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## Studies on the Labeling of Adrenocorticotrophic Hormone (ACTH) by $^{125}\text{I}$ Using Different Oxidizing Agents for Radioimmunoassay Purposes

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### ABSTRACT

Labeling techniques of adrenocorticotrophic hormone (ACTH) were described in details. Comparative study was constructed between all tracers that prepared using different oxidizing agents included chloramine-T, iodogen, N-bromosuccinimide and lactoperoxidase & urea- $\text{H}_2\text{O}_2$ . The radioisotope of  $^{125}\text{I}$  was used in radiolabeling as  $\text{Na}^{125}\text{I}$  in liquid phase. All tracers were purified using PD-10 column and the radiochemical purity was evaluated using paper chromatography electrophoreses charts. Specific activities of tracers were calculated in  $\mu\text{Ci}/\mu\text{g}$ . The shelf life of tracers in days were determined whereas the validity of radioimmunoassay (RIA) kit will required this factor. Immunoreactivity was examined using polyclonal antibody through RIA liquid phase technique. Maximum binding specific percent and binding nonspecific percent were evaluated. Displacement percent was calculated using minimum and maximum binding values. All the results obtained were used in comparative study included all types of tracers. The results show iodogen was preferred as oxidizing agent than chloramine-T and N-bromosuccinimide. On the other hand enzyme lactoperoxidase was filled completely as oxidizing agent. Because the iodogen is insoluble in water, it can be easily remove from the reaction mixture at the end of reaction without using reducing agent.

### INTRODUCTION

The biochemicals that lead to different jobs in the human body by sending a lot of messages over the blood to human organs, skin, muscles and other tissues are called hormones. All the messages make the human body predict when to do and what to do it. There is small gland, pea-sized gland find at lower of the brain beside the hypothalamus called pituitary gland. The pituitary gland is the one from endocrine glands. It is made of the anterior and posterior lobes. The anterior pituitary lobe produce and releases adrenocorticotrophic hormone (ACTH) [1].

Pituitary gland releases ACTH hormone which triggers the adrenal glands to produce the "stress hormone" that called cortisol hormone. Abnormal cortisol levels lead to appearance of many symptoms and the level of ACTH tests must be check in blood according to special conditions [2].

The basic role of ACTH is to stimulate the endocrine adrenal glands to produce cortisol. It is an important endocrine hormone that affects almost all organs and tissues in human body. It is widely known as the "stress hormone." However, it has different important effects and a

lot of functions throughout human body aside from regulating the body's stress response [3].

Also the ACTH hormone has a role in stimulating the adrenal glands to obtain the sex hormones (androgens). In addition to the role of ACTH in stimulating the production of biochemical substances that stimulate an increase a production of noradrenaline and adrenaline hormones. The concentration of cortisol and ACTH hormones in the human serum are highly levels in the first hours of the day and decrease with the day hours path, to reach the minimum level at midnight period. This pattern can change if the patient works at night shift or spent sleeping time at different hours of the day. So that ACTH or cortisol hormone levels prefer to measurement that in the morning blood draw samples. [4].

The normal values of ACTH concentrations in the blood human about 7.5 to 65 pg/mL from 7.30 a.m. to 10:30 a.m. It is important to know that the normal values can different from laboratory to laboratory, hour to hour and human to human. If there are need to get an ACTH test, the healthcare provider will evaluate the reports and may be need further testing [5-8].

In this study, the radiolabeling of ACTH using  $^{125}\text{I}$  radioisotope with different techniques were described. Chloramine-T, iodogen, N-bromosuccinimide and lactoperoxidase &  $\text{H}_2\text{O}_2$  were used as oxidizing agents. All tracers were purified and examined in different parameters. A comparative study between different tracers was constructed. The immunoreactivity of tracers was checked in order to radioimmunoassay purposes.

## MATERIALS AND METHODS

A lot of chemicals and reagents were required to prepare the components of the experimental. The chemicals are highly purified ACTH antigen and anti-ACTH-antibody (Scottish Antibody Production Unit, Scotland),  $\text{Na-}^{125}\text{I}$  (10 mCi/100  $\mu\text{l}$ ), pH 7-11 (Institute of isotopes Co., Ltd Budapest), chloramine-T, sodium metabisulphite, iodogen, N-bromosuccinimide, lactoperoxidase, urea hydrogen peroxide were purchased from Sigma Chemical Co., USA, BSA and some chemical were ordered from local manufacturers.

This study plan was gone according to following steps:

### Labeling of ACTH with $^{125}\text{I}$ :

Different oxidizing agents were used to produce  $^{125}\text{I}^+$  as a cation from  $\text{Na}^{125}\text{I}$  which easy to attack on the rich of electron moiety in a phenolic ring.

