Preparation of Radioiodine Labelled Human Follicle Stimulating Hormone for Radioimmunoassay Use

Jaerok Kim, Tae Ho Kim, and You Sun Kim

Radio-Organic Chemistry Laboratory Korea Atomic Energy Research Institute, Seoul

한국원자력 연구소

김 재 록·김 태 호·김 유 선

요 약

난포 자극 홀몬(hFSH)을 클로라민 티를 사용하여 방사성 요오드로 표지하였으며 평균표지수율은 대략 65%이었다.

표지홀몬을 방사면역측정용으로 사용하기 위하여 전분겔 전기영동과 겔 여과법으로 분리정제하고 그 분리정제효과를 분석한 결과 겔 여과법이 분리시간, 간편성, 항체와의 결합력등으로 보아 우수함을 알 수 있었다.

한편 유리 표지홀몬과 항체와 결합한 표지홀몬의 비율을 결정하기 위하여 이중 항체법을 크로마토 전기영동법과 비교하여 본 결과 이중항체법에 의해서만 효과적 비율결정이 가능하였다.

Abstract

Radioiodine labelled human follicle stimulating hormone has been prepared using chloramine-T, with the approximate labelling yield of 65%. The labelled product is purified by means of a starch gel electrophoresis, and a Sephadex gel filtration, and the separation efficiencies are assessed for the effective use in radioimmunoassay. The results indicate that the gel filtration is efficient in view of the separation time, simplicity and bindability of the labelled hormone to the antibody.

In determining the ratio of the free to the antibody bound labelled hormone, a double antibody technique is applied in comparison with a chromatoelectrophoresis. The ratio could be obtained only in the case of applying the double antibody technique.

1. Introduction

The introduction of the technique of radioimmunoassay by Yalow and Berson in 1960 and Hunter & Greenwood in 1962, as well as the method of labelling peptide hormones with the radioactive iodine by Greenwood et al have permitted the measurement of a wide variety of peptide hormones. A number of radioimmunoassay of human follicle stimulating hormone (FSH), human luteinizing hormon (LH) and human chorionic gonadotropin hormone (HCGH) in plasma has also been repoted and recently reviewed by Raiti & Davis (1969).[1] and Jaffe & Midgley (1969).[2] Saxena et al described the radioimmunoassay of FSH and LH using the chromatoelectrophoresis technique[3] of Berson et al (1956) for the separation of free and antibody bound labelled hormones. However, the up-to-date literatures mostly described the RIA data in clinical ground, and thus not emphasized the effective purification of the labelled antigen for the optimization of the RIA.

This paper describes the separation of the starch gel electrophoresis and the Sephadex gel filtration in the purification of the labelled FSH in view of the simplicity, seperation time and the bindability of

the labelled antigen to its antibody. In the free antigen from the antibody bound, the effectiveness of the double antibody technique is also proven in comparison with the chromatoelectrophoresis.

2. Experimental

(1) Preparation of ^{125}I Labelled FSH

(a) Reagents

1) Sodium phosphate buffer No. 1. ; 0.5M, pH 7.5; 61.75g Na_2HPO_4 and 8.18g NaH_2PO_4 in 1 liter water.

2) Sodium phosphate buffer No. 2. ; 0.05M, pH 7.5; Buffer No. I was diluted to 10 times with water.

3) Human follicle stimulating hormone (HFSH); highly purified, >3000 IU/mg, LH content;<60 IU/mg, dissolved in phosphate-T(Eastman Kodak) in the buffer NO. 2.

5) Sodium metabisulfite solution; fresh 0.24% sodiun of sodium metabisulfite (Yoneyama Chemicals) in the buffer No. 2.

6) Bovine serum albumin solution; fresh 1% solution of crystalline bovine serum albumin (Fraction V, Schwarz/Mann) in the buffer No. 2.

7) ^{125}I; carrier and reducing agent free for protein iodination, radioactivity concentration; 100 mCi/ml (Radiochemical Centre, Amersham, England).

