ISSN 0101-3084

CNEN/SP

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INSTITUTO DE PESQUISAS ENERGÉTICAS E NUCLEARES
SÃO PAULO – BRASIL

Série PUBLICAÇÃO IPEN

INIS Categories and Descriptors

C45.00

IMMUNE SERUM
IODINATION
PITUITARY HORMONES
PRODUCTION
RADIOIMMUNOASSAY

IPEN - Doc - 3048

Aprovado para publicação em 18/07/88

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ABSTRACT

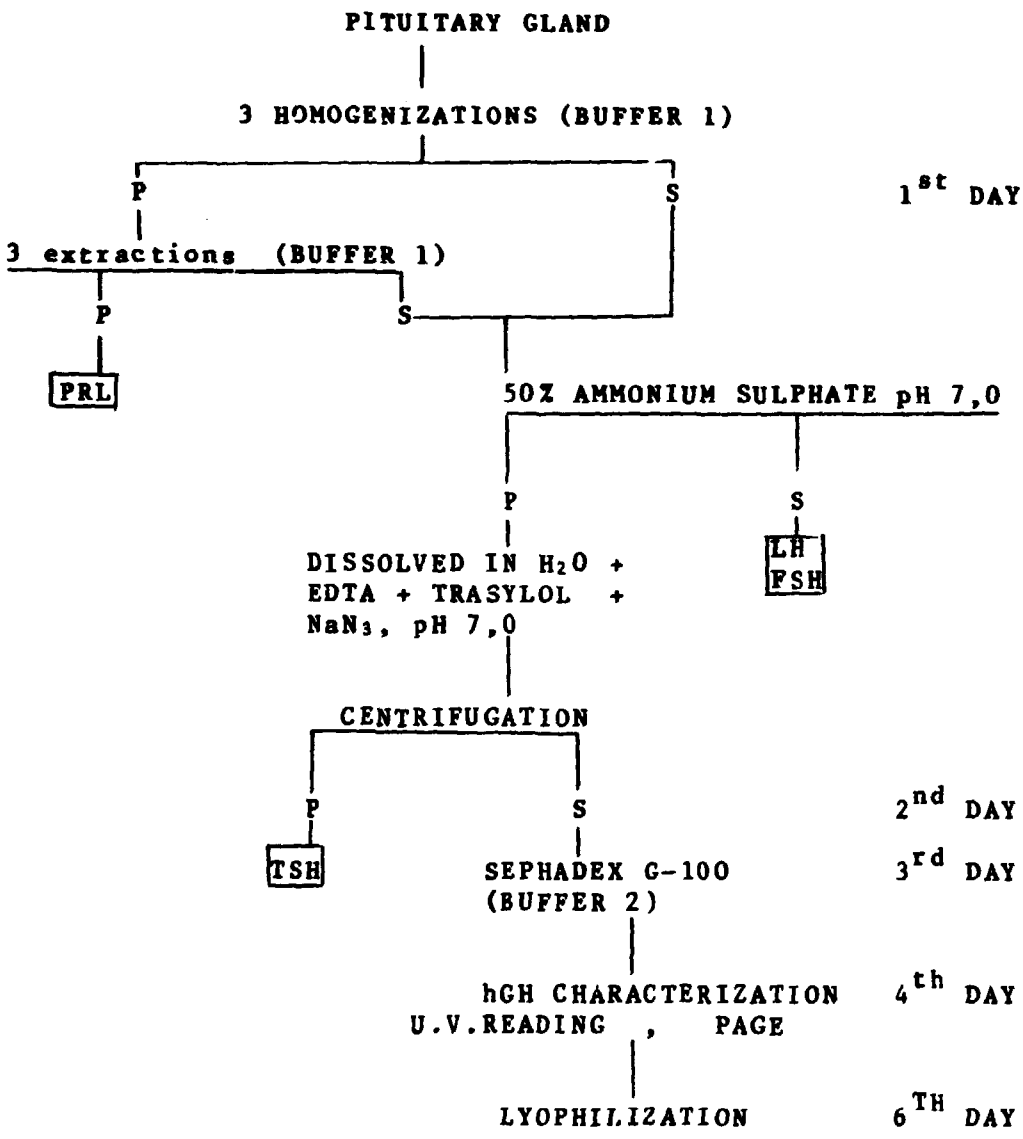
One of the main objectives of our laboratory is the national production of radioimmunoassay (RIA) reagents so to substitute the use of expensive imported commercial kits or in-bulk reagents such as the following: a) highly purified unlabelled hormones for radioiodination, useful also for preparing secondary standards and specific antisera; b) 125 I - labelled hormones; c) specific high titre antisera. The extraction and purification of human growth hormone (hGH) and human luteinizing hormone (hLH) has been already carried out. We have recently obtained quite purified, active and stable preparation starting from a small number of human pituitaries, the whole process being carried out in just one or two weeks for hGH an hLH respectively. For what concernes 125 I-labelling, we basically utilize two techniques: the Classical Chloramine T method described by Hunter and Roth's stoichiometric method, both followed by a quantitative and qualitative analysis of the reaction mixture, set up in our laboratory. The results of such comparison will be discussed. 125-I-hGH, 125-I-hLH, 125-I-hTSH and 125-I-h Calcitonin (unlabelled synthetic product donated by CIBA, Switzerland) have been prepared and tested in internal and external quality control, in comparison with good quality imported products. Satisfactory results have been obtained through the evaluation of these parameters: maximum binding to specific antiserum (Bo), nonspecific binding(NSB), mean effective dose(ED 50), sensitivity, precision and accuracy. Concerning antisera production, specific and high titre rabbit anti-hGH and anti-hLH antibodies have been obtained, while guinea-pig anti-h Calcitonin antiserum is being produced. Together with 125 I-Calcitonin it will be used in the setting up of the RIA of this hormone