#### 1- Chloramine-T method:

The  $^{125}\text{I}$ -ACTH tracer was performed using Ch-T as a powerful an oxidizing agent according to that was described by Hunter and Greenwood [6] with few modifications. 2.0  $\mu\text{g}$  of ACTH in 10  $\mu\text{l}$  phosphate buffer was added to, 10  $\mu\text{l}$  of 0.5M phosphate buffer (pH 7.4) into an eppendorf tube. 4  $\mu\text{l}$  of  $\text{Na-}^{125}\text{I}$  (400  $\mu\text{Ci}$ , 14.8 MBq) were added. The labeling was processed by using 10  $\mu\text{g}$  of Ch-T in 10  $\mu\text{l}$  of 0.05M phosphate buffer (pH 7.4). The reaction mixture was incubated 3 minutes with mix the mixture and to reach the end of reaction 10  $\mu\text{g}$  sodium metabisulphite in 10  $\mu\text{l}$  0.05M phosphate buffer (pH 7.4) were added. 100  $\mu\text{l}$  of 0.05M phosphate buffer (pH 7.4) containing 20  $\mu\text{g}$  potassium iodide were used as a carrier. The  $^{125}\text{I}$ -ACTH tracer product was separated using PD-10 sephadex column.

#### 2- N-Bromosuccinimide method

N-bromosuccinimide oxidation method for radiolabeling was described by Paul Reay [7]. This method was used with few modifications. To an eppendorf tube the reaction mixture containing 2.0  $\mu\text{g}$  of ACTH in 10  $\mu\text{l}$  assay phosphate buffer, 10  $\mu\text{l}$  of 0.5M assay buffer (pH 7.4) and 4  $\mu\text{l}$  of  $\text{Na-}^{125}\text{I}$  (400  $\mu\text{Ci}$ , 14.8 MBq). To start the reaction, 10  $\mu\text{l}$  of 0.05M assay buffer (pH 7.4) containing 12  $\mu\text{g}$  of N-bromosuccinimide was added. The reaction mixture

was proceeding for five minutes only and It was finished by addition of 10  $\mu\text{g}$  sodium metabisulphite in 10  $\mu\text{l}$  0.05M phosphate buffer (pH 7.4). 50  $\mu\text{l}$  of 0.05M phosphate buffer (pH 7.4) containing 10  $\mu\text{g}$  KI were added as a carrier. The purification of  $^{125}\text{I}$ -ACTH tracer was determined using sephadex column ( PD-10 column).

#### 3- Iodogen method

The solid phase iodogen is essentially mild oxidizing agent which insoluble in water. 4.0 mg of iodogen was dissolved in 1 mL chloroform in a clean glass V- shape tube then dried using nitrogen atmosphere. The thin film deposited into the interior the surface of the glass tube was iodogen. Radiolabeling reaction was carried out in this iodogen coated tube. 4  $\mu\text{g}$  of ACTH in 10  $\mu\text{l}$  of phosphate buffer and 10  $\mu\text{L}$  of  $\text{Na}^{125}\text{I}$  (400  $\mu\text{Ci}$ , 14.8 MBq) were added to iodogen coated tube. In a water bath, the reaction mixture was kept 10 minutes. Then reaction mixture was to plane eppendorf tube [8-13]. The  $^{125}\text{I}$ -ACTH tracer was purified using PD-10 sephadex column.

#### 4- Urea hydrogen peroxide method

The preparation of  $^{125}\text{I}$ -ACTH tracer was performed using enzymatic lactoperoxidase and urea hydrogen peroxide as oxidation technique that mentioned by El-Tawoosy et al 2011 [12]. To an eppendorf tube containing 2  $\mu\text{g}$  of ACTH in 10  $\mu\text{l}$  assay phosphate buffer, 10  $\mu\text{l}$  of 0.5M assay buffer (pH 7.4) and 4  $\mu\text{l}$  of  $\text{Na-}^{125}\text{I}$  (400  $\mu\text{Ci}$ , 14.8 MBq) were added.. The labeling was proceeding by addition of 10  $\mu\text{l}$  of 0.05M assay buffer (pH 7.4) containing 10  $\mu\text{g}$  urea hydrogen peroxide and 10  $\mu\text{l}$  of lactoperoxidase. The reaction mixture was allowed to proceed for 10 minutes, and then added 10  $\mu\text{l}$  of lactoperoxidase again. The reaction was stopped after 30 minutes by addition of 10  $\mu\text{l}$  0.05M assay buffer (pH 7.4) containing 10  $\mu\text{g}$  sodium metabisulphite. 100  $\mu\text{l}$  of 0.05M assay buffer (pH 7.4) containing 20  $\mu\text{g}$  KI were added and act as a carrier. The  $^{125}\text{I}$ -ACTH tracer was separated using sephadex PD-10 column [12].

