BR8919413

ISSN 0101-3084

IPEN-PUB--179

CNEN/SP

ipen Instituto de Pesquisas Energéticas e Nucleares

PITUITARY HUMAN GROWTH HORMONE: SMALL-SCALE PURIFICATION METHOD AND APPLICATION TO RADIOIMMUNOASSAY

Paolo Bartolini, Maria Teresa de Carvalho Pinto Ribela, Irene Schwarz and
Adir Janete Godoy dos Santos.

PUBLICAÇÃO IPEN 179 AGOSTO/1988

SÃO PAULO

PUBLICAÇÃO IPEN 179 AGOSTO/1988

PITUITARY HUMAN GROWTH HORMONE: SMALL-SCALE PURIFICATION METHOD AND APPLICATION TO RADIOIMMUNOASSAY

Paolo Bertolini, Maria Teresa de Carvalho Pinto Ribela, Irene Schwarz and
Adir Janete Godoy dos Santos

DEPARTAMENTO DE APLICAÇÃO EM CIÊNCIAS BIOLÓGICAS

CNEN/SP
INSTITUTO DE PESQUISAS ENRGÉTICAS E NUCLEARES
SÃO PAULO – BRASIL

Série PUBLICAÇÃO IPEN

INIS Categories and Descriptors

C45.00

STH
PURIFICATION
RADIOIMMUNOASSAY
IODINE 125

IPEN - Doc - 3047

Aprovado para publicação em 18/07/88.

Nota: A redação, ortografia, conceitos e revisão final são de responsabilidade do(s) autor(es).

PITUITARY HUMAN GROWTH HORMONE: SMALL-SCALE PURIFICATION METHOD AND APPLICATION TO RADIOIMMUNOASSAY

Paolo Bartolini, Maria Teresa de Carvalho Pinto Ribela, Irene Schwarz, Adir Janete Godoy dos Santos.

ABSTRACT

Human growth hormone (hGH) extracted from pituitary glands is often heterogeneous, presenting, besides the native isohormone-B (IH-B), up to four different isohormones (charge isomers). To obtain more homogeneous preparations, essential for reproducible radioligand assay results, the original method of Roos et al (Biochim. Biophys. Acta 74: 525, 63) was modified, so it could be done faster, on a small scale, and by adding bacteriostatic and enzyme inhibiting agents to all buffers (NaN_3, EDTA, Trasylol) in order to minimize isohormone formation. After 3 to 4 homogenizations and extractions of the frozen glands (10-20), hGH is precipitated by 50% ammonium sulphate immediatly purified by Sephadex G-100 molecular sieve chromatography, and hGH fractions are lyophilized. The whole process is completed in one week. Using 20 hypophyses, we obtained 1,3 mg hGH/gland; polyacrylamide gel electrophoresis analysed by UV scanning, revealed the predominance of IH-B with a small IH-C2 peak. Several labellings with 125-I were performed using this purified hGH stored for more than one year. Two labelling techniques were employed: the classical method of W. M. Hunter wich uses 50 µg of Chloramine T and the stoichiometric iodination described by Roth, which uses limintig amounts of Chloramine T; we emphasize that for this preparation only 1.5 µg Chloramine T were necessary to achieve the desired specific activity. All ^{125}I - hGH preparations presented good immunoreactivity: in a working antiserum dilution of 1:200.000, the binding ranged from 40 to 62%. Comparison of our ^{125}I - hGH preparations with commercial ^{125}I - hGH (Radioassay System), tested in the same RIA system, showed comparable results for non specific binding (NSB), maximum binding (B_0), Effective 50% Dose (ED_{50}) and serum control samples. The small-scale purification method proved to yield homogeneous hGH preparation, perfectly suitable for RIA purposes. It's major advantage is that is can be rapidly performed and, if necessary, repeated to attain desired hGH homogeneity. We thank for the support received from IAEA (4299/RB), FINEP Brazil (43.86.0351.00) and CNEN (Brazil).