8) Iobeads; inorganic iodine resin, Hycel Inc. U.S.A.

(b) Labelling Procedure

To a small polypropylene vial, 1 ug of FSH, 20 ul of the buffer No. 1 and 1 mCi of ^{125}I were introduced. Twenty ul of the fresh chloramine-T solution was added and the content was thoroughly mixed for 30 sec. At the end of which 70 ul of the sodium metabisulfite solultion and 10 ul of the bovine serum albumin solution were added in a quick succession. Approximately 100 mg of the Iobead was then added to the reaction mixture.

(2) Purification of the Labelled Product

The purification of the reaction mixture was performed by a gel filtration on Sphadex G 100 column (1×25 cm) according to the method applied by Saxena et al[3] or by a starch gel electrophoresis.[4]

(a) Sephadex gel Filtration

The whole reaction mixture was applied on the column which was pre-eqilibrated with 0.05M barbitone buffer, pH8.6, containing 0.25% bovine serum albumin. One tenth ml aliquot of the filtrate was collected in each tube, and the radioactivities were counted with a well type gamma scintillation conuter. The c/m in an aliquot of the filtrate were plotted against the elution volume as shown in Fig. 1. The approximate labelling yield was calculated from the Fig. 1. in connection with the radioactivity adsorbed to the Iobeads (Table 1).

(b) Starch Gel Electrophoresis

The whole reaction mixture was dropped in the holes of the starch gel plate which was prepared in advance of conducting the labelling as following; Thirty g of hydrolyzed starch powder (Sigma Chemicals) was mixed with 250 m of borate buffer (0.25M, pH 8.6) in 500 ml filtering with constant stirring bar[5]. As soon as the solution started to boil the heater was turned off, and a vacuum line was attached to the filtering flask to evacuate the air from the gel. The vacuum was removed and the gel was poured into a Perspex former (16×8×1 cm) with sample application holes and the filter paper wicks in position. The gel plate was transfered to a

Table 1. ^{125}I Labelling of FSH

Run No.	FSH(ug)	^{125}I (mCi)	Chloramine-T soln. (ul)	Reaction Time (sec)	Na metabisulfite soln. (ul)	1% HSA soln. (ul)	Labelling yield
1	1	1	20	30	70	10	60
2	1	1	30	30	80	10	65
3	1	1	30	40	80	10	68

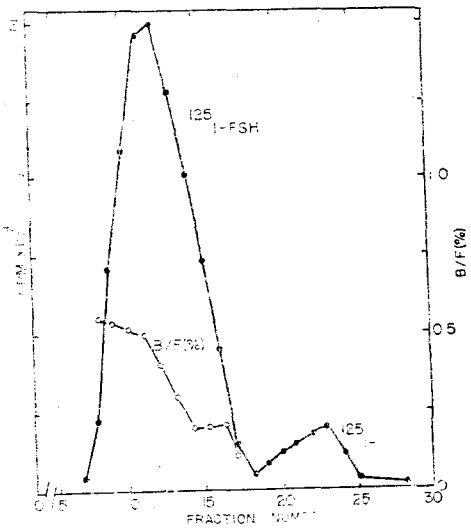

Fig. 1. Separation of the well-labelled FSH by a Sephadex filtration (Sephadex G-100 1×25cm column, eluent;0.05M veronal buffer, pH 8.6 containing 0.02% BSA)

refrigerator to cool and be set.

Electrophoresis was carried out in a refrigerator for 4 hrs at a constant voltage of 300 v. A borate buffer, 0.3M, pH 8.6, was used as an electrolyte. The gel plate was removed, and a radioautography was conducted. The gel which comes under the spots on the developed X-ray film was sliced into about 2 mm width in horizontal direction using a razor blade (Fig.. 2). Each slice was put into a test tube separately, and numbered. Then they were deep frozen. Using veronal buffer, pH 8.6, containing 0.25% bovine serum albumin, the labelled hormone was eluted from the gel as follows; One ml of the veronal buffer was put into each tube, and the gel was gently pressed with a round tipped Lorex glass bar and then the eluate was compressed out. Such manipulation was repeated three times for all of the tubes (usually, 8–10 tubes a new 1ml of the eluent for each elution. In some cases, the eluate was subsequently centrifuged to remove the trace amount of the starch gel came up with the eluate. After the measurement of the radioactivity of each fraction, the c/m were plotted against the fraction number as shown in Fig.. 3. The radioactivity in an aliquot of