### ACTH standards:

ACTH-standards were prepared using stock 1  $\mu\text{g}/\text{ml}$ . The hormone free serum was used as a matrix and to prepare serial dilutions. The stock standard was diluted to prepare different values of ACTH hormone ranged from zero to 200  $\text{pg}/\text{ml}$  [12]

## RESULTS AND DISCUSSION

### Radiochemical yield of $^{125}\text{I}$ -ACTH using PD-10 column:

ACTH was labeled with  $^{125}\text{I}$  using different oxidizing agents like chloramin-T, iodoen, N-bromosuccinimide or lactoperoxidase. Then the radioactive mixture was injected into sephadex PD-10 column. Fractions tube was collected

and the radioactivity was determined. Fig. (1) show that, the first peak was the  $^{125}\text{I}$ -ACTH tracer and the second one was the free of iodine. The fractions of  $^{125}\text{I}$ -ACTH was collected and diluted to obtain a suitable count for the assay.

### $^{125}\text{I}$ -ACTH tracer:

Using PD-10-chart, the radiochemical yield percentage of the  $^{125}\text{I}$ -ACTH tracer was estimated and equaled 35%, as shown in Fig (1). Paper electrophoresis was used to determine the  $^{125}\text{I}$ -ACTH tracer's purification degree as looks in Fig. (2). The radiochemical purity percentage was 96%. The data were used to compute the tracer's specific activity (32.5 Ci/g). By using the liquid phase RIA system, the immunoreactivity was calculated by using the maximum binding specific percent (% Bo) and binding non-specific (% NSB) which were 32.3% and 3.3%, respectively. These results in a good correlation with the other works [14-18].

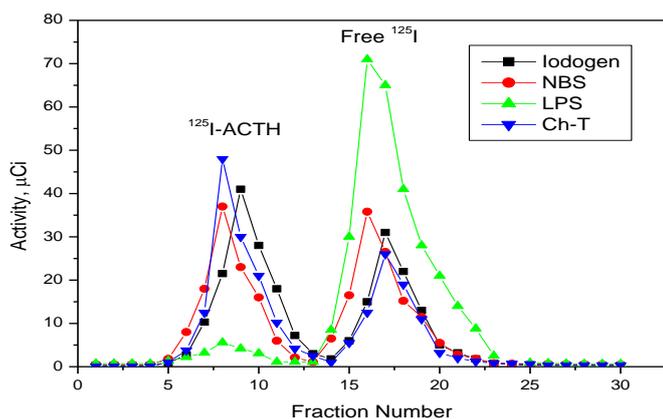


Fig. (1): Iodination of ACTH with  $^{125}\text{I}$  using PD-10 column.

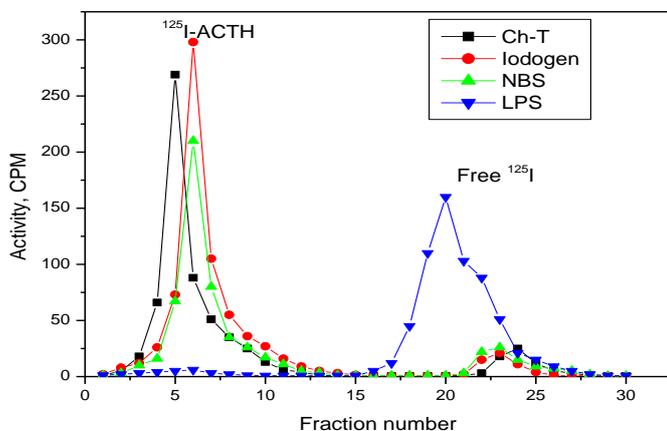


Fig. (2): The purity of  $^{125}\text{I}$ -ACTH using paper chromatography electrophoresis.

As indicated in Table (1), the labeling time consumed was different according to the type of oxidizing agent. Because of Ch-T is a powerful oxidizing agent, it consumed short time. The results are in a good agreement with some manuscripts [13 -20].