HORMÔNIO DE CRESCIMENTO HUMANO HIPOFISÁRIO: MINIEXTRAÇÃO E APLICAÇÃO AO RA - DIOIMUNOENSAIO

RESUMO

O hormônio de crescimento humano (hGH) extraído da hipófise é frequentemente heterogêneo, apresentando além de forma nativa (isohormônio B-IH-B) 4 ou mais diferentes isohormônios (isômeros de carga). Para obter preparações mais homogêneas, o que é essencial para resultados reprodutivos de ensaios radioligantes, o método original de Roos et al (Biochim. Biophys Acta 74: 525, 63) foi modificado tal que pudesse ser feito mais rapidamente, com menos hipófises e adicionando-se agentes bacteriostaticos e inibidores enzimáticos a todos os tampões (NaN_3, EDTA, Trasylol) a fim de minimizar a formação de isohormônio. Após 3 a 4 homogeneizações e extrações das glândulas congeladas (10 a 20) o hGH é precipitado por sulfato de amônia 50% e imediatamente purificado por cromatografia de exclusão molecular em Sephadex G-100 e as frações de hGH são liofilizadas. O processo inteiro é completado em uma semana. Usando 20 hipófises nós obtivemos um rendimento de 1,3 mg hGH/glândula; a eletroforese em gel de poliacrilamida, seguida de leitura UV revelou a predominância de isohormônio B com um pequeno pico de IH C_2. Várias marcações com ^{125}I foram realizadas usando o produto obtido conforme descrito e estocado por mais de um ano. Duas técnicas de marcação foram empregadas: o método clássico de W.M.Hunter que usa 50 µg de cloramina T e a iodação estequiométrica descrita por Roth que usa quantidades limitadas de Cloramina T; nós enfatizamos que para essa preparação apenas 1,5 µg de Cloramina T foi necessária para atingir a atividade específica desejada. Todas as preparações marcadas (^{125}I-hGH) apresentaram boa imunorreatividade: na diluição de trabalho do antisoro de 1:200.000, a ligação variou de 40 a 62%. A comparação do nosso produto marcado com produtos marcados comerciais (Radioassay System) testados no mesmo sistema de RIA, mostrou resultados comparáveis para ligação não específica(NSB), ligação máxima (Bo), dose correspondente a 50% de ligação (ED_{50}) e amostra de soros controle. A miniextração proposta provou fornecer preparações de hGH homogêneas perfeitamente adequadas para fins de RIA. A maior vantagem é que ela pode ser rápidamente realizada e se necessário repetida para obter a homogeneidade de hGH desejada.

INTRODUCTION

As it has been described (1) human growth hormone (hGH) extracted from pituitary glands is heterogenous, presenting, besides the native form (isohormone-B) several different isohormones (mainly charge isomers),all of them biologically active (2-3). How ever, the possibility that some of these forms are due to enzymatic cleavage and/or artefacts occurring during extraction, has not been completely eliminated yet.

For these reasons, to obtain more homogeneous preparations which is essential for reproducible radioligand assays, the original extraction method of Roos et al (4) was modified, so to be able to speed up the whole purification process, starting from a small number of pituitaries (5-20 units at the most). This way, also adding bacteriostatic and enzyme inhibitor agents to all buffers, we could always start from fresh material and possibly decrease the alterations which can occur when the material is kept in solution for several weeks.

MATERIALS AND METHODS

Sephadex G-100 was obtained from Pharmacia (Uppsala, Sweden) Acrylamide and Bisacrylamide were from INLAB (São Paulo - Brazil) Na ^{125}I free of carriers and reductants was purchased from New England Nuclear (Boston - USA). Reference preparations of hGH and anti-hGH rabbit antiserum were kindly provided by NIDDK (Baltimore - USA).

Sodium azide and EDTA were from Merck (São Paulo-Brazil) Trasylol from Bayer (São Paulo - Brazil) and PEG - 6000 from Atlas (São Paulo - Brazil).

The conventional labelling reaction was carried out, as described in previous work (5) and so was the determination of antibody titre (6) and the radioimmunoassay technique (7).

Gamma counting was done in a 200 sample Beckman Gamma 4000 while ultraviolet densitometric reading of polyacrylamide gels was carried out in a Joyce Loebl densitometer, model Scan 400 (London, UK).