wich allows the early detection of Medullary Thyroid Carcinoma.

RESUMO

Um dos principais objetivos do nosso laboratório é a produção nacional de reagentes para radioimunoensaios (RIE) com a finalidade de substituir os "Kits" comerciais e os resgentes "in bulk" importados de alto custo, tais como; a) hormônios frios de alta pureza para radioiodação, utilizado também na preparação de padrões secundários e anti-soros específicos; b) hormônios marcados com ^{125}I; c) anti-soros específicos de alto título. A extração e purificação do hormônio de crescimento humano (hGH) e do hormônio luteotrófico (hLH) já estão sendo realizados. Temos obtido preparações estáveis, ativas e puras à partir de um número reduzido de hipófise, durando o processo todo cerca de 1 a 2 semanas para o hGH e hLH respectivamente. Quanto às marcações com ^{125}I, utilizamos basicamente 2 métodos de marcação com Cloramina T: o método clássico de Hunter e Greenwood e o método estequiométrico de Roth, sendo a mistura de reação de ambos analisados quantitativa e qualitativamente. Os resultados da comparação entre esses 2 métodos serão discutidos. O ^{125}I-hGH, ^{125}I-hLH, ^{125}I-hTSH e ^{125}I-h Calcitonina (produto sintético frio doado pela CIBA, Suíça) vêm sendo preparados e testados em um sistema de controle de Qualidade intra e inter-laboratorial em comparação com produtos importados de boa qualidade. Tem-se obtido resultados satisfatórios na análise dos seguintes parâmetros: máxima ligação específica ao antisoro (Bo), ligação inespecífica (NSB), Dose efetiva média correspondente a 50% de ligação (ED_{50}), sensibilidade, precisão e exatidão. No que se refere à produção de anti-soro, conseguimos anti-soros anti-hGH e anti-hLH de alto título, em coelhos e o anti-soro anti-h Calcitonina está sendo produzido em cobaias. Esse, juntamente com a ^{125}I-h Calcitonina, será usado na montagem do radioimunoensaio para este hormônio, utilizado na detecção precoce do carcinoma medular da tireóide.