Table (1) Optimum conditions of labeling

	Labeling time (min.)	Oxidizing agent content(µg)	Radioactivity (µCi)	ACTH content (µg)	pH
<b>Ch-T</b>	3	30	350	2	7.8
<b>Iodogen</b>	10	50	360	2	7.7
<b>NBS</b>	5	30	370	2	7.5
<b>LPS</b>	20	35	398	2	7.4

The yield percentage was highly reduced by using LPS method and this due to the  $\text{H}_2\text{O}_2$  may be effect on ACTH. Ch-T method reveals a high radiochemical yield percentage (65 %) as shown in Table (2)

Table (2): Radiochemical yield % and radiochemical purity%

	Yield%	Purity%
<b>Ch-T</b>	<b>65</b>	<b>92</b>
<b>Iodogen</b>	<b>57.8</b>	<b>96</b>
<b>NBS</b>	<b>47</b>	<b>90</b>
<b>LPS</b>	<b>5.8</b>	<b>5</b>

The specific activity was calculated as indicated in Table (3). The results show high specific activity (32.5, µCi/µg) was obtained by using Ch-T other than oxidizing agents.

Table (3): Specific activity

	Validity(d)	Sp. Activity( µCi/µg)
<b>Ch-T</b>	<b>70</b>	<b>32.5</b>
<b>Iodogen</b>	<b>80</b>	<b>28.9</b>
<b>NBS</b>	<b>50</b>	<b>23.5</b>
<b>LPS</b>	<b>1</b>	<b>2.5</b>

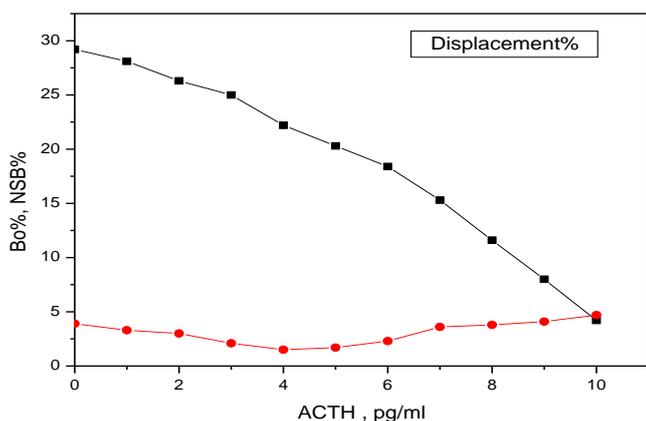
### Liquid phase RIA system

To produce a trustworthy assay, it is necessary to adhere to the fundamental ideas of assay optimization. 100 µl of the purified anti-ACTH with an initial dilution of 1:1000, 100 µl of the ACTH standard or sample, and 100 µl of the  $^{125}\text{I}$ -ACTH tracer were used in the test. The all tubes' contents were combined, and then incubated at 37 °C for three hours. All of the reagents are in the non-specific binding (NSB) tubes except of the first antibody. With the exception of the tubes marked with a

(TA), second antibodies (100 µl goat anti-rabbit IgG 1/80), normal rabbit serum 1/200, and 500 µl of PEG-8000 (12%) were added to each tube and incubated for 30 min at room temperature following the separation agent second antibodies. Centrifugation of the tubes was place for 10 min. at 4 °C and 4000 rpm. The decantation step for all tubes was proceeding and the fractions were read using a gamma counter.

### Immunoreactivity and Displacement %:

The displacement % of tracer that prepared using N-bromosuccinimide was calculated As shown in Fig (3). The displacement is the largest distance between binding specific (Bo) and binding non specific (NSB). The greater degree of distance value, it means the better displacement% between the different values of standards and the tracer was able to differential between different concentrations of ACTH. [16-18].



**Fig. (3): The displacement % using N-bromosuccinimide as oxidizing agent.**

The comparative displacement results were presented in Table (4). These numbers show, the binding percent (Bo%) and the displacement percent were highly increased when the iodogen or Ch-T was used. But the binding percent and the displacement percent were reduced using N-bromosuccinimid and show no binding occurs with enzymatic oxidizing agent [19- 21].

**Table (4): Immunoreactivity and Displacement%:**

	Bo%	NSB%	B <sub>min</sub> .%	Displacement%
<b>Ch-T</b>	28	1.1	4.1	85.3
<b>Iodogen</b>	34	1.0	5.6	83.5
<b>NBS</b>	25.2	1.4	5.8	76.9
<b>LPS</b>	1.8	1.6	1.5	11

### CONCLUSION

In summary, the solid phase iodogen oxidizing agent was preferred to labeling of ACTH tracer. This technique provides greater benefits than other oxidizing agents methods. In addition to high purity and low non-specific binding tracer also a high sensitivity and displacement were observed by using N-bromosuccinimide as oxidizing agent than Ch-T or NBS. On the other hand, LPS method non suitable to labeling ACTH which obtain very low yield and no binding percent.

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