APPLICATION OF A HUMAN THYROTROPIN PURIFIED FROM HYPOPHYSES AT IPEN-CNEN/SP IN THE PREPARATION OF THE RADIOIMMUNOASSAY TRACER: [^{125}I] hTSH.

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SUMMARY

This paper reports the isolation of thyrotropin (hTSH) from a side-fraction obtained during the purification of growth hormone at IPEN (Somatormon®) and stored at -20ºC since 1981.

It also evaluates the adequacy of the hTSH-IPEN aslabelled reagent in the radioimmunoassay (RIA) method in comparison to imported hormone,supplied by the National hormone and Pituitary Program (Baltimore, Md., U.S.A.).

The results suggest the fitness of long time stored crude preparations in the attainment of pure hTSH for labelling purposes and consequent use as reagent in RIA.

INTRODUCTION

The present study form part of a project developed to prepare biological reagents for the radioimmunoassay of human hypophyseal hormones in order to replace imported products with confirmed quality.

In this way, growth hormone(hGH) and lutropin have been isolated from hypophyses and employed in the development of specific radioimmunoassays at IPEN (1,2), while the purification of follitropin and prolactin are in progress.

This paper reports the isolation of thyrotropin (hTSH) from a side-fraction obtained during the purification of hGH in the begining of 1980's. It also evaluates the adequacy of this purified hTSH for labelling with ^{125}I in the preparation of the radioimmunoassay tracer.

MATERIALS AND METHODS

Preparative methods:

The starting material was a concentrated hTSH obtained at IPEN in 1981 as a side-fraction during the processing of whole frozen human hypophyses for the production of hGH (Somatormon®). The procedure involves homogenation of the glands, extration of the homogenate, ammonium sulfate fractionation and chromatography on Sephadex G-100.

The adequacy of this lyophilized long time stored preparation in the attainment of pure hTSH was evaluated previously(3), according to the procedure described by Roos et al(4).

Thus, the hTSH purification was perfomed following the same procedure, starting from a larger amount of crude material by successive chromatographies, as shown in Table I.

The absorbance of eluant fractions in the different fractionation steps was determined at 280 nm by use of a Zeiss Model PMQ-II spectrophotometer.

Analytical methods:

The protein determination of the samples submitted to the different chromatographies was performed by absorbance measurements, assuming that an absorbance of 1.0 in 1-cm quartz cells at 280 nm corresponds to 1 mg protein per ml solution(4).

The method of Lowry et al (5) was employed in the protein measurement for the estimation of the hTSH specific activities from the sucessive purification steps.

The hTSH content was determined by specific radioimmunoassay (RIA) developed at our laboratory. The reagents employed, purified hTSH-I-6 for iodination and anti-hTSH-3 antiserum, were provided by the National Hormone and Pituitary Program (NHPP-Baltimore, Md., U.S.A.) and the 63/14 MRC Research Standard A by the National Institute for Medical Research (Holly Hill, London, U.K.).

The purified hTSH (approximatlly 5 ug) was labelled with $Na^{125}I$ (0.8 mCi) by classical method employing chloramine T (50 ug) and metabisulfite (200 ug). The iodinated hormone was purified on a Sephadex G-100 column (2 x 40 cm) at 4ºC and eluted with 0.05 M phosphate buffer pH 7.4 containing 0.1% BSA.

The peak-related fractions from the tracer purification were identified by its distribution coefficients (Kd) and evaluated by its reactivity to excess of antiserum.

The RIAs were carried out for 48 h in phosphate-buffered saline containing 0.1% BSA by a non-equilibrium method, in which the tracer addition is delayed by 24 h. The free and bound $[^{125}I]$ hTSH were separated by precipitation of the latter with polyethyleneglicol 6000 (PEG) or with a second antibody (SA) purchased form the Radioassay System Laboratories (Carson, Calif., U.S.A.), calculating the percentage of $[^{125}I]$ hTSH bound to its antiserum.