hGH - MINI EXTRACTION



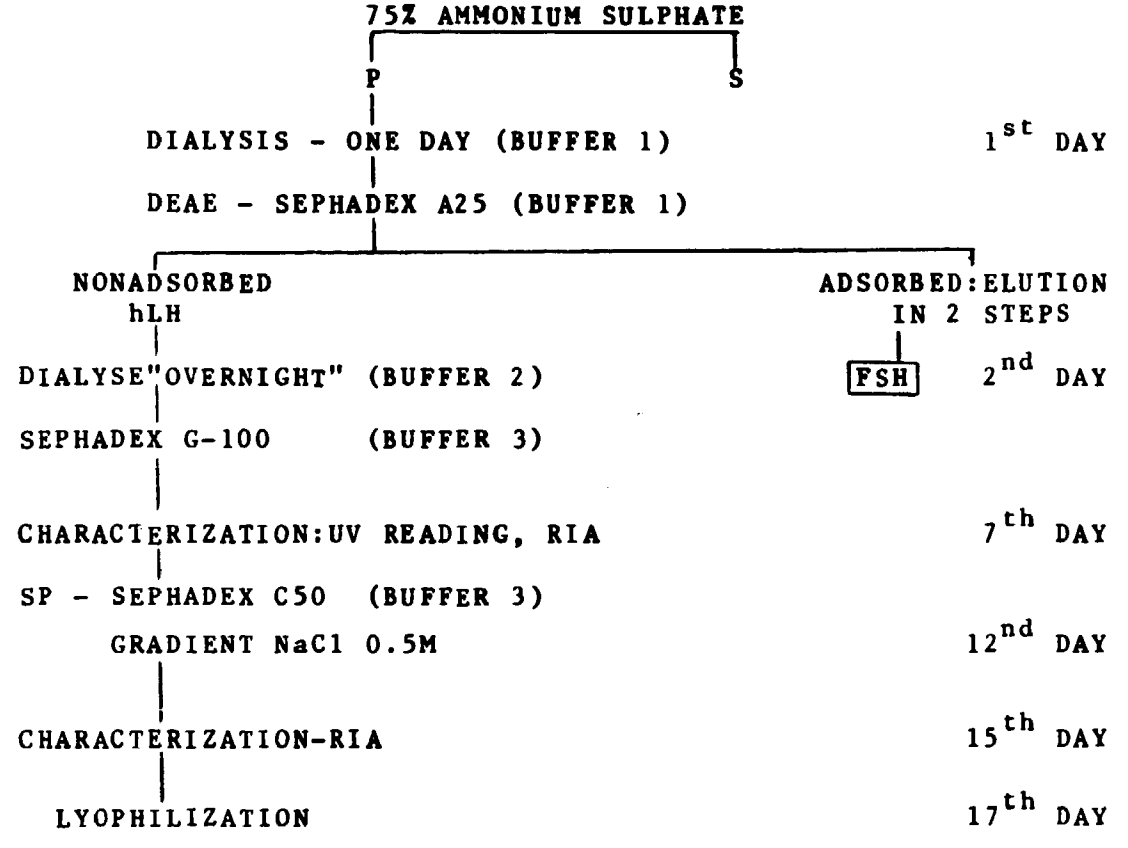
BUFFER 1 = Na Phosphate 0,03 M pH 6,2 EDTA 5 mM NaN_3 0,02%
Trasylol 50 UI/ml

BUFFER 2 = Glycine-Phosphate 0,5 M pH 7,2 (STERILE)

FIG. 1

hLH - MINI EXTRACTION

STARTING MATERIAL:AFTER 50% AMMONIUM SULPHATE PRECIPITATION FROM hGH EXTRACTION



BUFFER 1: TRIS-HCl 0.045 M pH=7.0

BUFFER 2: Na ACETATE 0.2 M pH=4,4 + NaCl 0.5M

BUFFER 3: Na ACETATE 0.02M pH=6.5

FIG.2

1) EXTRACTION AND PURIFICATION OF HUMAN GROWTH HORMONE (hGH) AND HUMAN LUTEINIZING HORMONE (hLH).

Hormone extraction from anterior pituitary glands is a quite laborious multi-step process. We tried to speed up the whole purification process starting from a small number of pituitaries: 5-20 units at the most. This way we could always start from fresh material and greatly decrease the possibility of alterations which can occur when the material is kept in solution for several weeks.

In Fig.1 we can see the flow chart of the hGH-small extraction set up in our laboratory. Considering that about six milligram of hormone are present in each human gland, the process can be applied to as few as 2-5 glands, lasting only 1 week.

In Fig.2 the same small-extraction is presented for hLH. Since only 20-50 µg of this hormone are present in each gland, at least 10-20 pituitaries have to be used. The process is more laborious, hLH quantification can be carried out only via Radioimmunoassay due to the extremely limited amount of hormone, and the whole purification takes about two weeks.

2) RADIOIODINATION OF HUMAN POLYPEPTIDE HORMONES VIA THE CLASSICAL AND STOICHIOMETRIC CHLORAMINE T METHODS.

In Fig.3 the flow chart of the classical Greenwood & Hunter labelling technique (Ref.1), slightly modified in our laboratory (Ref.2), is presented. This method always provided good quality tracers (Ref.2) but is uses a relatively high concentration of oxidizing (Chloramine T) and reducing (sodium metabisulfite) agents. This can damage some type of protein molec., especially glycoproteins (hLH, hFSH, hTSH) and those containing disulfide bridges. For this reason a second method, derived from a work of Roth (Ref.3) was set up in our laboratory (Fig.4). This allows the use of much smaller amounts of Chloramine T, which is added step by step until the desired 125-I incorporation is obtained. Its main advantage is the fact that the resulting specific activities (number of Iodine atoms/molecule) can be better predicted and controlled and that the reductant is completely omitted. In Table 1, we can observe a comparison between the two radioiodination tech

niques when applied to the labelling of five different protein hormones. As we can see the Chloramine T concentration was greatly diminished (8 to 30 times), most of the times obtaining also higher yields.