RESULTS

In Fig. 1 we can see an example of hGH isohormone distribution pattern, in stained polyacrylamide gel electrophoresis (PAGE) gels. NIDDK preparation is the purest one while KABI - Crescormon and hGH - IPEN present at least four isomers. In Fig. 2 a comparison betweem NIDDK preparation

(hGH-I-1; AFP 4793 B) and a hGH - IPEN preparation of completely different isohormone distribution is shown, this time after UV scanning densitometry. It is interesting to observe that the IPEN preparation, composed of at least five isomers, was even found with a higher bioactivity (2).

In Fig. 3 we present in a resumed flow chart, the main steps of the small-scale hGH extraction. It is possible to observe the addition of EDTA, sodium azide and trasylol to the buffer and the reduced duration of the purification process: approximately four days. The yield was of ~1.3 mg hGH per hypophisis.

In Fig. 4 one can see the densitometric profile of hGH obtained after the described mini-extraction: at least three isohormones are still present but the native form (isohormone-B) is clearly predominant.

This same hGH preparation was radioiodinated through the conventional and the stoichiometric method presented in Fig. 5. The latter permits the use of much smaller amounts of Chloramine T (down to 1.5 µg instead of 50 µg), metabisulfite elimination and a better prediction and control of iodine incorporation. In Fig 6 and Fig. 7 the Sephadex - G 100 chromatograms relative to a conventional and a stoichiometric labelling are also shown.

The obtained ^{125}I - hGH was tested in antibody titration curves with anti-hGH antiserum from IPEN and NIDDK, determining with 1:200,000 dilution of our antibody specific bindings of the order of 40 to 62%.

In Fig. 8 the radioimmunoassay curve obtained with ^{125}I-hGH extracted and radioiodinated in our laboratory, is compared to an analogous preparation imported from Radioassay System Laboratories (Carson, CA, USA). We cas observe that the two curves are practically superimposable, their median effective doses (ED_{50}) presenting very close values. In Table I the same preparations (IPEN and Radioassay) are analyzed further, considering also other parameters: specific and non-specific binding, high, medium and low quality control sera and precision limits (ED_{20} and ED_{80}).

DISCUSSION

The small-scale hGH extraction has proved to be efficient for the obtainement of a relatively homogeneous preparation via a cheap process requiring only one week.

Through a fast UV scanning densitometry of PAGE samples the quality of the obtained preparation can be confirmed in a nondestructive way. This same technique can provide a further purification step, as described in an

accompanying paper.

hGH so obtained was labelled via the conventional or stoichiometric technique, the latter having provided, for this particular hormone, better control of iodine incorporation, higher yields with obviously, much lower oxidizer concentration and elimination of the reductant.

^{125}I - hGH prepared this way was perfectly comparable to an expensive imported product, when used in the setting up of radioimmunoassay curves. The same unlabelled hGH can also be used for the preparation of secondary standards and of specific antisera.

Table 1 - Inter Laboratorial Quality Control of Human Growth Hormone (hGH) Extracted and Radio iodinated at the IPEN-CNEN (S.Paulo)

Tracer	Labelling Method	Mon-Specific Binding (%)	Specific Binding (%)	ED_{50} (ng/ml)	Precision Limits 80-20% Binfing	Specific (ng/ml)	Quality Control Sera (ng/ml) low (0.5-0.9)	medium (10.5-16.9)	high (25.37)
Radioassay		5	27	5.2	2.05 -	13.5			
IPEN 1	Chloramine T (classical)	5	31	5.0	1.85 -	15.0			
IPEN 1	Chloramine T (classical)	5	30	6.5	1.90 -	16.0			
Radioassay		8	24	5.8	1.45 -	18.5	0.9	14.2	38.7
IPEN 2	Chloramine T (classical)	6	28	6.2	1.20 -	25.0	0.5	15.3	50
Radioassay		10	32	6.2	1.70 -	21.5	0.5	13.0	50
Radioassay		6	32	6.2			0.5	17.6	52.4
IPEN 3	Chloramine T (stoichiometric)	7	45	5.0	1.00 -	14.0	0.9	21.0	50
IPEN 3	Chloramine T (stoichiometric)	6	39				0.5	13.3	50
Radioassay		9	37	7.5	1.80 -	23.5	0.8	14.7	57.5
IPEN 4	Chloramine T (stoichiometric)	5	40	7.2	2.25 -	19.0	0.9	18.5	50
IPEN 4	Chloramine T (stoichiometric)	5	29	7.3	1.85 -	21.5	0.8	16.0	57.5