TABLE I

PURIFICATION PROCEDURE OF HUMAN HYPOPHYSEAL THYROTROPIN

CRUDE THYROTROPIN (1.5 g)

DIALYSIS
0.1 M buffer pH 7.0 + 0.3 M NaCl

SEPHADEX G-100 CROMATOGRAPHY (248 mg)
column size, 5 × 70 cm
flow rate, 40 ml/h and fraction volume, 8.5 ml
elution with the same dialysis buffer

CONCENTRATION BY ULTRAFILTRATION

DIALYSIS
0.03 M buffer pH 7.0

HYDROXYAPATITE* CHROMATOGRAPY (73.5 mg)
column size, 5.2 × 6 cm
flow rate, 8 ml/h and fraction volume, 15 ml
stepwise elution with 0.03 - 0.05 - 0.2 - 0.5 M buffer pH 7.0

DIALYSIS
0.005 M buffer pH 7.0

SP-SEPHADEX C-50 CHROMATOGRAPHY (37.7 mg)
column size, 1.6 × 20 cm
flow rate, 9 ml/h and fraction volume, 2 ml
stepwise elution with 0.005 - 0.02 - 0.06 - 0.5 M buffer pH 7.0

All buffers were prepared with potassium phosphate and the columns were equilibrated with the same buffer employed in the precedent dialysis.

* The hydroxyapatite was prepared at our laboratory(3) according to Tiselius et al(7).

RESULTS AND DISCUSSION

Fig. 1 shows the elution pattern of one occasional chromatography on Sephadex G-100 of hGH purification. The fractions within the hatched area were pooled, lyophilized and stored at -20ºC during 7 years until be submitted to hTSH purification.

Figs. 2 to 4 illustrate the elution profiles of the hTSH purifications on Sephadex G-100, hydroxiapatite and SP-Sephadex C-50, respctively. Hatched areas indicate the hTSH fractions pooled for subsequent processing.

Sephadex G-100 chromatogram (Fig.2) shows a small protein peak of a high molecular component (possibly an aggregate), not evidenced in the previous experiment using a shorter column(3).

The active material was eluted in the further purifications (Figs.3 and 4) at the same buffer concentrations determined in the previous experiment by RIA(3). However, a protein peak not observed in those purifications was eluted immediately before the hTSH peak, indicating a probable alteration in the stored material.

In this final chromatography (Fig.4) the hTSH was separated in two peaks, having the latter higher protein content (1.85 against 0.81 mg) and higher activity (1.01 against 0.17 units/mg). Therefore, this second peak corresponds to the hTSH-IPEN.

The activities and recoveries from the sucessive purifications are summarized in Table II. It can be seen that the hTSH increased 7 times during the purification, according to the previous experiment (8 times). However the increment founded by Roos et al starting from fresh material was 46 times (4).

The low hTSH potency of our preparation (1.01 units/mg) was around 1/5 (6) and 1/10 (4) of other preparations, also determined by RIA employing the same Standard A. This lower activitie can be attributed to the long storage period of the glands before the hGH extration summed to the several years of storage of the hTSH crude preparation.

In spite of its low activity, when the hTSH-IPEN was labelled with ^{125}I it presented in the Sephadex G-100 purification (Fig.5) Kd value (0.25) of the same order of those revelead by the hTSH-NHPP (mean value ± SD of 0.26 ± 0.01 from 5 experiments). On the other hand, the [^{125}I] hTSH is masked by an excess of BSA which carries part of the free ^{125}I. Besides, the small peak observed at the void volume (Kd=0) indicates the presence of [^{125}I] hTSH degradation products.

Fractions corresponding to hatched area from the chromatogram, which presented higher binding to the antiserum by PEG precipitation (from 29 to 32%), were pooled and stored at -20ºC .

This tracer was tested in a antiserum tritation curve by SA separation (Fig. 6) determining the 1:1.000.000 dilution for use in the RIA.