TABLE 1

HORMONE	CLASSICAL METHOD		STOICHIOMETRIC METHOD	
	Chloramine T	Yield%	Chloramine T	Yield%
hGH	50 µg	30	1,5 µg	43
hLH	50 µg	19	6,0 µg	43
hTSH	50 µg	46	6,0 µg	45
hPRL	50 µg	20	6,0 µg	23
hCT	50 µg	37	6,0 µg	51

CLASSICAL METHOD

40 µl PO_4 0,5M pH=7,5
~ 1 mCi ^{125}I
5 µg hormone (10 µl)
50 µg Chloramine T (10 µl)

30 seconds

200 µg Sodium metabisulfite (20 µl)

200 µg KI (200 µl)
1000 µg Blue-Dextran (1 ml)

PURIFICATION (Sephadex G-50 or G-10C)

FIG. 3

STOICHIOMETRIC METHOD

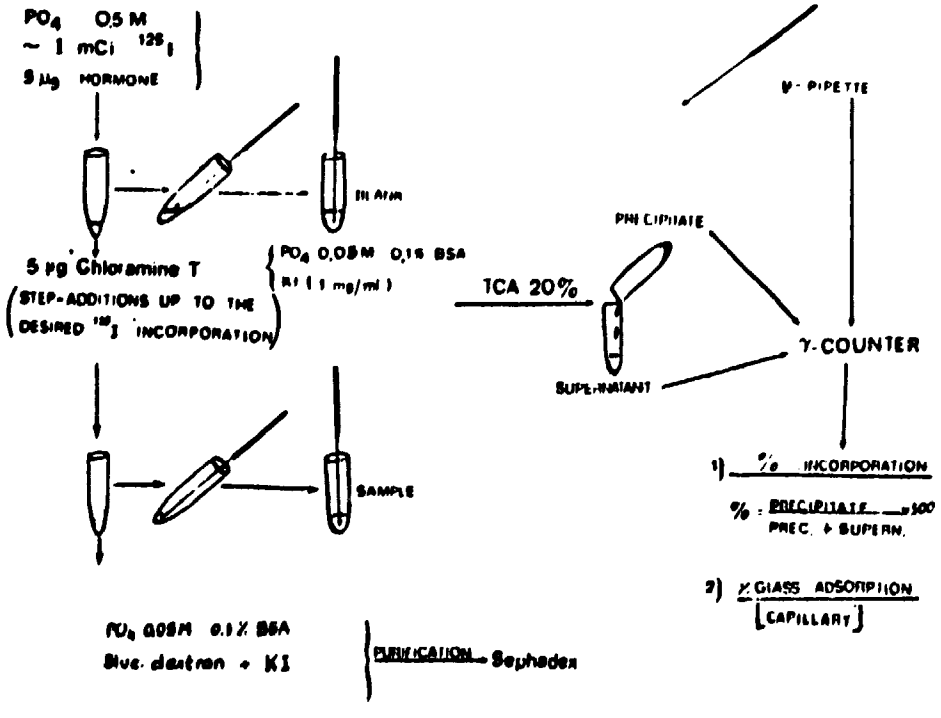


FIG. 4

3) QUALITATIVE AND QUANTITATIVE ANALYSIS OF THE LABELLING REACTION MIXTURE:125I-hGH,125I-hLH,125I-hTSH AND 125I-hCT

The technique of purification of our radioiodinated tracers was studied so to allow also a qualitative and quantitative analysis of all desired and "undesirable" components which are present in the labelling mixture (Ref.4). The methods finally permits also a non-immunological determination of tracers specific activity.

In Fig.5 an example of this analysis of the reaction mixture applied to 125-I-hGH purification in presented. In this Sephadex G-100 chromatogram several different components can be identified. PIa is aggregated tracer, PIb non-specifically labelled BSA, PIc a dimeric form of the tracer, PII is desired monomeric 125-I-hGH and PIV, free 125-I. On this same column, in separate run, also 125-I-BSA was chromatographed, to confirm the position of this interfering peak.

