PREPARATION OF HIGH-QUALITY IODINE-125-LABELLED PITUITARY HUMAN FOLLICLE-STIMULATING HORMONE (hFSH) FOR RADIOIMMUNOASSAY: COMPARISON OF ENZYMATIC AND CHLORAMINE-T IODINATION

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Cidade Universitária "Armando de Salles Oliveira"
SÃO PAULO – BRASIL

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PREPARATION OF HIGH-QUALITY IODINE-125-LABELLED PITUITARY HUMAN FOLLICLE-STIMULATING HORMONE (hFSH) FOR RADIOIMMUNOASSAY: COMPARISON OF ENZYMATIC AND CHLORAMINE-T IODINATION*

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ABSTRACT

A method is described for the enzymatic radioiodination of human follicle-stimulating hormone (hFSH) by a system consisting of lactoperoxidase, hydrogen peroxide and $Na^{125}I$. It was compared with the Chloramine-T modified technique. A satisfactory specific activity of the labelled hormone was obtained with the enzymatic iodination, with much greater immunoreactivity and stability than after Chloramine-T.

INTRODUCTION

The degradation of the labelled hormone is presently one of the major difficulties encountered in the radioimmunoassay (RIA) and receptor assay of gonadotropins. The Chloramine-T method for radioiodination of polypeptide hormones combining high efficiency with simplicity is the most widely used. It may, however, induce oxidative changes in the protein to be labelled.

FSH is known to be easily damaged by Chloramine-T, producing iodohormones with anomalous physicochemical and immunochemical characteristics, the former being studied by chromatoelectrophoresis, where the damaged fractions are in greater proportion with increasing concentrations of the powerful oxidizing agent.

Electrolytic iodination[12] has the advantage of not affecting the protein structure to any appreciable extent but it has proven technically difficult to obtain the required high specific activities when handling very small volumes.

In this paper we report our experience with enzymatic iodination[7,17] of hFSH by lactoperoxidase, producing a labelled hormone which appears quite stable over a 3-week period, as well as having great affinity to the specific antisera. Also, a comparative study was performed with the Chloramine-T method.

MATERIALS AND METHODS

Lactoperoxidase (Calbiochemical Co., U.S.A.) was prepared as a stock solution at 5 $\mu g/\mu l$ in 0.05 M sodium phosphate buffer (pH 7.5) and stored at $-20^{\circ}C$. Before use the stock solution was diluted 1 : 10.

* Area of Isotope and Radiation Applications in Medicine, C A B R R., Instituto de Energia Atômica and Diabetes and Adrenal Unit, Hospital das Clínicas, Universidade de São Paulo, São Paulo – Brasil.

** To whom correspondence should be adressed

Hydrogen peroxide 30% (Perhydrol®) was obtained from Merck, A.G., G.F.R.

$Na^{125}I$ from Behringwerke A.G., G.F.R. with a specific activity of at least 200 mCi/ml, specified as carrier free without reducing agents, was used.

BioGel P-60 (100 – 200 mesh) was obtained from BioRad Labs., Los Angeles, U.S.A.

Hormone preparation. Purified hFSH (LER 1575-C) was obtained from the National Pituitary Agency (N.P.A.) through the Hormone Distribution Office of the National Institute of Arthritis, Metabolism and Digestive Diseases.

Standard hFSH (LER 907) prepared from human pituitaries (N.P.A.) was used as the hFSH standard (1 mg LER 907 = 35 I.U., by immunoassay) and our results are reported as mI.U./ml.

Antisera prepared in rabbits and preadsorbed with hCG, were obtained from the National Pituitary Agency (N.P.A.) through the Hormone Distribution Office of the National Institute of Arthritis, Metabolism and Digestive Diseases.

a. Enzymatic Radioiodination of hFSH

The reaction was carried out at room temperature (25°C) in small polystyrene tubes (11 x 68 mm). There were added in the following order:

1) 2.5 μg (25 μl) of hFSH (LER 1575-C).

2) 30.0 μl of 0.5 M sodium phosphate buffer pH 7.6.

3) 200 μCi of $Na^{125}I$ (3 – 5 μl).

4) 10.0 μg of lactoperoxidase.