Fig. 7 illustrates the displacement of the IPEN tracer by the Standard A in comparison with the NHPP hormone radioiodinated at our laboratory by the same method. The very similar standard curves indicate that our hTSH preparation is equally suitable as labelled reagent in the RIA method as the imported hormone with

confirmed quality.

Nevertheless, some problems as the presence of BSA in the tracer must be solved, needing additional investigation.

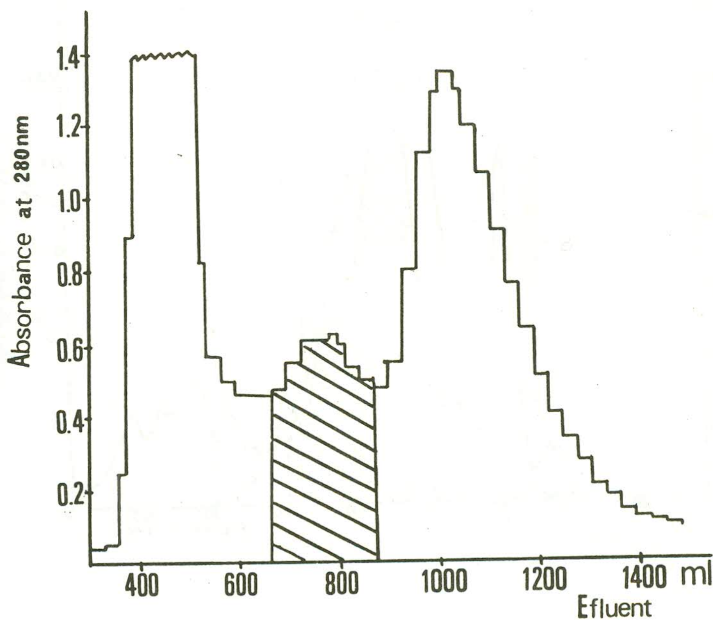

Fig.1. Molecular sieve chromatography on Sephadex G-100 of $(NH_4)_2SO_4$ precipitate (1.2 g) from human hypophyseal extract. Column (5 x 90 cm) equilibrated and eluted with 0.5 M glycine-phosphate buffer pH 7.2. Flow rate, 43 ml/h and fraction volume, 15 ml.

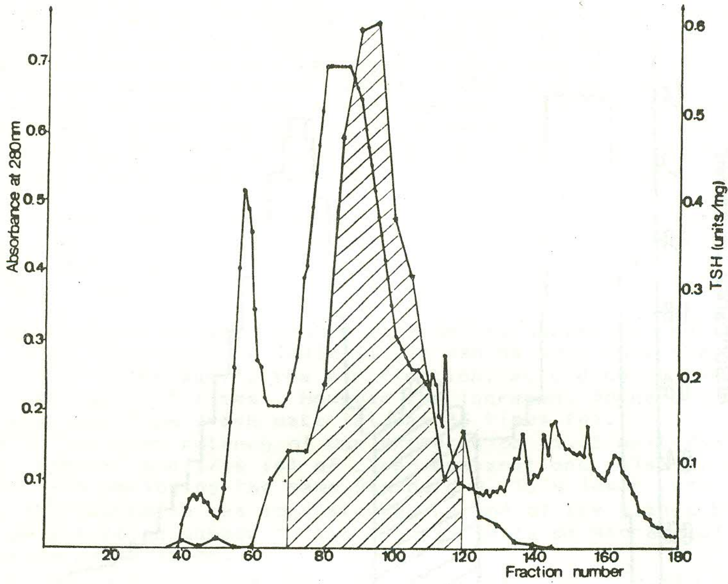

Fig.2. Molecular sieve chromatography on Sephadex G-100 of hTSH fractions. ●——● , absorbance at 280 nm; ★——★ , thyrotropin activity.

