth hormone (Intern. Std. WHO) and the other three groups with three different daily doses (0.25; 0.5; 1.0 mg) of IEA growth hormone (Figure 8).

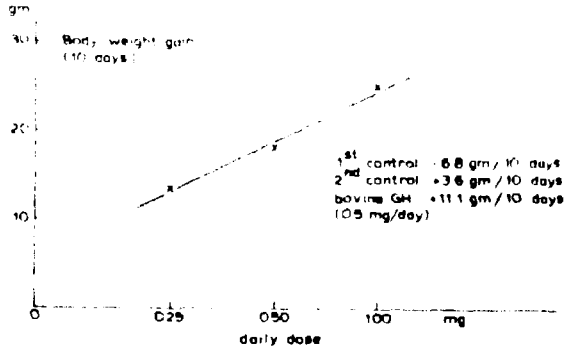

Figure 8 – Response of normal rats at plateau to different daily doses of IEA HGH preparation. Period of administration: 10 days.

The plot shows an evident relationship between dose and response, since the weight gain presents a linear increase in accordance with the daily hormone administration. The two control groups showed no weight gain or even negative values, whereas the group treated with bovine growth hormone presented a weight gain which, compared with the average growth of the three groups treated with HGH-IEA, determined for the latter a biological activity of 1.71 I.U./mg. Of course, given the low reproducibility usually offered by this kind of assay, more tests should be done in order to obtain a certain degree of confidence in this value.

DISCUSSION

Using the PAGE technique for the separation of the bound from the free antigen, the findings presented by the AB-KABI Research Dept.[2] regarding the immunoactivity of HGH extracted according to Roos et al.[1] are confirmed. Moreover, a very good agreement is found with the stated content of commercial Crescormon for clinical use. This demonstrates that agreement is possible between different laboratories using a different assay technique, provided the standard of reference is the same.

A global value of 1.59 I.U./mg is found for the pooled HGH (peak II + peak III, Figure 2), and a maximum of 2.24 I.U./mg is achieved in the central fraction of peak III (Table I). This allows the use of the pooled hormone for clinical purposes, and of the central fraction for the labelling and as a standard in the production of kits for radioimmunoassay.

Also interesting is the immunoactivity presented by NIH ultrapurified Growth Hormone, which might indicate a considerably higher level of purification, compared with the WHO-HGH 1st IRP 66/217, which, as has been said, was prepared in the same laboratory. Dr. Wilhelmi himself admitted this possibility (Wilhelmi, A. E. (1976) personal communication) and it is therefore advisable, in this kind of assay, to use always the same reference preparation if comparable values are required.

The relative agreement found in our hormone between bioactivity and immunoactivity needs further confirmation, but the use of non-hypophysectomized rats at plateau might really give greater accuracy than that of hypophysectomized young rats, as is mentioned in the original work[14].

Also confirmed is the lower or practically non-existant antigenicity produced during the treatment with HGH extracted through this method. Regarding this assay, it is our opinion that the PAGE technique represents a better tool, for the detection of antibodies in human serum than the traditionally used paper chromatoelectrophoresis or electrophoresis[20]. It also permits a higher number of assays to be run simultaneously, which is very useful, especially in the determination of antibody titre and binding capacity.

Concerning the radioimmunoassay itself, this technique proved to be most suited to our purpose, and indicated interesting possibilities for a further study on the nature and behaviour of the damaged antigen, for its complete separation from the formed complex on the gel itself and also on the formation and behaviour of the various antigen-antibody structures originating at different antibody concentrations (work to be published).

For routine purposes, especially when dealing with hundreds of serum samples, this technique might be considered too laborious and impractical, although we believe it to be of reasonable applicability. Considering its good accuracy, sensitivity, reproducibility and the possibility of repeating five tests at five different times from the same incubate, even a laboratory doing clinical work might find it useful. We found it without doubt the best technique to be used for research purposes. One must remember, in fact, the considerable advantage of not using a second antibody which, as has been said[4], can alter the kinetics of the immunological reaction between the first antibody and the antigen, thus introducting a factor of uncertainty in the assay itself. In this way it is therefore possible to determine directly the two values corresponding to the bound and free antigen which, knowing the total amount of radioactivity put on the gel, offers the possibility of a double check of the B/F correctness.

Regarding the problem of simultaneously running of larger number of samples, it might be interesting to consider the use of gel slabs, where one can run up to 40 samples together, or of a better adapted routine application of particularly large electrophoretic cells, using the system described in Figure 1b. Use of the latter gave results, on the bound and free determination, very close to those determined by extracting and slicing the rods of gel.

Finally, interesting are also the results obtained on the labelling with the adopted technique of purification and re-purification, which produced a [^{125}I] - HGH that could be used up to four months in our radioimmunoassay technique, still presenting a very limited amount of damaged antigen.

RESUMO

Hormônio de crescimento humano foi extraído e purificado de acordo com o método de Roos e cols. Realizou-se um primeiro controle de sua purificação e integridade através da determinação do peso molecular pelo gel eletroforese em Sephadex G-100 e gel eletroforese em poliacrilamida. Sua atividade biológica foi confirmada pelo crescimento promovido em ratos não hipofisectomizados em platô. O principal objetivo, entretanto, foi estabelecer um método preciso e reprodutível que pudesse fornecer os valores mais "absolutos" e comparáveis do conteúdo imunoensaiável de HGH em perfeita concordância com os resultados obtidos por outros laboratórios. Isto foi conseguido através de um sistema de radio-imunoensaio que usa o HGH marcado com ^{125}I e onde a separação entre livre e complexado é alcançada pela gel eletroforese em poliacrilamida, conforme método de Davis modificado. Os valores resultantes, extremamente próximos daqueles estabelecidos pelos laboratórios Kabi (Stockolm), apesar de obtidos em condições completamente diferentes de incubação, concentração de anticorpos e sem emprego do segundo anticorpo, representam uma aproximação fiel para uma medida comparável deste hormônio em extratos e que também pode ser estendida para determinação no plasma.

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