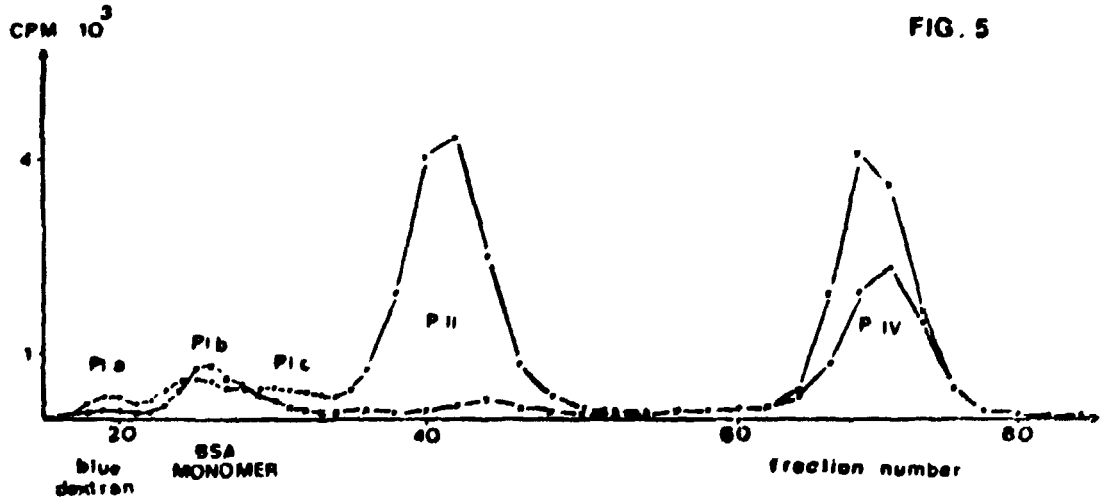


FIG. 5

In Figs. 6, 7 and 8 the same type of analysis is carried out for human luteinizing hormone (hLH), human thyrotropin (hTSH) and human Calcitonin (hCT). The "undesirable" components can be identified now the same way. 125-I-BSA and aggregates are presented in all cases though in different amounts. As it is known hLH is contaminated also by smaller components:125-I-hLHs repre

senting the labelled alpha and beta sub-units of hormone. Radioiodinated Calcitonin, which has a much smaller molecular weight (3400 d) had to be purified on Sephadex G-50 M, the aggregate peak being unresolved from 125-I-BSA. Unlabelled synthetic human Calcitonin was kindly provided by CIBA (Switzerland).