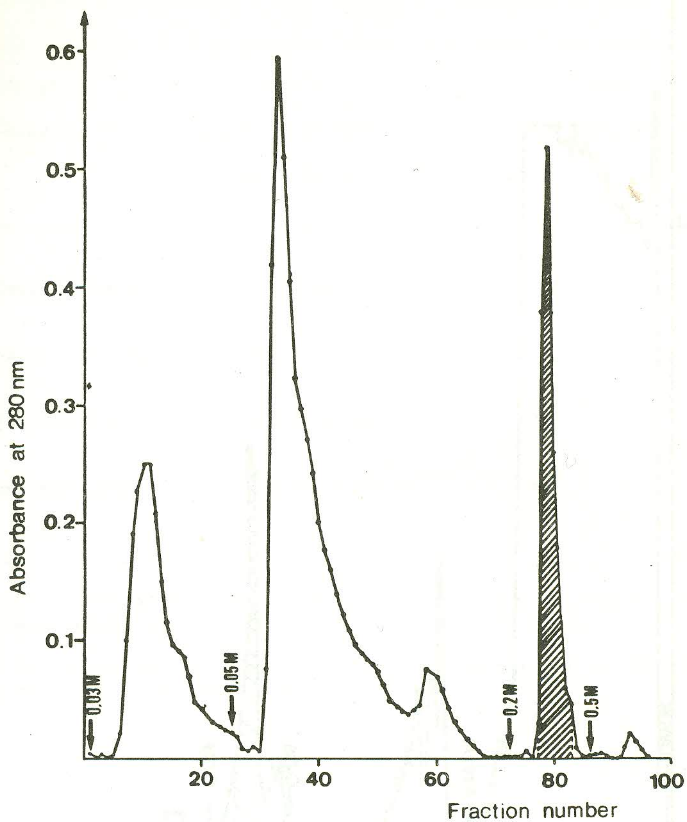

Fig. 3. Hydroxiapatite chromatography of hTSH preparation following gel filtration on Sephadex G-100. Stepwise elution is indicated by arrows.

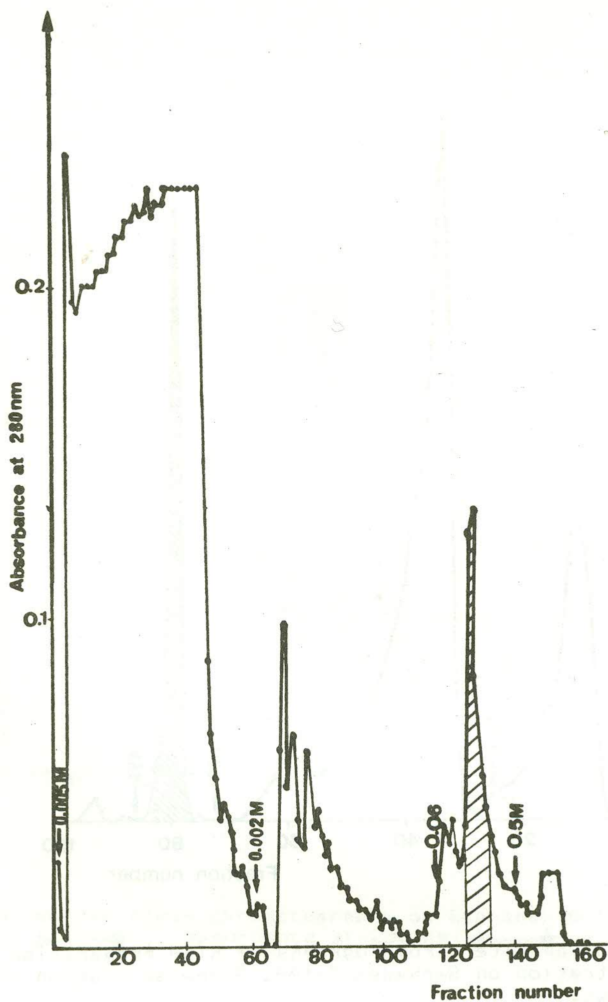

Fig. 4. Ion-exchange chromatography on SP-Sephadex of hTSH fractions from hydroxiapatite chromatography. Stepwise elution is indicated by arrows.