5) 300 ng (10 μl) of hydrogen peroxide added twice, at 2-min intervals

6) At the end of the incubations, the reaction was stopped by dillution with 0.5 ml of 0.05 M phosphate buffer (pH 7.5), containing 0.1% bovine serum albumin.

b. Radioiodination of hFSH by Chloramine-T Method

A standardized method, using Chloramine-T, was modified in our laboratory, with reduction in the amount of the oxidative agent to 1 μg of Chloramine-T/1 μg of hormone, to minimize overoxidation and iodination. The reagents were added as follows:

1) 2.5 μg (25 μl) of hFSH (LER 1575-C).

2) 25 μl of 0.5 M sodium phosphate buffer (pH 7.4).

3) 200 μCi of $Na^{125}I$ (3 – 5 μl).

4) 2.5 μg of Chloramine-T (2.5 μg/μl of sodium phosphate 0.05 M pH 7.4).

After 20 seconds, were added:

5) 25 μg of sodium metabisulfite (5 μg/μl of 0.05 M sodium phosphate (pH 7.4)).

6) 100 µl of "blue plasma" (plasma + bromophenol)

The reaction was carried out at 4°C, in small glass tubes (11 x 68 mm)

A small aliquot of the iodination mixture, obtained by each method was removed for the assessment of the radioiodinated hormone (chromatoelectrophoresis). Part of the remainder was immediately applied to starch gel electrophoresis and part was purified in a BioGel P-60 column.

c. Purification of the Labelled Hormone

To purify the labelled hormone preparation freeing it from unreacted iodine and from damaged components, the following methods were employed:

1. Starch gel electrophoresis[3,13] The starch gel was prepared by the method of Smithies[15,16], using Connaught hydrolyzed starch. The autoradiography of ^{125}IFSH on starch gel showed one fraction migrated behind the region of the albumin stain after Chloramine-T. However, the electrophoretic behaviour of the hormone labelled by enzymatic iodination, was slightly different (less migration to the anode) from that after Chloramine-T[6].

2. Purification by BioGel P-60 filtration (1 x 25 cm column). An aliquot of the iodination mixture also purified on a BioGel P-60 column (100 – 200 mesh) previously washed with 2 ml of 2% bovine serum albumin (Sigma, U.S.A.) in 0.05 M PBS (sodium phosphate buffer with 0.14 M sodium chloride) (pH 7.5), and subsequently equilibrated with PBS.

Aliquots of 1 ml were collected in tubes containing 0.05 ml of BSA-PBS 2% and counted in a well-type gamma scintillation counter. The specific activity was calculated on the basis of the amount of radioactivity incorporated into FSH fractions isolated by gel filtration.

3. Characterization of the labelled hormone. Aliquots of labelled hFSH were taken before and after purification and submitted to paper chromatoelectrophoresis[5,17], allowing us to determine some physicochemical characteristics of the iodohormone, specially the damaged fraction and the degree of purification.

Immunoassay (RIA)

hFSH was measured by the double-antibody radioimmunoassay (RIA) technique[10], with previous testing of the immunoreactivity of the purified fractions of the labelled hormone, choosing the best one for the assay.

100 µl of serum sample or a known amount of hFSH (LER-907) were added to 100 µl of anti-human FSH serum; the dilution of the latter (1 : 10 000) was sufficient to bind 30 – 40% of the [^{125}I]FSH, in the absence of unlabelled hFSH. The tubes were incubated for 24h at 4°C and then 100 µl of [^{125}I]hFSH (10 000 cpm or 50 pg) were added. After a 3-day incubation at 4°C, 100 µl of a potent sheep anti-rabbit serum (dilution 1 : 4) were added. The tubes were incubated for an additional 24 h at 4°C and then centrifuged for 20 min at 3500 rpm (2500 x g) and the supernatant decanted. The precipitate was counted in a well-type gamma counter. The binding was expressed as the percentage of the added radioactivity recovered in the precipitate.

RESULTS

Radioiodination

Considering that hFSH labelling with Chloramine-T (Figure 1) produces a more highly substituted labelled hormone with more damage on incubation and providing a less sensitive radioimmunoassay, labelling with lactoperoxidase despite its low yield (Figure 2) has the advantage that it is possible to control the degree of iodination with changes in the exposure time and amounts of the oxidizing agent. This can be seen in Table I where the results are indicated of 6 labelling procedures with lactoperoxidase, showing that the immunoreactivity (percentage binding to excess of antibody) is greater with a shorter exposure time to the oxidizing agent. Besides, on chromatoelectrophoresis of the labelled hFSH (Figure 2) it is clearly indicated that the damaged fractions increase with the longer reaction time. Furthermore, it can be noticed that if the reaction is not stopped, by dilution or adding an enzymatic inhibitor (Table II), there is a greater yield but with a reduction in immunoreactivity, and a labelled hormone is obtained with similar characteristics to that obtained after Chloramine-T (Table III).