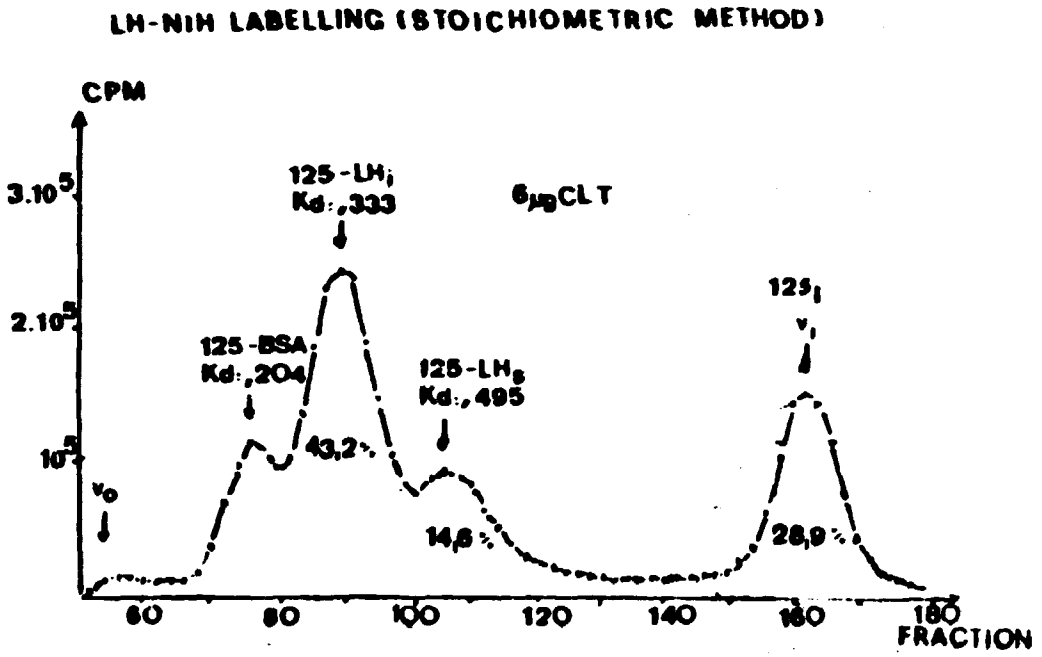


FIG 6

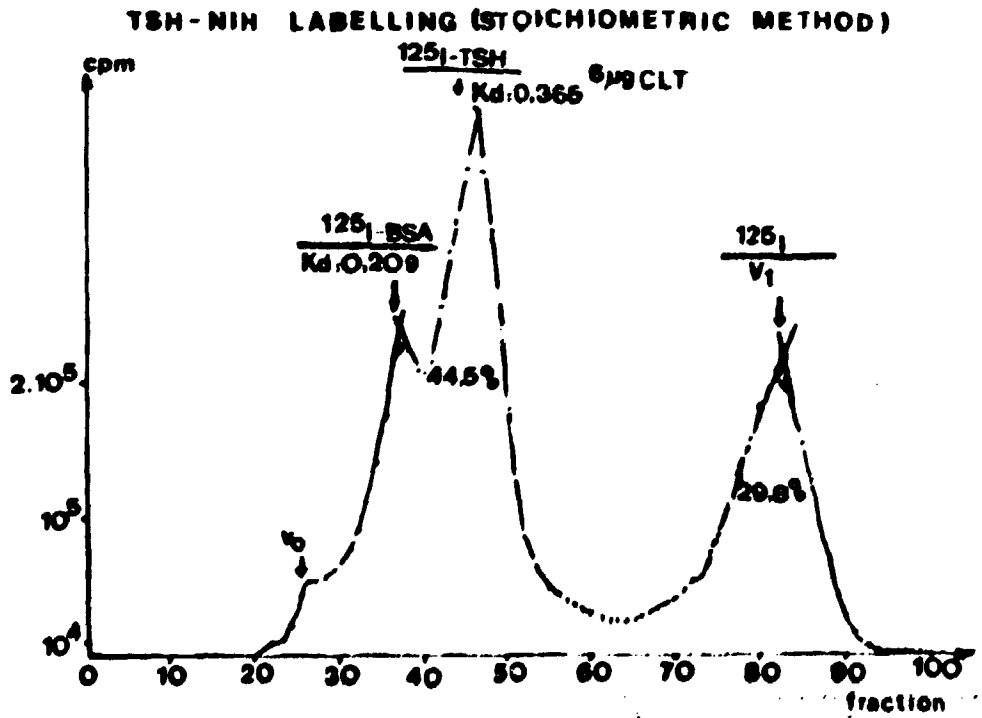


FIG. 7

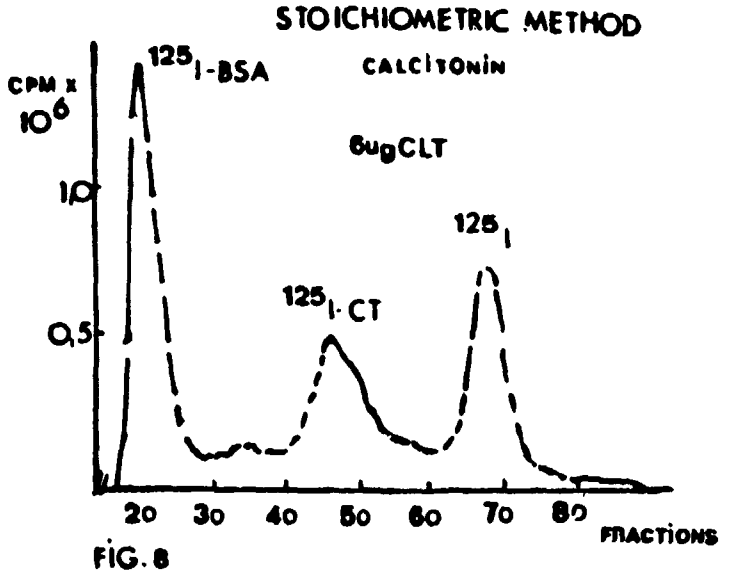


FIG. 8

4) INTRA AND INTER-LABORATORIAL QUALITY CONTROL OF IPEN-RADIOIODINATED HORMONES AND IPEN-ANTISERA

^{125}I-hGH, extracted and radioiodinated in our laboratory was distributed to different laboratories and tested in comparison with a good quality imported tracer. In Fig.9 an example is shown of the equivalence between the two corresponding Radioimmunoassay curves. Other RIA parameters, like Maximum Specific Binding, Non-specific Binding, ED50, Precision and Accuracy were confirming the equivalence between the two products.

