ISSN 0101-3084

IPEN - PUB -- 180

CNEN/SP

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PUBLICAÇÃO IPEN 180 AGOSTO/1989

SÃO PAULO

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SÃO PAULO – BRASIL

Série PUBLICAÇÃO IPEN

INIS Categories and Descriptors

C45.00

LH
PITUITARY HORMONES
RADIOIMMUNOASSAY

IPEN - Doc - 3046

Aprovado para publicação em 18/07/88.

SMALL-SCALE PURIFICATION OF HUMAN PITUITARY LUTROPIN (hLH) FOR USE IN RADIOLIGAND ASSAYS

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ABSTRACT

Human lutropin (hLH) is a relatively unstable protein, which even in lyophilised form tends to dissociate into subunits during long storage periods. Considering also the limited disposibility of human pituitaries and the minimal amounts of hormone necessary for radioas - says, we proposed to overcome this problem by developing a small- scale extraction method. Based on our previous experience with the method des cribed by Roos et al. (Biochim. Biophys. Acta 405: 363, 75). The origi- nal procedure was modified as follows: 1. homogenization and 3 to 4 extractions of the frozen glands in phosphate buffer; 2. 50% ammonium sulphate precipitation (growth hormone fraction); 3. 75% ammonium sul- phate precipitation (glycoprotein fraction); 4. overnight dialysis against 0.045 M TRIS - HCl buffer pH 7.0; 5. DEAE-Sephadex A25 chroma- tography; 6. overnight dialysis of the unadsorbed fraction against 0.2 M sodium acetate buffer pH 4.4 + 0.5 M NaCl; 7. Sephadex G100 chro- matography run with 0.02 M sodium acetate buffer pH 6.5; 8. SP-Sephadex C50 chromatography, linear gradient elution with 0.02 M to 1.0 M sodium acetate buffer pH 6.5. After steps 7 and 8, hLH in the eluted fractions was determined by radioimmunoassay (RIA). Since a highly purified hormo ne was desired, the given RIA results are expressed in terms of the pure hLH-NIDDK-I-3 for labelling. Starting from 10 and 20 hypophyses , after Sephadex G 100 purification we obtained about 10 µg hLH/ gland , with approximate 10% purity. This product is equipotent to the less pu- rified Reference Preparation LER - 907, and so could be considered for use as a standard for hLH RIA. After the last purification, hLH recov - ery was od 1.5 µg/gland. Major losses were attributed to the observed adherence (about 80%) of the diluted hormone to the glass tubes used for fraction collection. The proposed method is considered effective and recoveries may be improved by reducing the above mentioned losses ,

for which efforts are in progress. An additional advantage lies in the rapidity of the process, that con supply fresh hormone in 10 to 14 days and be easily repeated when needed.

MINIEXTRAÇÃO DO HORMÔNIO LUTEOTRÓFICO HUMANO (hLH) PARA USO EM ENSAIOS RADIOLIGANTES

RESUMO

A lutropina humana (hLh) é uma proteína instável que mesmo liofilizada tende a dissociar-se em subunidades durante períodos longos de estocagem. Considerando também a limitada disponibilidade de hipófises humanas e as quantidades pequenas de hormônio que são necessárias para os radioensaios, para superar este problema, nos propusemos, desenvolver um método de extração em pequena escala (miniextração). Baseados em nossa experiência anterior com o método descrito por Roos et al (Biochim. Biophys. Acta 405: 363, 75), o procedimento inicial foi modificado como segue: 1- homogeneização e 3 a 4 extrações das glândulas congeladas em tampão fosfato. 2- precipitação por sulfato de amônia 50% (fração do hormônio de crescimento) 3- precipitação por sulfato de amônia 75% (fração da glicoproteína) 4- diálise, "overnight" em tampão 0,45 M tris-HCl pH 7,0.5. Cromatografia em DEAE-Sephadex A 25 6- diálise, "overnight" da fração não absorvida em tampão acetato de sódio 0,2 M pH 4,4 + 0,5 M NaCl. 7- cromatografia em Sephadex G 100 em tampão acetato de sódio 0,02 M pH 6,5. 8- cromatografia em SP-Sephadex C50, eluição por gradiente linear com tampão acetato de sódio pH 6,5 de 0,02 M a 1,0 M. Após as etapas 7 e 8 o hLH nas frações eluidas foi determinado por radioimunoensaio (RIA). Desde que o que se deseja é um hormônio altamente purificado os resultados do RIA são expressos em termos do hLH-NIDDK-I-3 utilizado para marcação. Partindo de 10 e 20 hipófises, após purificação em Sephadex G 100 nós obtivemos 10 µg de hLH/glândula com aproximadamente 10% de pureza. Este produto é equipotente com o padrão de referência menos purificado LER-907 e, portanto, poderia ser usado como padrão para RIA de hLH. Após a última purificação, a recuperação do hLH foi de 1,5 µg/glândula. As perdas maiores fo

ram atribuidas a aderência observada (cerca de 80%) do hormônio diluído às paredes dos tubos de vidro usados na coleção das frações. O método proposto é considerado efetivo e as recuperações podem ser melhoradas reduzindo as perdas mencionadas o que está sendo tentado. Uma vantagem adicional, é a rapidez do processo que pode fornecer hormônio fresco em 10 a 14 dias e pode ser facilmente repetido quando necessário.