TABLE II

PURIFICATION OF HUMAN HYPOPHYSEAL THYROTROPIN

Purification step	hTSH specific activity (units/mg)*	hTSH recovered activity units	%
Sephadex G-100(#1)	0.15 ± 0.01 (4)	37.0	100
Sephadex G-100(#2)	0.15 ± 0.02 (4)	11.1	30
Hydroxyapatite	0.26 ± 0.04 (4)	9.7	26
SP-Sephadex C-50	1.01 ± 0.17 (4)	1.9	5

* Activities are given as means ± SD with the number of hTSH radioimmunological determinations in parentheses.

* 0.05 units is the radiommunological activity of the contents of one ampoule of Standard A.

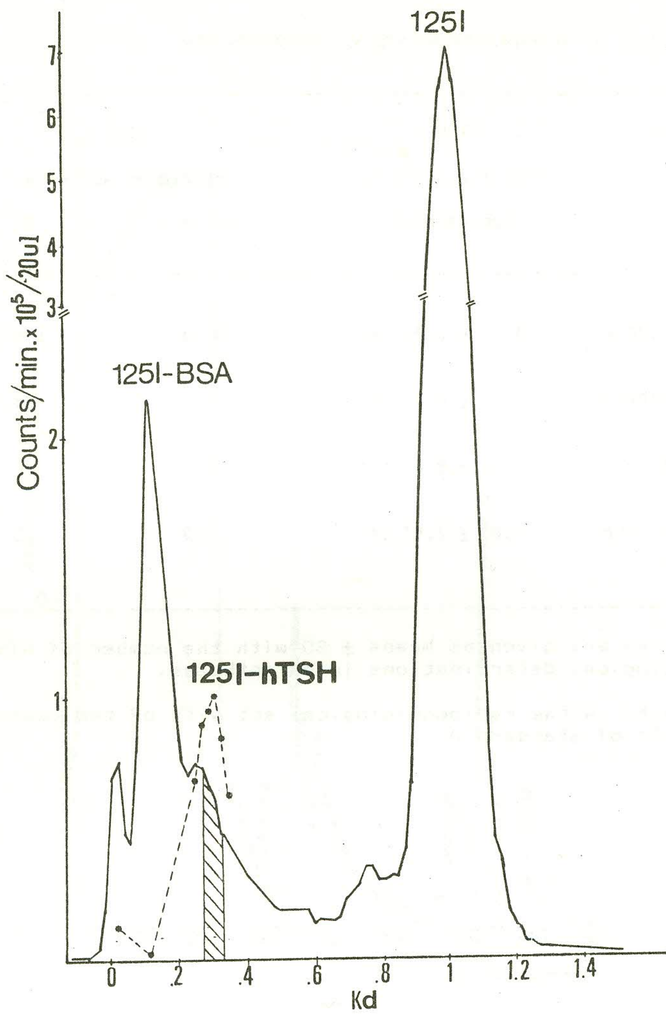

Fig. 5. Molecular sieve chromatography on Sephadex G-100 of [^{125}I] hTSH-IPEN. The dotted line shows the specific bindings of the fractions to the anti-hTSH antiserum.