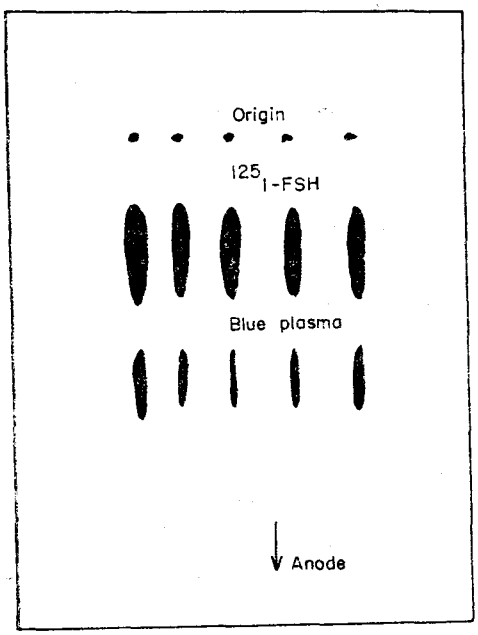

Fig. 2. Typical radioautogram sketch

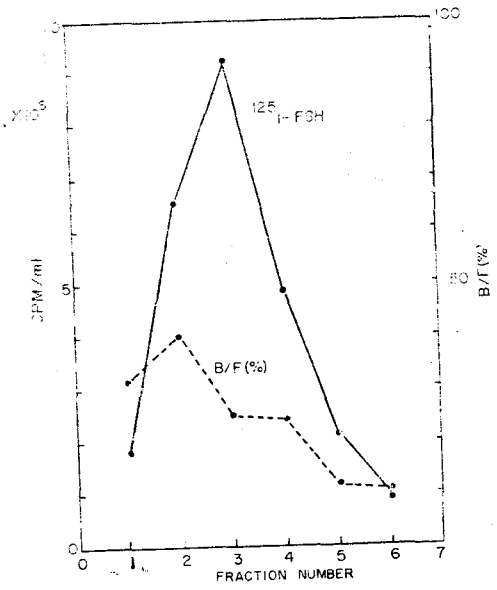

Fig. 3. Separating of the well-labelled fractions of ^{125}I-FSH by a starch gel electrophoresis.

the anode side electrolyte to was also counted to check the labelling yield (Table 1). In some cases, an aliquot of the crude reaction mixture was taken out and the

labelled FSH was separated from the unbound radioiodide by a paper electrophoresis. Five hundredth M vercnal buffer' pH 8.6, was used as an electrolyte. Electrophoresis was conducted at a constant voltage of 300V for 80 min in a refrigerator. The paper strips were cut in 1 cm width, and the the distribution of the radioactivity in each strip was measured to check the labelling yield (Table 1). The same method was also applied to the radiochemical purity check for the Sephadex gel filtered product (Fig. 4). A radio paper chromatography technique was also applied to check the radiochemical purity of the purified FSH ^{125} I. Whatman No. 1 paper and the solvent system of 75% methanol was used. The c/m in each sliced section of the chromato-paper was plotted against the developed length (Fig. 5).