INTRODUCTION

Intact human lutropin (hLHi) is a pituitary glycoprotein hormone consisting of two peptide chains (hLHs), known as alpha- and beta-subunits and held together by non-covalent interactions. Human pituitary thyrotropin and follitropin have protein structures closely related to hLH, and the alpha sequence is essentially identical for each hormone , biological specificity being determined by tne beta-subunit (3). Kourides (1) reported excess free alpha-subunit in the normal pituitary. For these reasons, a hLHi preparation highly purified and free of subunits is needed for radioassay purposes.

In our experience, hLH dissociation into its subunits is temperature-dependent (7), even ocurring during long storage periods of the lyophilised hormone.

To obtain hLH for use in radioassays, we started extracting a large number of hypophyses by the method of Roos (4). Considering the very limited supply of glands, which had to be stored for long periods before extraction, we decided to adapt the original procedure to a rapid small-scale extraction method, whose preliminary results are presented. Thus, a sufficient amount of hormone (of the order of 100 to 500 µg) could be prepared for radioassay purposes, avoiding long storage periods for pituitaries and for the purified hormone.

MATERIALS AND METHODS

Extraction method:

Extraction and purification of LH from frozen pituitaries was performed by the method described by Roos (4), modified for small-scale purification as shown on Table 1. All fractionation steps were perform-

ed at 4ºC; centrifugations were done at 20.000 x g for 15 minutes. On the fractions eluted from the chromatographic columns, optical den - sities (O.D.) were read at 280 nm and hLH estimated by specific radioim munoassay (RIA). Fractions for further processing were pooled accord - ing to the O.D. or RIA results.

Two extractions were performed, starting from 20 and 10 pitu - itaries. The final preparations obtained are referred to as hLH IPEH.

Protein quantification:

The protein content in the extracts obtained in the different fractionation steps was estimated by the method of Lowry (2). When this method was not applicable, due to very low protein concentration. The hLH yields were estimated by RIA.

hLH RIA:

hLH RIA reagents were the highly purified hLH-NIDDK-I-3 prepara- tion for radioiodination, also used as standard preparation, and rabbit anti-hLH antiserum from NIDKK (batch nº 2), kindly provided by the Na - tional hormone and Pituitary Program, Maryland, USA. Immunopotency of this hLH preparation was estimated by us to be 10 to 20 times that of Reference Preparation LER 907, also provided by NIDDK. In the figures, NIDDK reagents are denominated NIH preparations.

The buffer used for incubations and reagent dilutions was 0.01 M phosphate pH 7.4 + 0.14 M NaCl + 0.1% BSA. 0.4 ml incubations were car- ried out at 4ºC for 48h. The bound fraction was precipitated by the addition of 1ml 17% PEG 6000 + 40 µl normal human serum to the incu - bate, followed by centrifugation at 3.000 x g for 20 minutes, at 4ºC . Immunoreactive hLH content of the fractions was determined on logit-log standard curves calculated by least squares regression lines. Parallel ism of RIA curves was tested by a t-test.

^{125}I-labellings of hLH were performed by the stoichiometric Chlo ramine T method described by Roth (5),using from 12 to 24 µg Chloramine T/~5 µg hormone. The labelling mixture was purified on a long Sephadex G100 column (2.5 x 90 cm), at 4ºC, eluted with 0.05 M phosphate buffer pH 7.4 + 0.1% BSA. Intact hLH (LHi) and its subunit (hLHs) peaks were identified by their distribution coefficients (Kd) of about 0.27 and 0.45, respectively. The immunoreactivity of the labelled hormone was tested by incubating different fractions of the eluted ^{125}I-LH with

excess antiserum.

RESULTS

Table 2 shows the mean protein yields (µg/gland) of hLH fractions in the various purification steps, obtained in the two small-scale extraction lots.

Fractionation of the dialysed 75% ammonium sulphate precipitate on DEAE ion-exchange chromatography is shown in Fig. 1. The shaded area indicates the hLH fractions pooled for subsequent processing. Immunoactive hLH is not retained by the gel, together with most of the eluted proteins, as shown by the O.D. profile.

Sephadex G100 cnromatography of the dialysed DEAE-hLH pool (Fig. 2), shows that it contains a complex mixture of proteins. Although a relatively large column was used, hLH fractions can be identified only by RIA and no 'clean' hLH peak is defined by A_{280} readings.

Fig. 3 shows the final purification step on SP-Sephadex C50. At this stage, O.D. readings are impossible due to the extreme protein dilution. RIA profile shows that hLH is retained by the gel and eluted with approximately 0.3 M acetate buffer.