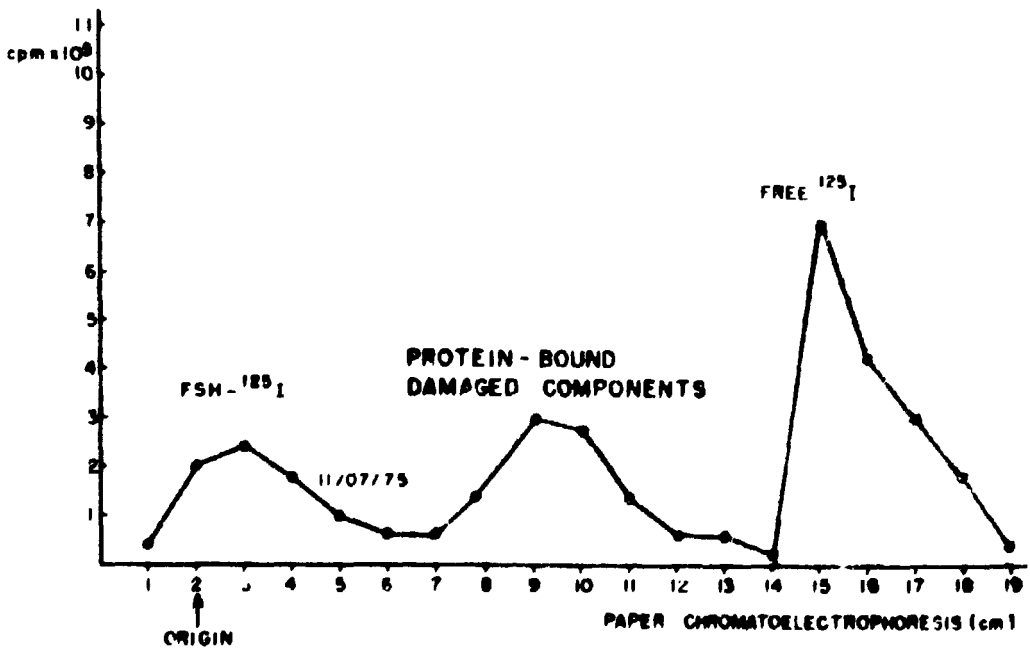

Figure 1 – Chromatoelectrophoresis of ^{125}I-hFSH immediately after Chloramine–T iodination