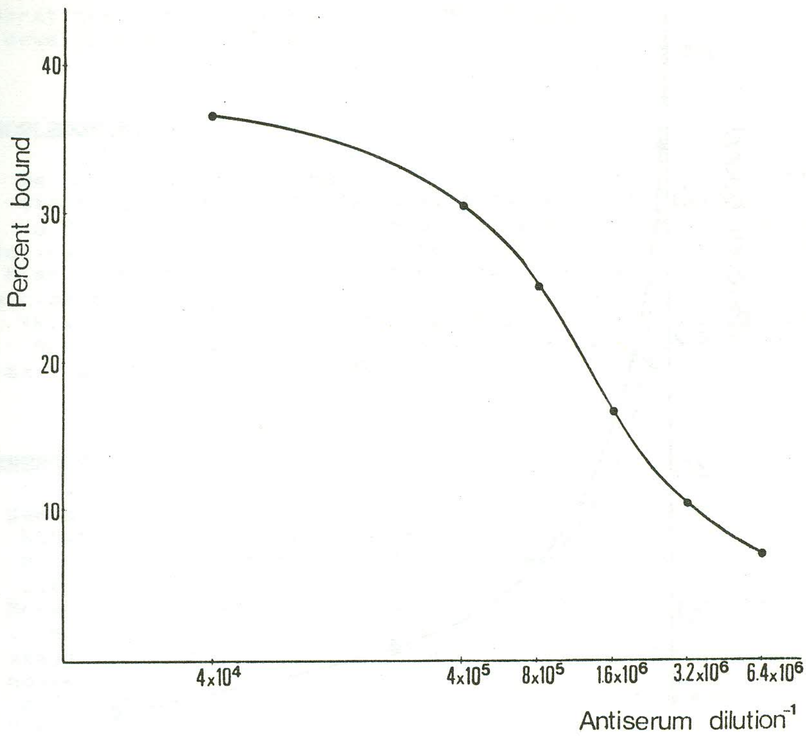

Fig. 6. Titration of anti-hTSH antiserum by RIA employing the [125I] hTSH-IPEN.

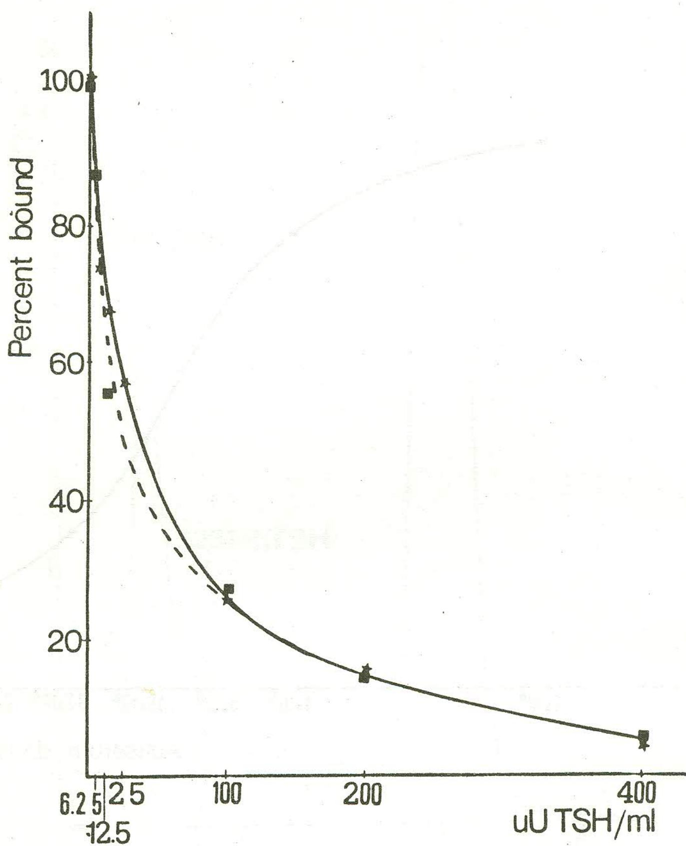

Fig. 7. Comparison of the displacement of [^{125}I] hTSH from different preparations by Standard A and by SA precipitation; ★——★, hTSH-IPEN; ■-----■ hTSH-NHPP.

CONCLUSION

The hTSH isolated at IPEN from long time stored crude preparation when labelled with ^{125}I and tested for its use as reagent in RIA was comparable to imported product, provided by the NHPP.

Considering the restricted supply of hypophyses, old crude preparations are an alternative hormonal source to be employed in the development of hTSH RIA.

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