In the first extraction lot, no glycine was added to the tubes before collection. After elution, one aliquot of the hLH fraction was immediately diluted and frozen in 0.1% BSA RIA incubation buffer, while another was stored frozen without modifications. RIA of several dilutions of these two aliquots together with the NIDDK-I-3 standard curve, is shown in Fig. 4. The 80% loss of immunoactivity of the aliquot stored without BSA is clearly evident.

A freeze-dryed aliquot of hLH-IPEN from the second extract was labelled with ^{125}I and the purification on Sephadex G100 is shown in Fig. 5A. Specific binding to excess antiserum (Bo) and related non-specific bindings (NSB) of different fractions (between Kd = 0.24 and Kd = 0.45) are shown on the same figure. In this case the maximum specific binding of the labelled intact hormone (Kd = 0.31) was 27%. The ^{125}I-hLHi peak masked by an excess ^{125}I bound to BSA, while a significant ^{125}I-hLHs peak is evident. The fraction corresponding to Kd=0.31 (typical of hLHi was repurified on the same column, as shown in Fig. 5B. The

antiserum titration curve of this repurified ^{125}I-hLH is shown on Fig. 6.

To eliminate the interferent ^{125}I-BSA, a second aliquot of the same hLH-IPEN preparation was iodinated and purified with-out BSA in the elution buffer, adding 1% gelation to the sample. This way an in - creased specific binding of 40% was obtained for the intact ^{125}I-hLH.

DISCUSSION

The A_{280} profiles of the first two chromatographies (Fig. 1 and 2) and of the immunoactive hLH elution from the last chromatography (Fig. 3), were comparable to those previously obtained in two large-scale extraction lots following the original method of Roos (6).

The main protein yields in the purification steps from 175g per litre ammonium sulphate precipitation to Sephadex G100 chromatography (Table 2) tended to be higher in the small-scale extraction method when compared to those obtained in the large-scale extraction lots, although no significant difference could be demonstrated ($p=0.05$), except for protein recovery after DEAE-chromatography, which was about three times higher.

After Sephadex G100 purification, the central fractions of the hLH peak had a mean content of 0.09 ± 0.02 µg LH/µg protein, as determined by RIA. RIA curves raised with this impure hLH, were parallel to the NIDDK standard, and so this fraction could be suitable for use as a standard preparation for RIA, although further tests are required.

The final immunoactive hLH yield of 1.4 ± 1.0 µg/gland is also not significantly different from the 0.76 ± 0.24 µg/gland previously found in the large-scale extractions.

RIA curves of the final product (hLH-IPEN) were parallel to the highly purified hLH-NIDDK ($p=0.05$) (Fig. 4), thus demonstrating its identity to hLH, with no cross-reacting proteins or interfering impurities.

Roos (4), starting from 5 to 6g of 75% ammonium sulphate precipiptate, reports a yield in pure hLH of approximately 7 µg/gland, based on O.D. readings at 280 nm. For the large-scale extractions, our lower recoveries (2.02 ± 0.54 µg/gland, determined by the method of Lowry ,

can be attributed to the very long storage periods (several years) of the pituitaries and of the starting material for hLH purification, which was accumulated from several growth hormone extractions. Losses in the small-scale purifications, especially occurring in the last purification step, seem to be associated with the great protein dilution, which apparently causes hLH adherence to glass tubes and/or favors its degradation during freezing and thawing. Concerning the loss of immunopotency of hLH stored without BSA, glycine, added to the collection tubes before elution of the last chromatography could represent an attempt to solve the problem. Immunoreactive hLH yield was better indeed: 2.06 µg/gland, against 0.67 µg/gland for the first extraction.

In both radioiodinations, the preliminary data on incorporation of ^{125}I into the hormone presented quite low values (Fig. 5A). The high specific binding presented by the second tracer could be related to the different purification technique. Further experiments will be carried out studying the influence of the labelling technique and of the purification system.

We conclude that the time- and work-reducing modifications for the small-scale extraction method can provide an hLH preparation suitable for radioassays, although problems like protein losses, correct protein quantification in dilute solutions and stability of the hormone during radioiodination should be better resolved and are being investigated.

We thank for the support received from FINEP (43.86.0351.00) and the IAEA (4299/RB).