Legends

Fig. 1 Polyacrylamide gel electrophoresis on different hGH preparation . From left to right: hGH from NIDDK; KABI-Crescormon;hGH-Secondary STD - IPEN (~30 μg); hGH Secondary STD-IPEN (~100 μg). Acrylamide concentration: 10%. Protein bands stained with 0.05% Comessie Blue.

Fig. 2 UV densitometric scanning of two hGH preparationsrun on PAGE
A) hGH - I-1 (AFP - 4793 B) from NIDDK
B) hGH - Secondary STD - IPEN
Acrylamide concentration: 7%
The figures refer to the migrationrates (Rm)
TB = Tracking bye (Bromophenol blue) position

Fig. 3 Flow chart of hHG small-scale purification method.

Fig. 4 UV scanning densitometry of hGH - IPEN prepared via a small scale extraction. The figures refer to the migration rates (Rm).

Fig. 5 Detailed Chloramine T stoichiometric labelling technique.

Fig. 6 Sephadex G-100 chromatogram of small-scale extracted hGH, labelled via the conventional method.
Qualitative analysis based on the distribution coefficients (kd)

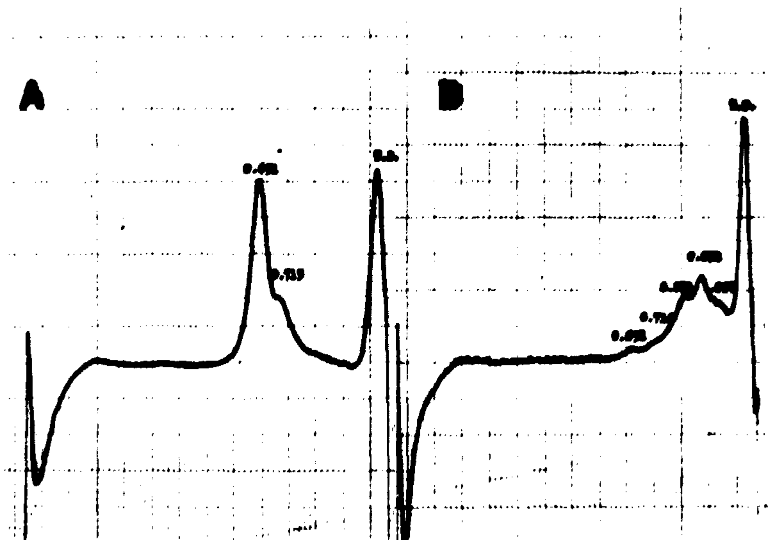
$$Kd = \frac{V_e - V_o}{V_t - V_o}$$

Fig. 7 Sephadex G-100 chromatogram of the same hGH preparation as in Fig. 6, labelled via the stoichiometric method.

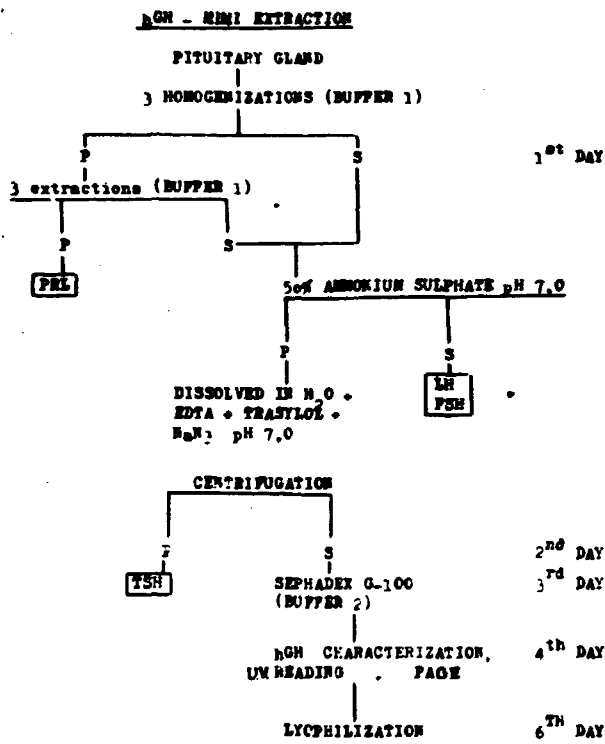
Fig. 8 Comparison between the Radioimmunoassay curves obtained using ^{125}I - hGH from IPEN and from Radioassay System Laboratories (Carson, CA, USA).