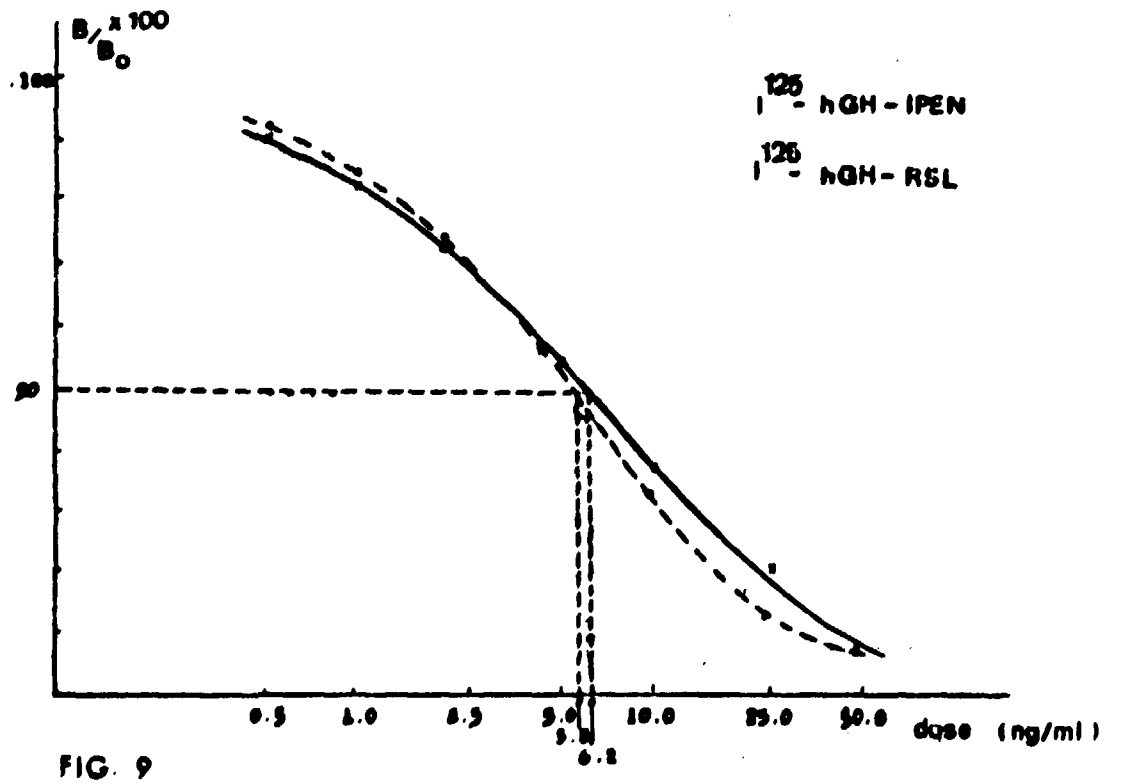


FIG. 9

^{125}I-hLH and ^{125}I-hTSH. The same type of study was also performed for radioiodinated luteinizing hormone (Fig.10) and thyrotropin, obtaining similar results and quality. For these two hormones, however, an imported polypeptide hormone preparation was used for radioiodination, since the purity of our own preparations is still being tested.

Antisera anti-hGH, hLH and anti-hCT, obtained in rabbits and guinea-pigs were also prepared in our laboratory of IPEN-CNEN/SP with titres that can be useful for the setting up of RIA curves. In Fig.11 a study is presented comparing the anti-hLH antiserum produced at the IPEN, with an imported high titre antiserum kindly provided by the National Institute of Health (USA). Even with a lower titre, the IPEN-antiserum presents an identical behaviour.

Similar comparison were carried out for anti-hGH and anti-hCT antisera, the latter being still under testing, especially for what concerns the possibility of obtaining the extremely high sensitivities which are necessary for its clinical use.