TABLE 1 - hLH EXTRACTION / PURIFICATION METHODS

ROOS*	MODIFIED FROM ROOS
FROZEN PITUITARIES EXTRACTIONS: 3 to 4, in Potassium phosphate	FROZEN PITUITARIES EXTRACTIONS: 3 to 4, in * Potassium phosphate + EDTA + Trasylol
50% Ammonium sulphate (hGH fraction)	50% Ammonium sulphate (hGH fraction)
Ammonium sulphate : 175g/l (75%)	Ammonium sulphate : 175g/L (75%)
DIALYSIS 0.02M Potassium phosphate pH 7.0	*DIALYSIS 0.045M TRIS-HCl pH 7.0
DEAE-CELLULOSE - Stepwise elution Potassium phosphate pH 7.0: 0.02 - 0.06 - 0.5 M	*DEAE-SEPHADEX A25 - Stepwise elution 0.045M TRIS-HCl pH 7.0 + NaCl 0.06 - 0.5 M
DIALYSIS : 90 - 92h 0.2M Sodium acetate pH 4.4 + NaCl 0.5M	*DIALYSIS : Overnight 0.2M Sodium acetate pH 4.4 + NaCl 0.5M
SEPHADEX G100 0.2M Sodium acetate pH 4.4 + NaCl 0.5M	*SEPHADEX G100 0.02M Sodium acetate pH 6.5
SEPHADEX G100 - Same buffer as before	
DIALYSIS 0.01M Potassium phosphate pH 7.0	
SP-SEPHADEX C50 - Stepwise elution Potassium phosphate pH 7.0 : 0.01 - 0.02 - 0.05 - 0.1 - 0.5 M	*SP-SEPHADEX C50 - Gradient elution a: 0.02M Sodium acetate pH 6.5 b: 1.0 M Sodium acetate pH 6.5
TOTAL TIME : 16 days	TOTAL TIME : 8 days

* ROOS et al.- Biochim.Biophys.Acta, 1975

* modified procedure

TABLE 2 - PROTEIN (hLH) YIELDS OF TWO hLH EXTRACTION LOTS

PURIFICATION STEP	MEAN*S.D. (µg/gland)	METHOD
50% Ammonium sulphate	6,300 ± 2,800	LOWRY
175g/L Ammonium sulphate	6,000 ± 1,200	"
DEAE-SEPHADEX A25	2,000 ± 200	"
DIALYSIS	1,400 ± 100	"
SEPHADEX G100	130 ± 100	"
SP-SEPHADEX C50	1.4 ± 1.0	RIA (NIH-I-3 STD.)

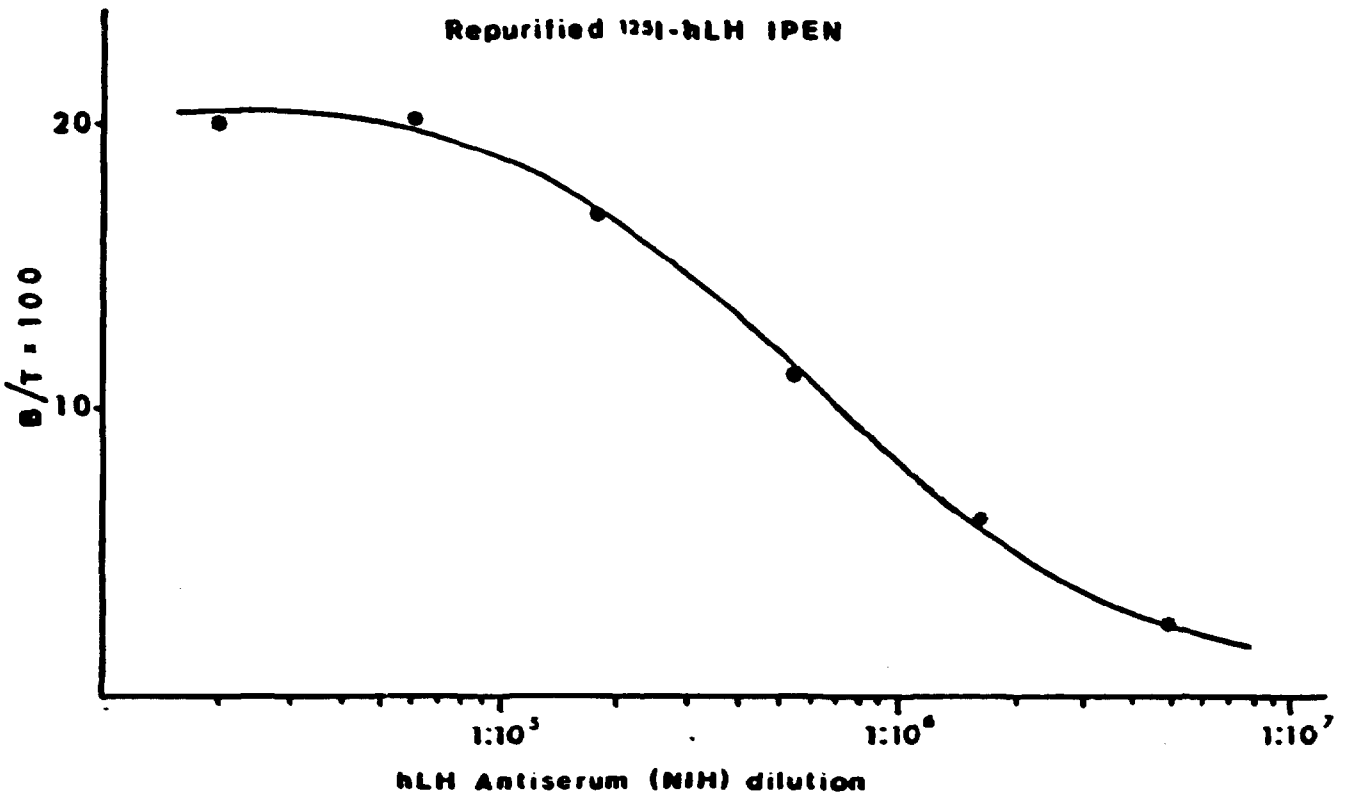

FIG. 6 - Titration of NIH anti-hLH antiserum by RIA, using repurified ^{125}I-hLH-IPEN.