1

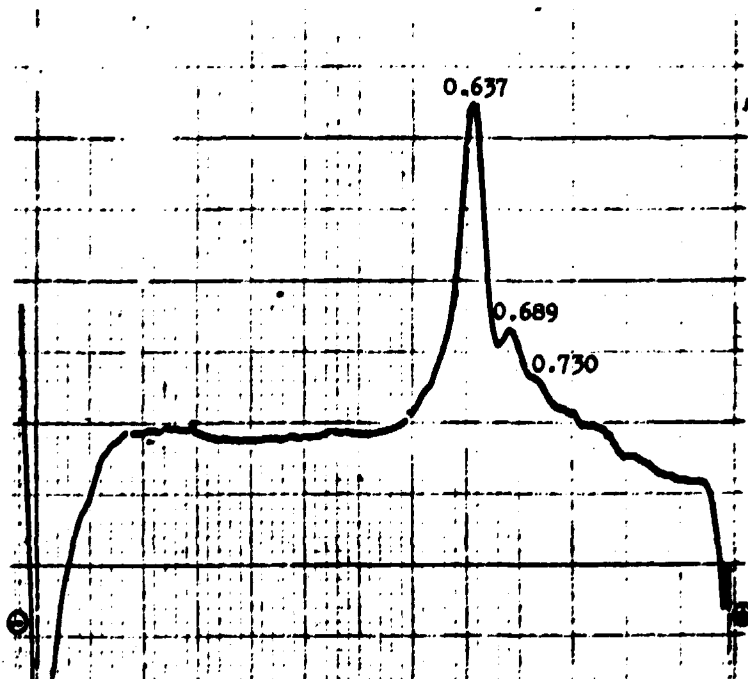
2



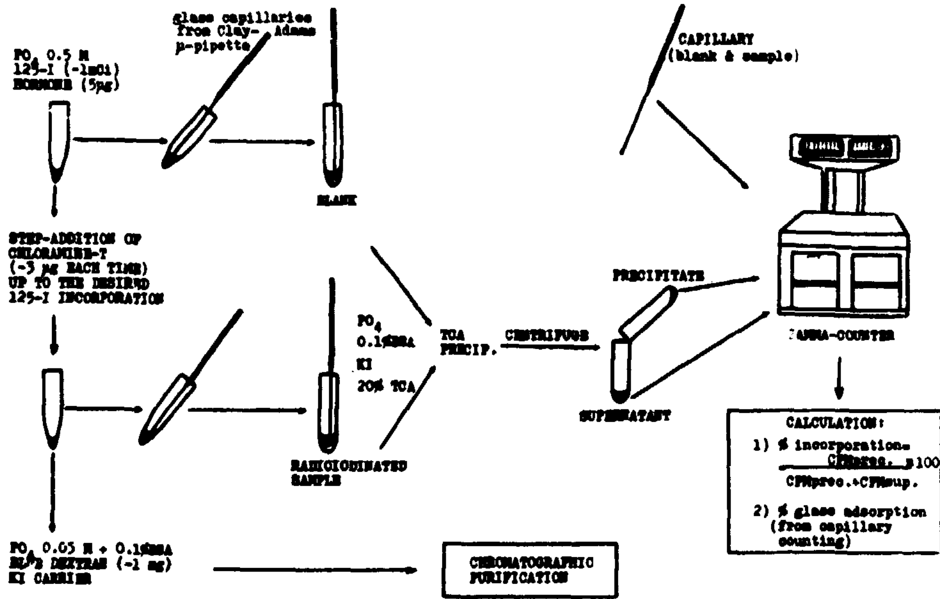
3



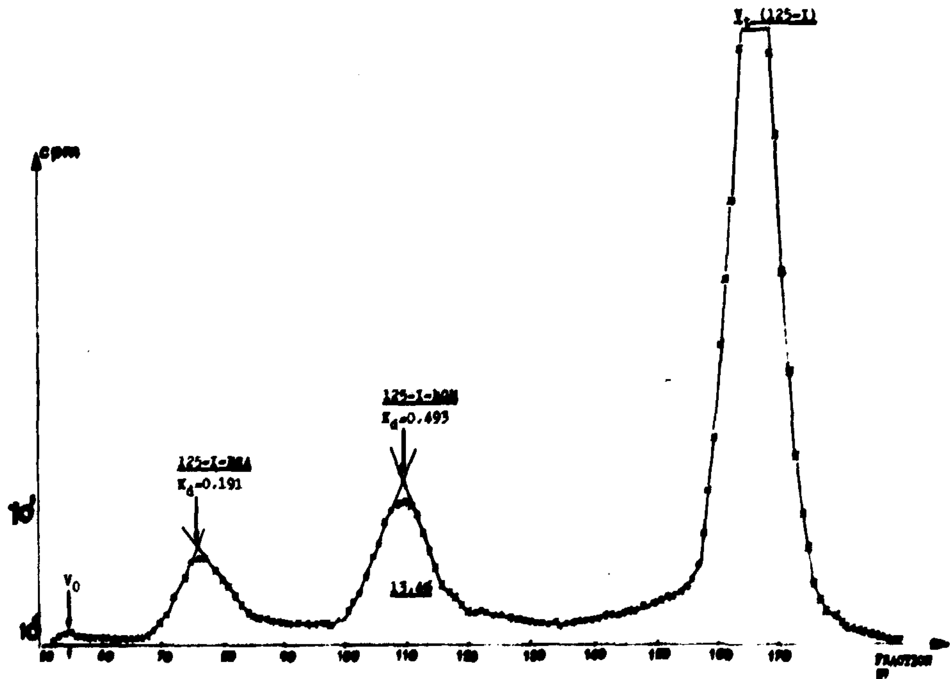
4



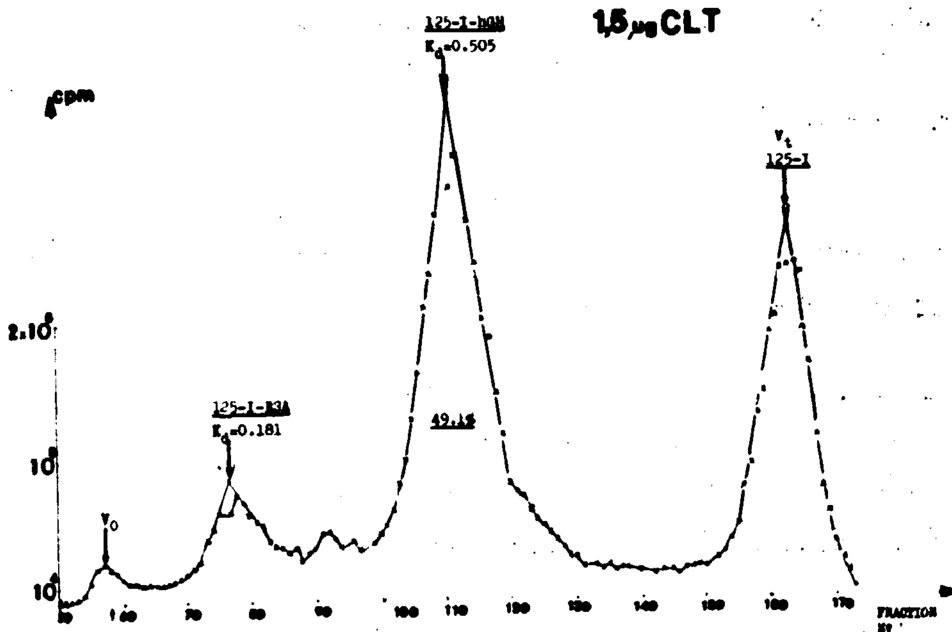
5



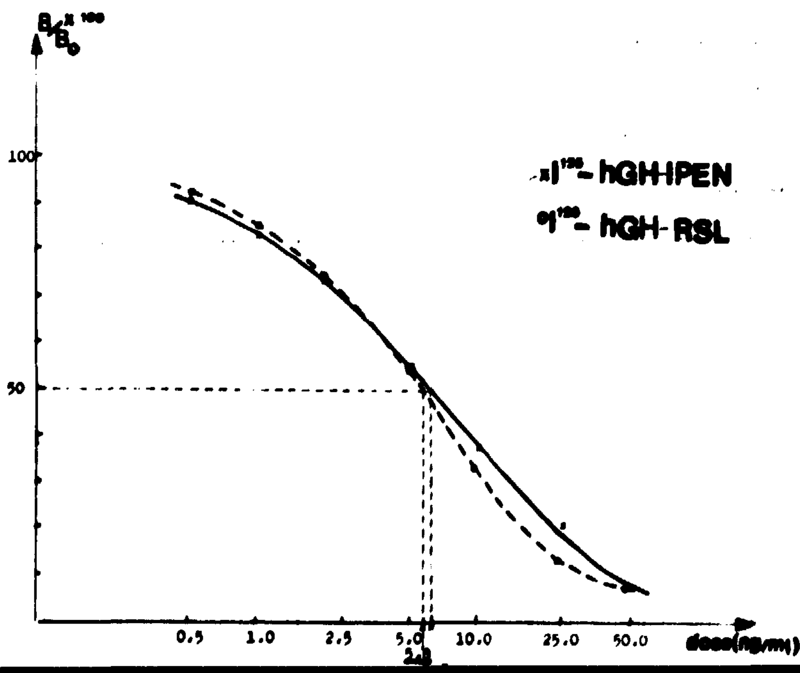
6



7



8



REFERÊNCIAS BIBLIOGRÁFICAS

1. LEWIS, U.J.; PENCE, S.J.; SINGH, R.N.P.; VANDERLAAN, W.P. Enhancement of the growth promoting activity of human growth hormone. Biochem.Biophys. Res. Commun. 67:617-24, 1975.

2. LEWIS, U.J.; PENCE, S.J.; SINGH, R.N.P.; VANDERLAAN, W.P. Bioassay of hGH: standardization of more economical assay designs. Biochem. Biophys. Res. Commun., 67:617-24, 1975.