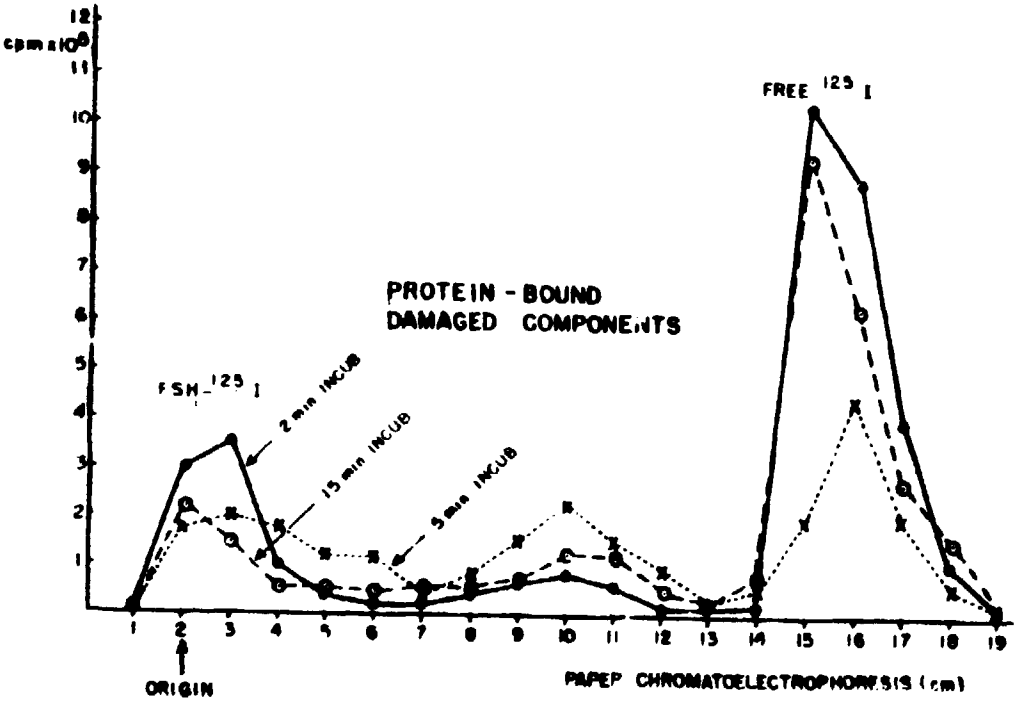

Figure 2 — Chromatoelectrophoresis of ^{125}I-hFSH immediately after enzymatic iodination

Table I

Conditions for Radioiodination of FSH Using Lactoperoxidase (10 μg)

Date	Efficiency of iodination** (%)	Time of incubation (min)	Spec. act. (μCi/μg)	Efficiency after purification*		
				Adsorption to talc (200 mg)	Binding excess Ab(%)***	Adsorption talc after 7 days
74-11-26	55	15	100	68	18	61
74-12-22	47	10	108	70	22	64
75-01-16	51	15	105	69	17	59
75-01-30	36	5	100	79	36	74
75-02-13	30	2	105	85	50	81
75-05-06	28	2	108	90	46	86

* BioGel P-60 column.

** % of iodide-125 incorporated.

*** Dilution of anti-FSH serum, 1:1000. Time of incubation, 24 h.

Table II

Comparison of Immunoreactivity of Labelled hFSH
After Enzymatic Radioiodination,
With and Without Stopper

Date Date	Binding to excess of Ab (%)	
	With stopper*	Without Stopper
75-02-13	50	22
75-05-06	46	17

* After the last incubation with H_2O_2, 0.5 ml of 0.01 M PBS (pH 7,5) was added.

Table III

Comparison of Immunoreactivity of Labelled hFSH
Eluates from BioGel P-60, After Iodination by
Chloramine-T and Lactoperoxidase

Fraction Fraction	Binding to excess of Ab (%)	
	Chloramine-T	Lactoperoxidase
4	16	30
5	19	43

Purification of the Labelled Hormones

Starch gel electrophoresis. The fraction extracted from gel was tested for the percentage of total radioactivity in that section and for "purity" (undamaged [^{125}I]hFSH has the percentage of total radioactivity in gel section adsorbed to plasma-coated talc) When lactoperoxidase was compared to Chloramine-T, it was evident that the percentage of total radioactivity and "purity" of the extracted fraction were greater with the latter but the immunoreactivity evaluated in a double-antibody system was greater with lactoperoxidase, as expected (Table IV)

2. **BioGel P-60 (100 – 200 mesh) filtration**. The purification on BioGel P-60 column presented 3 radioactive components (Figure 3), similar to those observed on paper chromatoelectrophoresis. Undamaged [^{125}I]hFSH, corresponding to about 25% of the total radioactivity, was eluted as a peak in fractions 4 – 6, in the same elution volume previously described for non-labelled hFSH[11]. In fractions 1 – 3 the components with greater molecular weight (molecular aggregates) are found, corresponding to the damaged fractions present in the labelled preparations. For better characterization of the radioiodinated product in relation to the molecular integrity and homogeneity, the effluents from BioGel P-60 column were tested by paper chromatoelectrophoresis (Figure 4). In the eluates numbers 4 and 5 (Figure 3), the greatest contents of the undamaged hormone are concentrated, adsorbed to the paper at the site of application (Figure 4). Fraction 4, having a greater percentage of radioactivity

Table IV

Starch Gel Purification of [^{125}I]-FSH Labelled With Chloramine-T and Lactoperoxidase