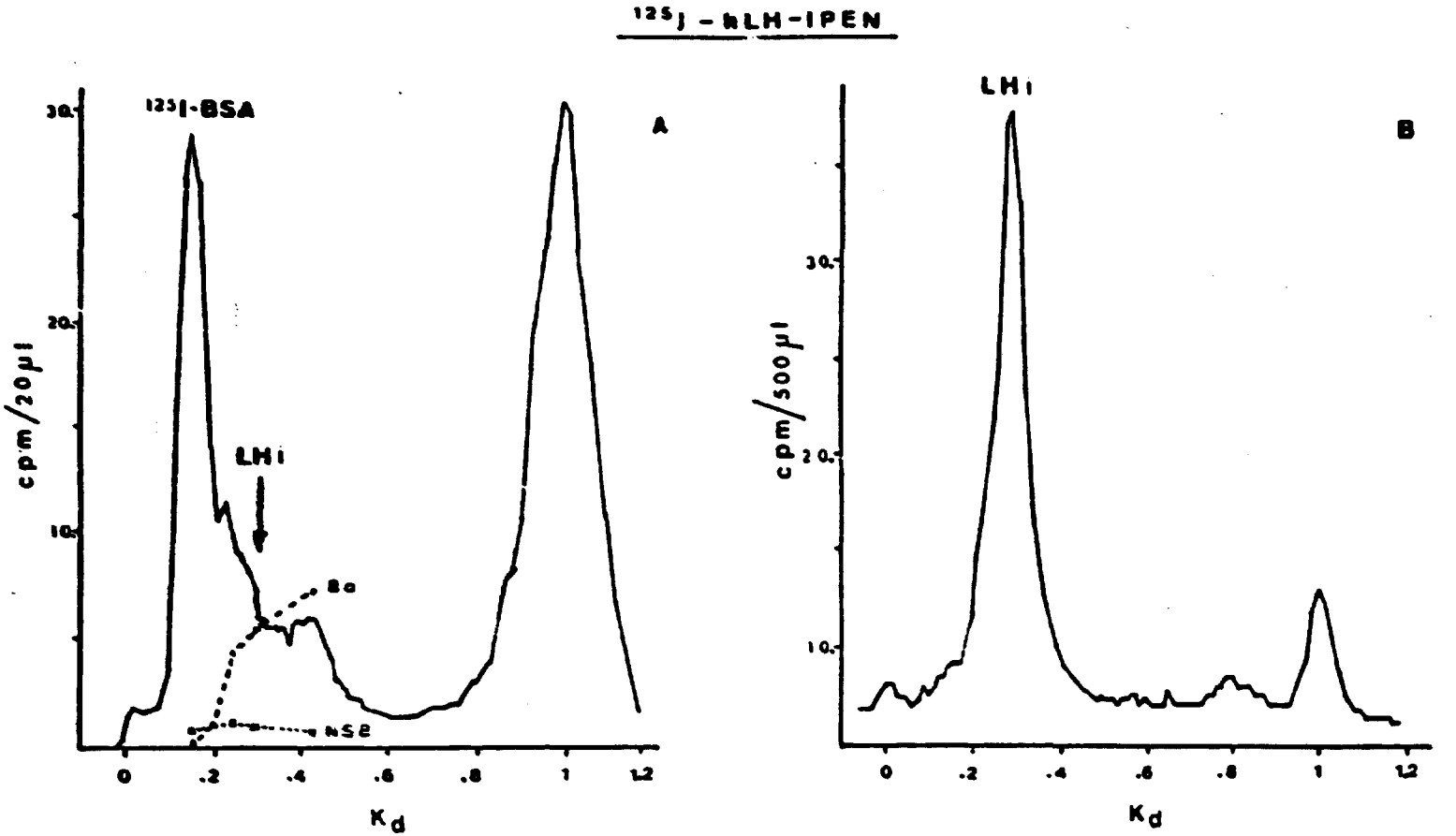

FIG. 5 - SEPHADEX G100 chromatographies (2.5 x 90 cm, fractions = 2.5ml, at 4°C) of ^{125}I-hLH-IPEN. A: Labelling mixture; immunoreactive hLH was eluted between Kd = 0.24 and 0.45, as shown by specific binding (Bo) to hLH anti-hLH antiserum. B: Repurification of Kd = 0.31 fraction.