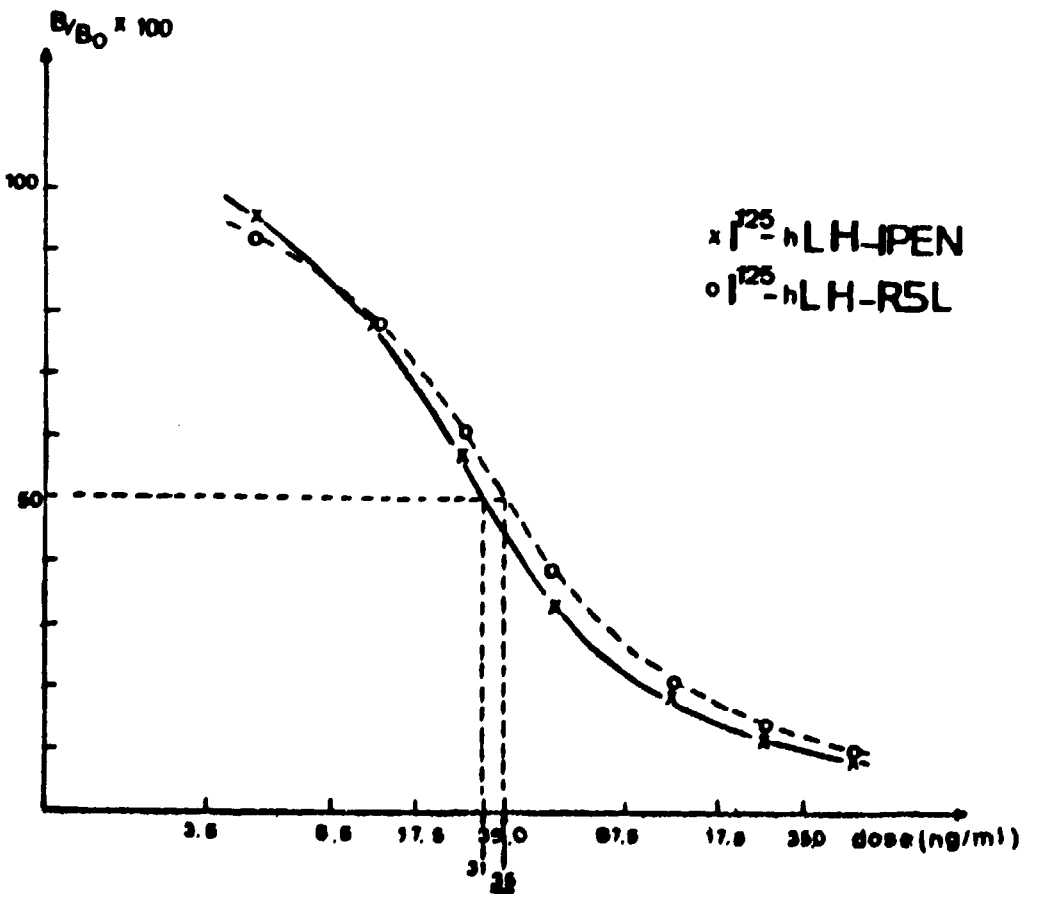


FIG. 10

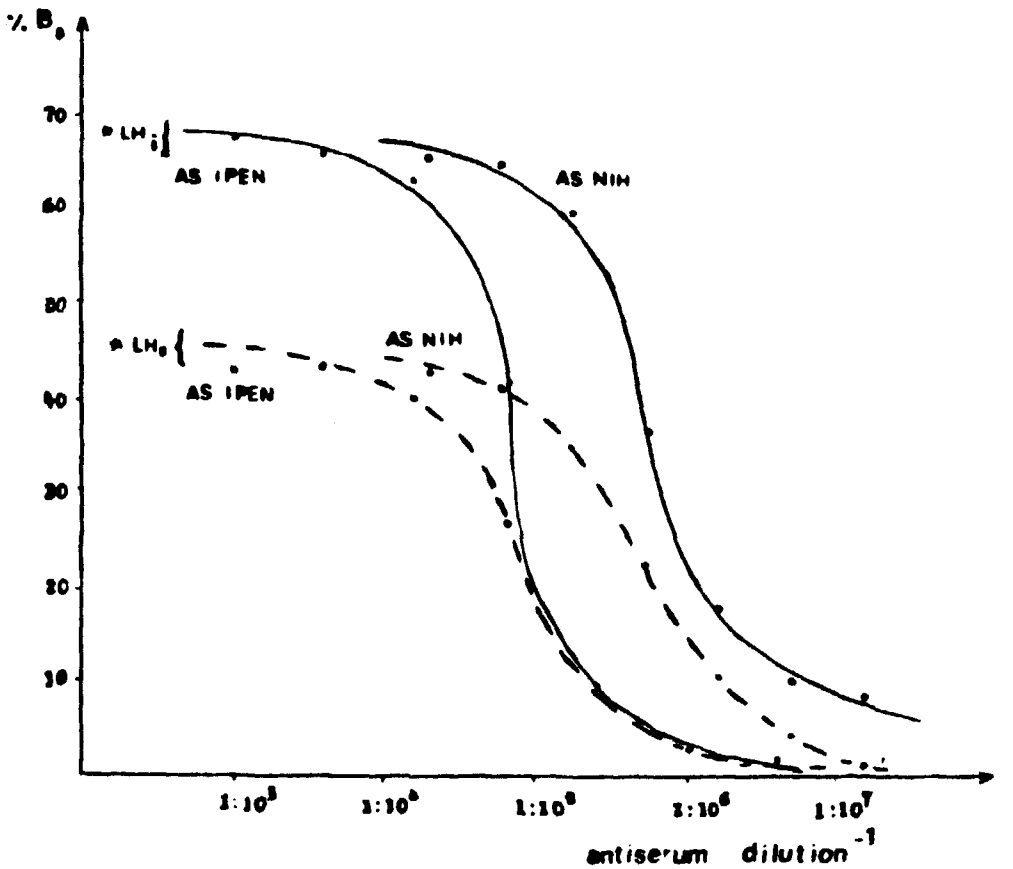


FIG. 11

ANTI-hLH ANTISERA (NIH AND IPEN) TITRE DETERMINATION USING INTACT MONOMERIC 125-I-hLH (*LHi) AND 125-I-hLH. SUB-UNITS (*LHs).

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