Method	% Total radioactivity extracted	% "Purity" (talc adsorption)	Spec. act. (μCi/μg)	Binding to excess of Ab (%)
Chloramine-T	72%	71%	105	18
Lactoperoxidase	35%	60%	108	49

however has more damaged components than fraction 5, for both iodination techniques. Immunoreactivity of the labelled hormone, determined by addition of excess of antibody in the double-antibody system, indicated that fraction 5 is better for radioimmunoassay (Table III). Furthermore, as it can be seen, the immunoreactivity was greater with lactoperoxidase. Eluates from fractions 7 – 10, representing 70 to 80% of the total radioactivity after lactoperoxidase and from 40 to 50% with Chloramine-T iodination (Figure 3), tested by chromatoelectrophoresis and adsorption to an ion-exchange resin (Ioresin-Abbott) corresponding to free, unreactive ^{125}I, did not show immunoreactivity, as expected.

Purified ^{125}I-FSH, with a specific activity of 0.5 mol ^{125}I/mol hormone, was diluted to a concentration of 30 – 50 pg of the labelled preparation in 0.1 ml buffer, to avoid damage of the radioiodinated hormone and subsequent loss of sensitivity of the standard curve. Radiochemical damage was detected by adsorption to plasma-coated talc (200 mg talc + 0.1 ml of blood Bank plasma)[10] and by binding to an excess of antiserum. Besides, it was noticed that there is a loss of about 10% of the molecular integraty and 20% in the immunoreactivity, after 15 days of storage, with the enzymatic labelling of the hormone. However, after Chloramine-T, there was already a loss of 25% in molecular integrity and 33% in immunoreactivity after 7 days of storage. It was also evident that the fraction of [^{125}I]FSH diluted to 30 – 50 pg/0.1 ml buffer and kept at 4°C was more stable than if kept undiluted and frozen.

In Figure 5, a typical standard curve is indicated for human FSH in the range 1 – 50 mI.U./ml of LER 907 or 44 to 1750 ng/ml, using [^{125}I]hFSH prepared with lactoperoxidase and Chloramine-T. It is evident that, within the range studied, the curve obtained with enzyme-iodinated hFSH sensitive and precise whereas after Chloramine-T there was no precision, at higher levels of hFSH. The lower limit of sensitivity is 4.4 ng, corresponding to a plasma concentration of 44 ng/ml (1.2 mI.U./ml).

DISCUSSION

More highly iodinated hFSH preparations usually obtained with Chloramine-T are less satisfactory when used as a tracer in RIA procedures, because of lesser immunoreactivity and greater susceptibility to damage.

The purpose of this paper was to devise a method for the radioiodination of hFSH that would give specific activities high enough for radioimmunoassay but without loss of immunoreactivity.

We have tried to modify the Chloramine-T method[2,4] for iodination of hFSH making it suitable for our purpose by varying the amount of the oxidizing agent and/or the reducing agent, the reactions being carried out either at 4°C or at room temperature. The time exposure of the hormone to the oxidizing agent varied from a couple of seconds up to 10 minutes and the specific activity of the

radioiodinated hormone from 40 to 120 μCi/μg. The purification procedures were also variable, sometimes three techniques being used in sequence* starch gel electrophoresis, Sephadex gel filtration and cellulose chromatography. The results were uniform, excellent incorporation of the label into protein but poor immunoreactivity (10 to 20%), decreasing rapidly on storage. However, the use of small amounts of Chloramine-T and sodium metabisulfite appears to be more efficacious for iodination of hFSH[14]. The immunoreactivity is further improved when the RIA is performed on the same day of iodination but the "incubation damage", despite being lower, is still high enough.