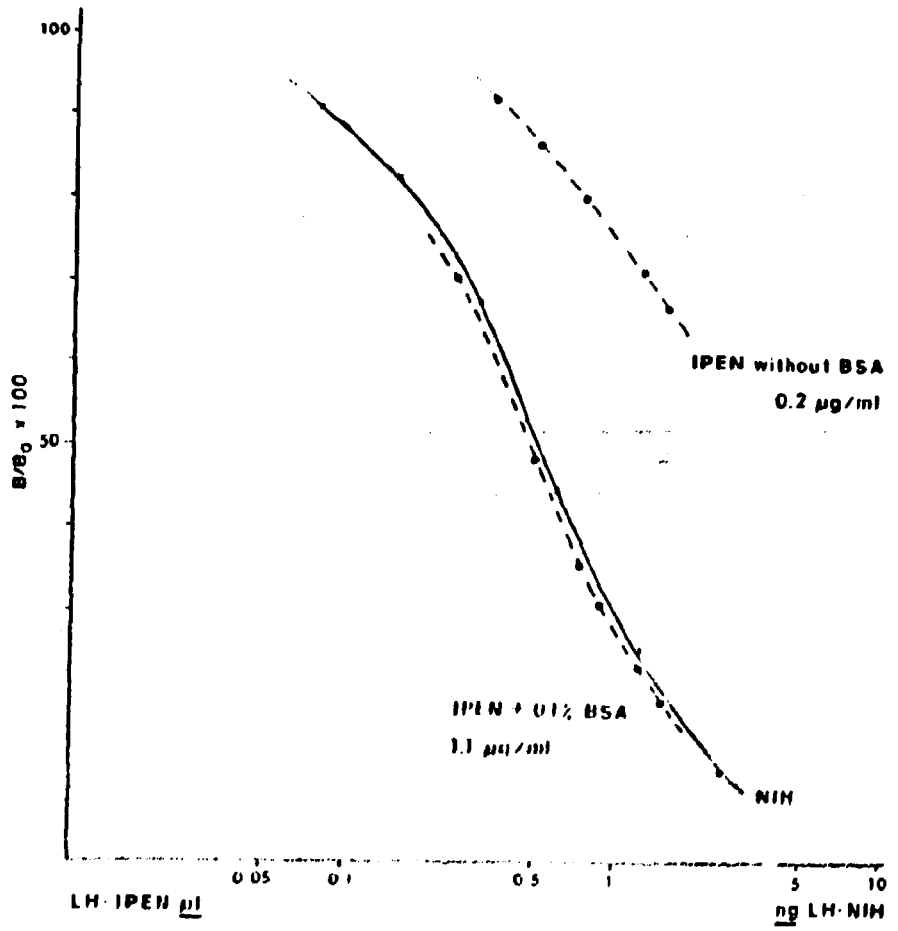

FIG. 4 - RIA potency estimation of hLH-IPEN against hLH-NIH-I-3 standard. hLH-IPEN stored without BSA lost about 80% immunopotency.