(3) Seletion of the Well-Labelled Fractions

To select the maximum binding fractions of the labelled FSH to its antibody, the fractions of the

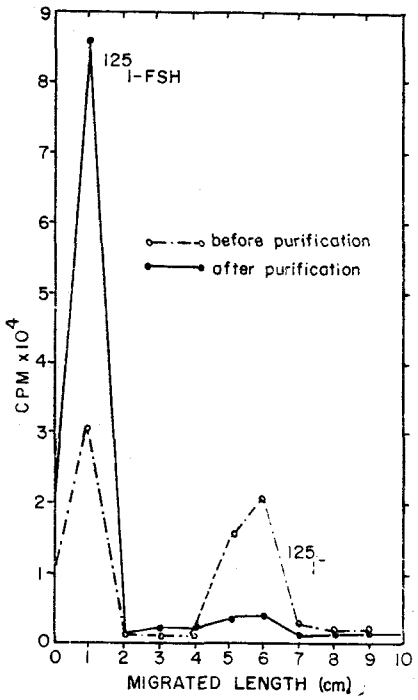

Fig. 4a. Determination of radiochemical purity of ^{125}I-FSH purified by a Sephadex G-100 filtration

Table 2. Protocol of the Incubation Mixture for Seletion of the Well-Labelled Fractions

Fraction No.	Diluent (ul)	Anti-FSH serum (1 : 150) (ul)	FSH-^{125}I (ul)
1	400	0	100
1′	300	100	100
2	400	0	100
2′	300	100	100
3	400	0	100
3′	300	100	100
6	400	0	100
6′	300	100	100

eluate from the column or the starch gel were diluted to yield about 3×10^5 c/m per ml using a diluent (buffer No. 2 containing 0.01% bovine serum albumin, 1:10000 merthiolata and 0.01 M disodium EDTA). An incubation mixture was perpared as shown in Table 2 and incubated for 24 hrs at 4C. At the end of the incubation period, 100 ul of the second antiserum (anti-rabbit gamma globulin, goat) was added to each type and the mixtures were allowed further for 24 hrs at 4° C. The mixtures were then centrifuged at 4000 rpm speed for 30 min in the cold. The radioactivities of the supernatant and the precipitate were separately counted and the net counts were expressed as free (F) and bound (B) hormone, respectively. The B/ F ratios were plotted against each fraction (Fig. 1, 3).

(4) Determination of Antiserum Dilution Ratio and Plot of Standard Dose-Response Curve

By using the well labelled fraction which was selected according to the afore-described procedure, the incubation mixture was made as shown in Table 3. After 24 hrs' incubation at 4° C, 100 ul of the second antiserum was added to each tube and incubated for further 24 hrs. The B/F ratios were determined in the same way as described in section 3 (Fig. 6). For plot of stand dose-response curve, the incubation mixture was prepared as shown in Table 4 using the selected well-labelled fraction of FSH and

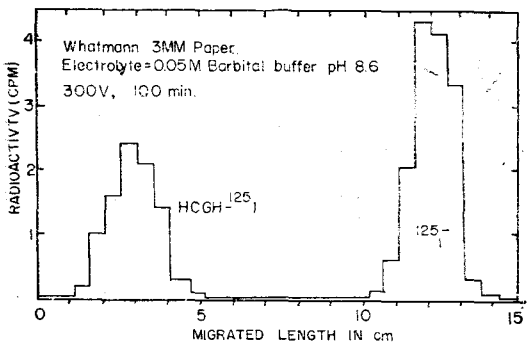

Fig. 4b. The Determination of the Labelling yield by a chromatoeleotrophoresis and Radioactivity Counting

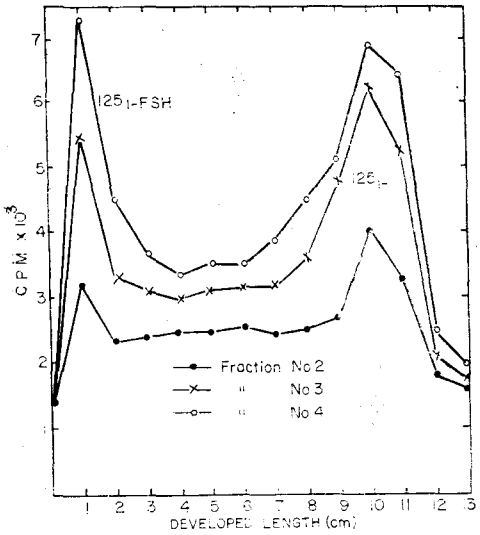

Fig. 5. Determination of the radiochemical purity of Sephadex filtered ^{125}I-FSH by paper chomatography