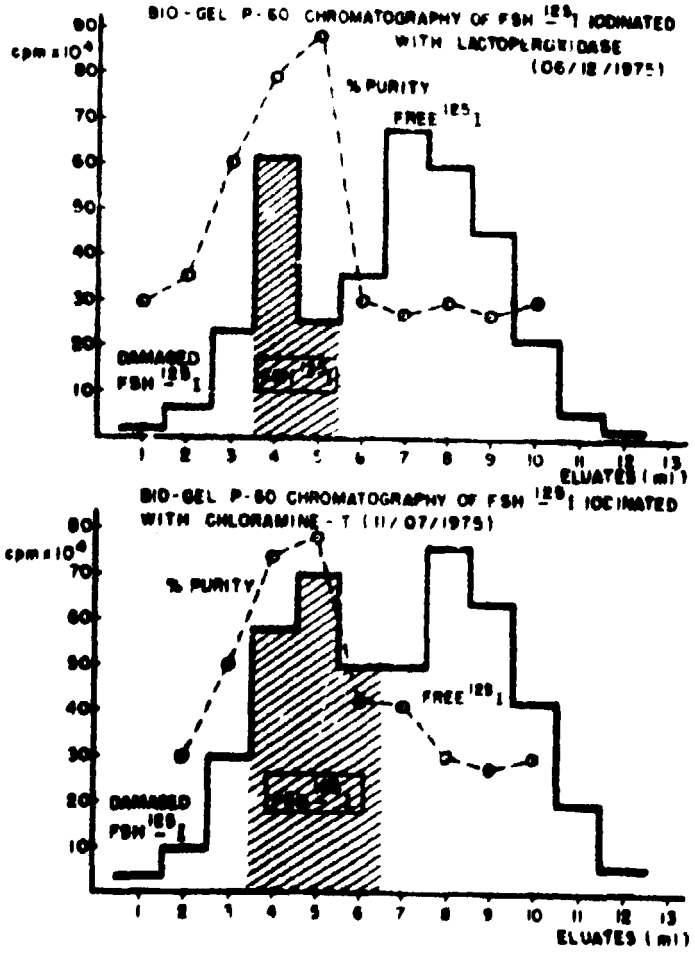

Figure 3 – BioGel P-60 chromatography of ^{125}I-hFSH

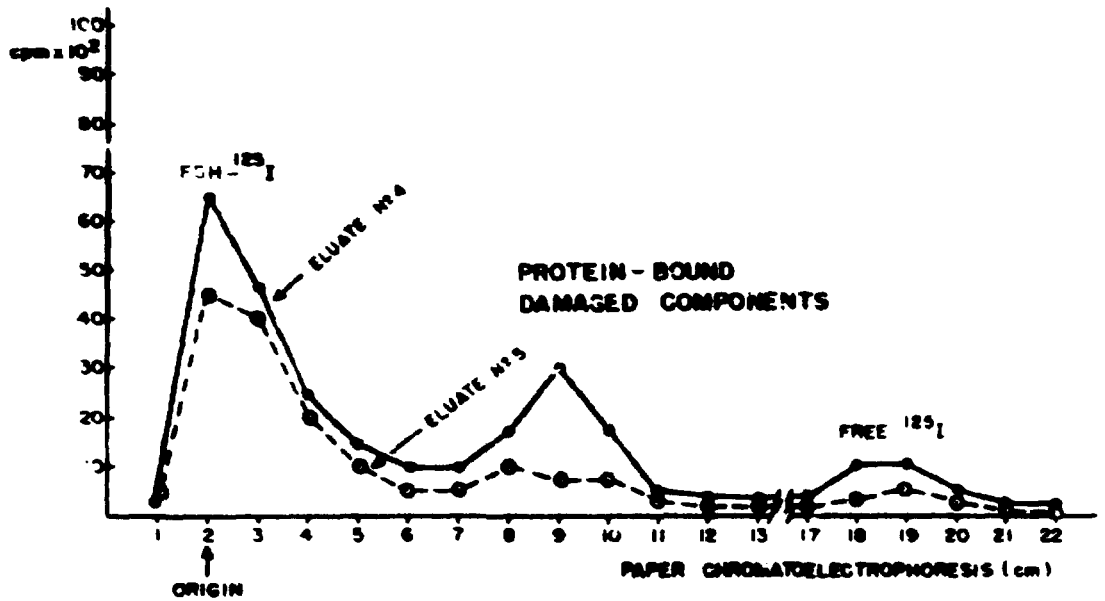

Figure 4 — Paper chromatoelectrophoretogram of fractions 4 and 5 of ^{125}I-hFSH (lactoperoxidase iodination) eluted from BioGel P-60 column.