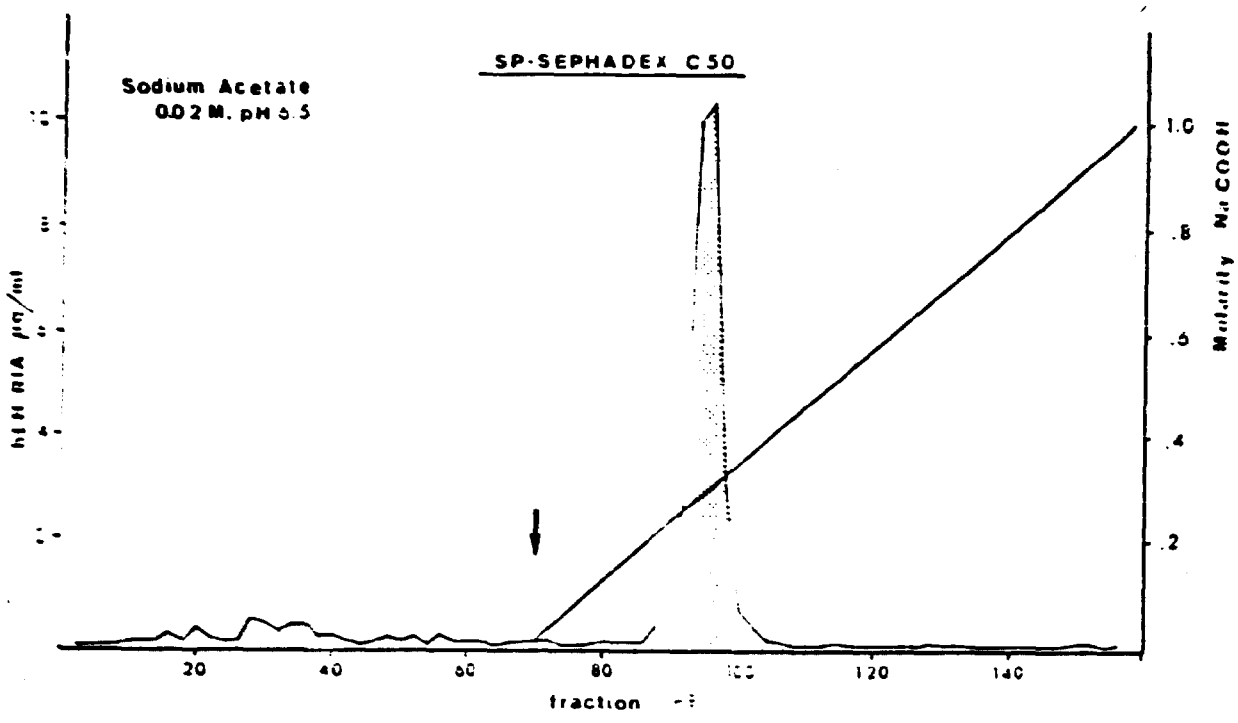

FIG. 3- Ion-exchange chromatography of previous Sephadex G100 pooled fractions on SP-SEPHADEX C50.Column 2.5 x 5cm (25ml), flow = 10ml/h, fractions = 2ml. 0.2 mg Glycine/tube before collection. Linear gradient elution from 0.02 M to 1.0M Sodium acetate pH 6.5. hLH estimated by RIA eluted at about 0.3 M buffer.

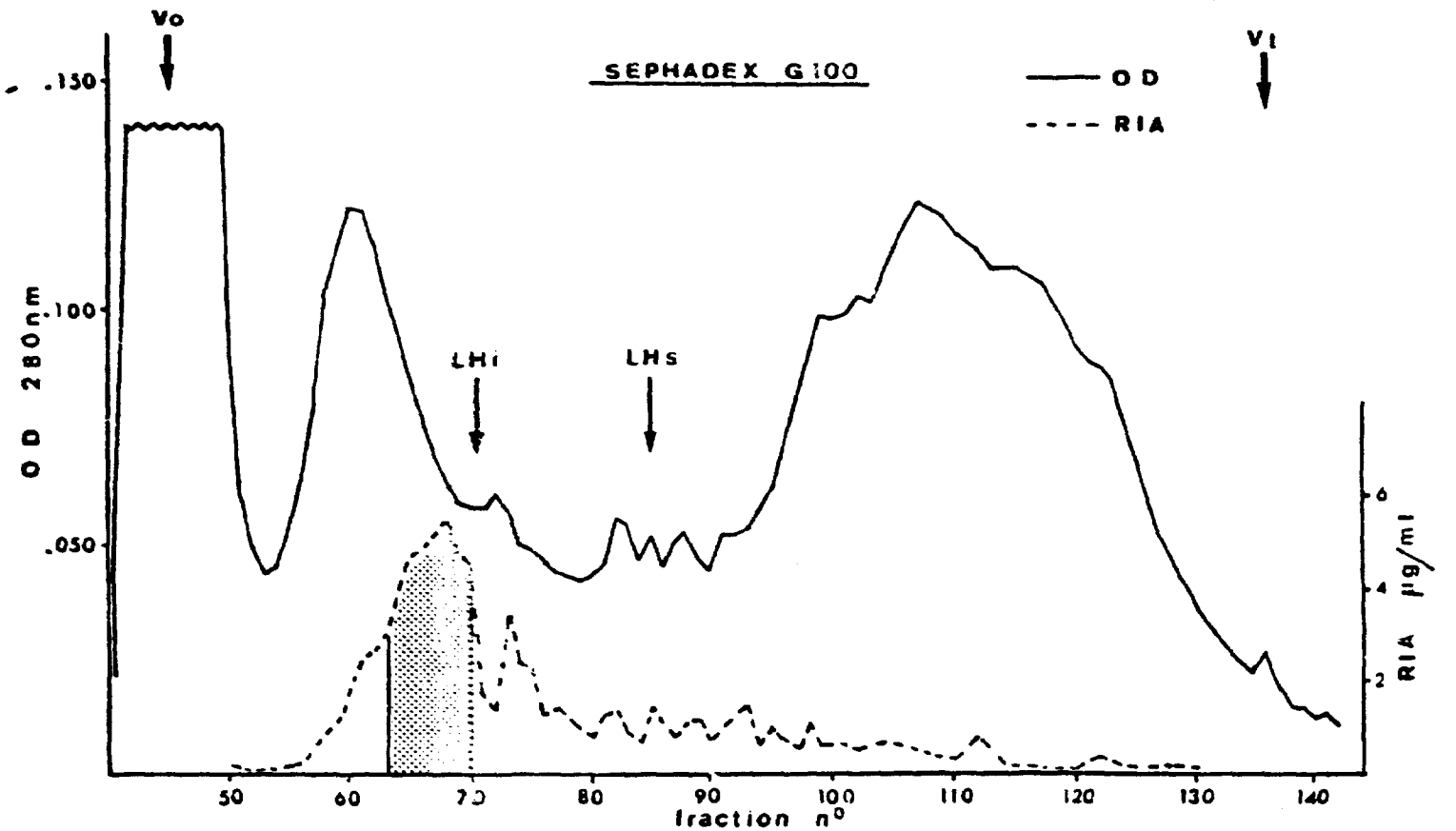

FIG. 2 - Chromatography of DEAE pooled fractions on SEPHADEX G100. Column: 2.5 x 90 cm, flow - 12ml/h, fractions = 3 ml. Elution buffer: 0.02M sodium acetate pH 6.5 The hLH fractions were pooled according to the RIA results.