3. HABER, E.P.; DALMORA, S.; SILVA, E.N.D.; MURAMOTO, E.; BARTOLINI, P. Bioensaio de hormônio de crescimento humano por incremento de peso de ratos hipofisectomizados: análise computadorizada de esquemas fatoriais 2x2. Arq. Bras. Endocrinol. Metabol., 31:10-4, 1987.

4. ROOS, P. FEVOLD, H.R.; GEMZELL, C.A. Reparation of human growth hormone by gel filtration. Biochim. Biophys. Acta, 74:525-31, 1963.

5. BARTOLINI, P. & RIBELA, M.T.C.P. Influence of chloramine T iodination on the biological and immunological activity or the molecular radius on the human growth hormone molecule. J. Immunoassay, 7:129-38, 1986.

6. BARTOLINI, P.; ASSIS, L.M.; SCHWARZ, I.; PIERONI, R.R. 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. Clin. Chim. Acta, 79:223-36, 1977.

7. BARTOLINI, P.; CAMILLO, M.A.P.; CARVALHO, E.I.; MURAMOTO, E.; SCHWARZ, I. Quality control in the potency determination of hGh extracts: radioimmunoassay and radioreceptor assay with standardized labelling and assay conditions. In: INTERNATIONAL ATOMIC ENERGY AGENCY. Radioimmunoassay and related procedures in medicine: proceedings of an international symposium on..., held in Vienna, Austria, 21-25 June, 1982. Vienna, 1982. p. 540.