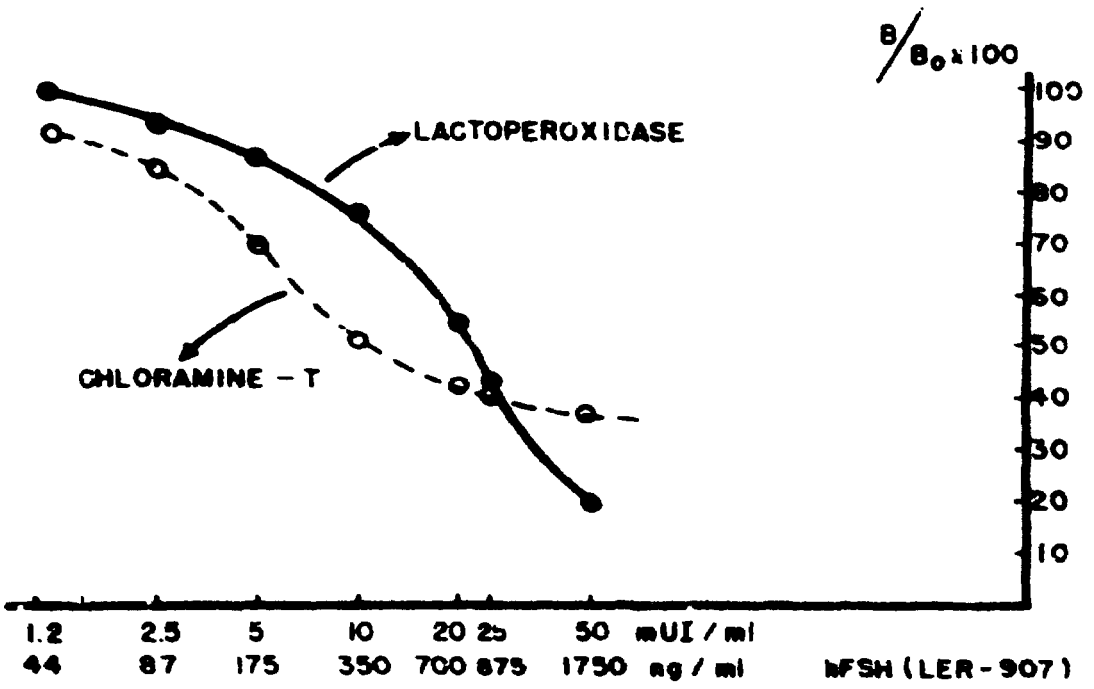

Figure 5 – Standard curves for RIA of hFSH Each point was obtained in triplicate (75.00.04).

Comparison of the radioiodination using Chloramine-T modified and the enzymatic procedures revealed a greater binding affinity to the antibody by the enzyme-iodinated hFSH (Table IV).

Several important characteristics of the enzymatic technique must be indicated:

1. During the iodination procedure, the effect of hydrogen peroxide was more pronounced with longer incubation time, increasing the efficacy of iodination while decreasing the immunoreactivity. Since Reichert has shown that the pituitary glycoproteins are susceptible to inactivation by H_2O_2[11] the amount of hydrogen peroxide should be kept at the minimum amount (300 ng) required for activation of lactoperoxidase, acting for a very short period of time (2 – 3 min) and then a "stopper" is needed, diluting the reaction mixture (Table II).

2. The rate of incorporation of iodine has been shown to vary considerably with pH[9]. The pH used in our studies was 7.4, at variance with that employed by the several authors[8], consistent with sufficient rate of labelling and maximal stability of the protein hormone solution[7,17].

3. For the purification method, it was evident that either by starch gel electrophoresis or BioGel P-60 column, the immunoreactivity of the eluted [^{125}I]hFSH was similar when using the lactoperoxidase method while the BioGel P-60 is preferable after Chloramine-T iodination (Tables III and IV).

4. The RIA standard curve obtained, with the [^{125}I]FSH labelled with lactoperoxidase gives a more sensitive and precise assay (Figure 5).

The present study revealed that a gonadotropin, FSH, radioiodinated using lactoperoxidase, is suitable for use in RIA for the hormone. Since biological activity was probably preserved (molecular integrity) this preparation is adequate for many studies.

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RESUMO

Descreveremos nesta publicação, um método para a radioiodação enzimática do hormônio folículo estimulante humano (hFSH), e sua comparação com a técnica clássica da Cloramina-T, modificada.

A reação enzimática consiste de um sistema de lactoperoxidase (peroxidase do leite), peróxido de hidrogênio (diluído de 1:15000) e Na-^{125}I e com isto obtemos uma boa atividade específica (100 μCi/ μg) para o FSH-^{125}I, bem como imunorreatividade molecular bem maiores do que as obtidas com a oxidação química com Cloramina-T.

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