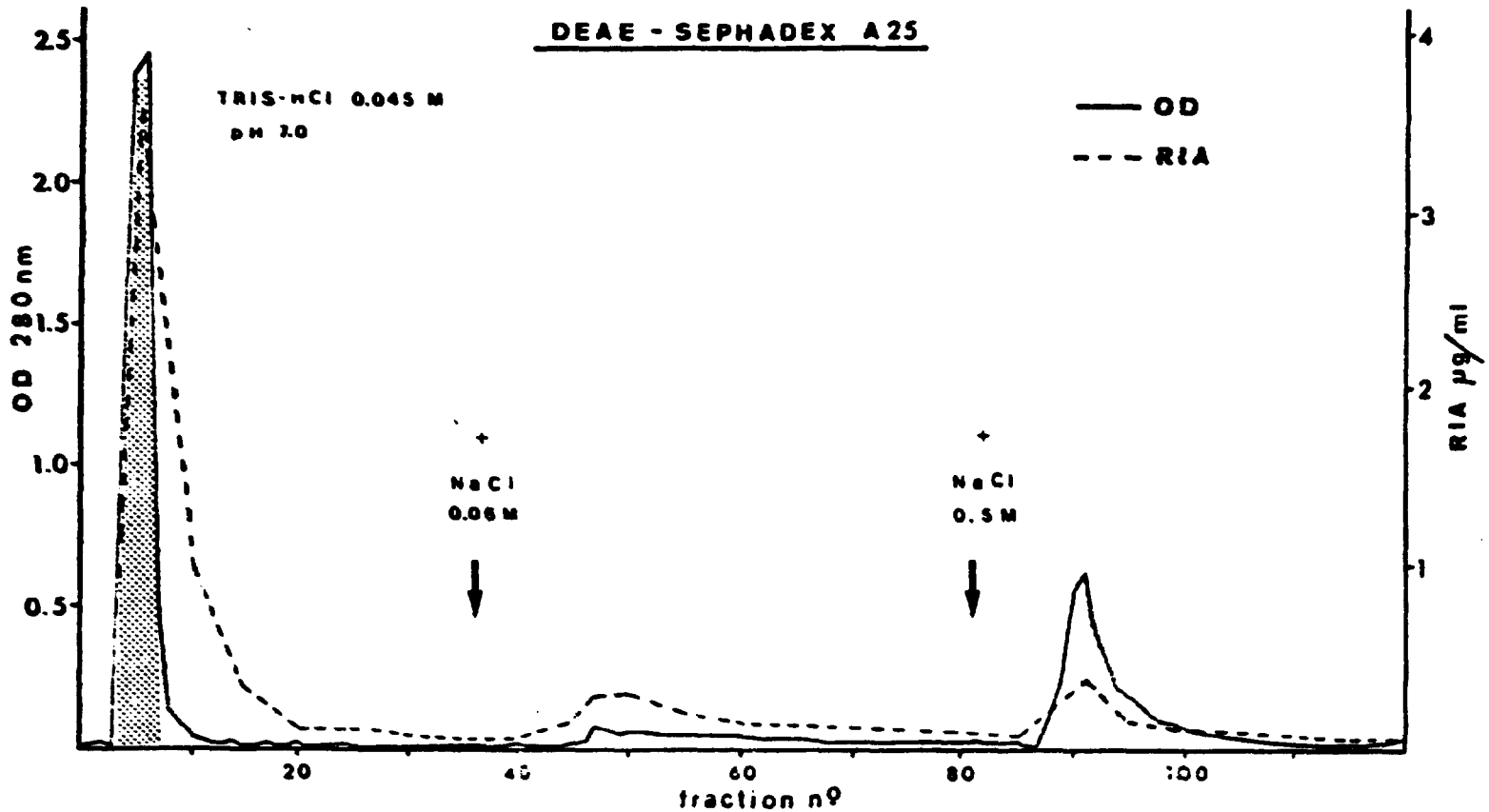

FIG. 1- Purification of the pituitary glycoprotein extract on DEAE-SEPHADEX A25 column: 1.1 x 27cm, 12ml/h, fractions = 3ml. Stepwise elution with 0.045 M TRIS-HCl pH 7.0 with 0.06M and 0.5M NaCl added as indicated by arrows. hLH was eluted on the unadsorbed fraction and pooled according to the RIA results.

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