Table 3. Preparation of Incubation Mixtures for the Determination of Anti-FSH serum Dilution Ratio

Tube No.	FSH-^{125}I (ul)	Anti-FSH serum dilution in 100 ul vol.	Diluent (ul)
1	100	1 : 25	300
2	100	1 : 50	300
3	100	1 : 100	300
4	100	1 : 200	300
5	100	—	400

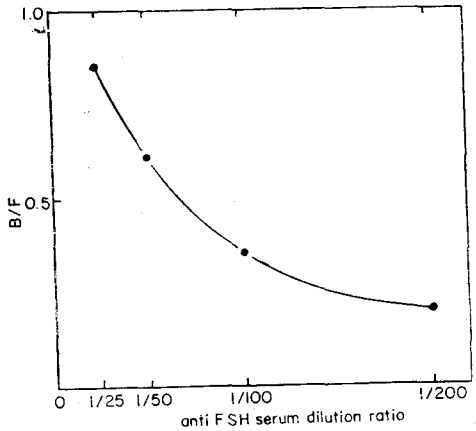

Fig. 6. Determination of FSH antiserum Dilution Ratio.

FSH antiserum (of which dilution ratio was determined as described). After pre-incubation for 72 hrs, the labelled FSH was added, and after 24 hrs therefrom the second antiserum was added, and incubated further for 24 hrs. The B/F ratios were determined as afore-described method and plotted against the standard dose FSH (Fig., 7).

Table 4. Preparation of Incubation Mixtures for the Plot of Dose-Response Curve

Dileuent (ul)	FSH Standard (ng)	(ul)	Anti-FSH serum(ul)	^{125} I-FSH (ul)	2nd Antiserum (ul)
400	0	0	0	50	50
300	0	0	100	50	50
290	0.010	10	100	50	50
275	0.025	25	100	50	50
250	0.050	50	100	50	50
225	0.075	75	100	50	50
290	0.100	25	100	50	50
250	0.250	25	100	50	50
250	0.500	50	100	50	50

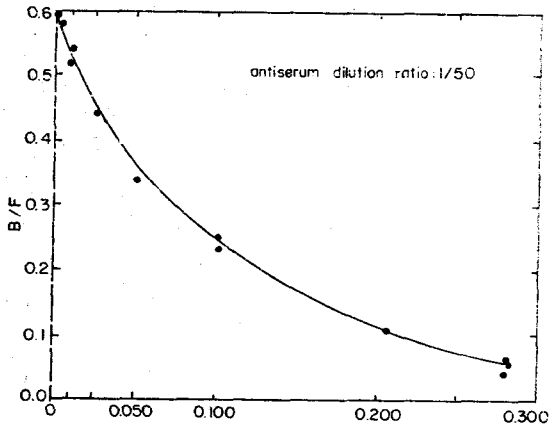

Fig. 7. Standard dose rseponse curve.

3. Results and Discussion

In labelling FSH, the chloramine-T method has only been applied since it is the most generally known method of labelling proteic homone so far.[6] In case of using iodine monochloride as oxidizing agent, the inactive iodine can be incorporated into the antigen which may somehow be contradictory to the concept of the labelling antigens for RIA use. As Table 1 shows, the average labelling yield is 65%. The influence of increasing the amount of chloramine-T or the influence of slight extension of the reaction time on the labelling yield is not so much, and the ratio of the damaged fraction in varying the two labelling conditions was not changed much.

In the sepration, the Sephadex G 100 filtration is preferable to the starch gelelectrophoresis in both of the simplicity and the immunological points of view. The time needed for the Sephadex filtrating is about 2 hrs while for the starch gel electrophoresis 10-12 hrs over-all. Thus, the labelled FSH is not able to be processed in the day of labelling if the latter separation technique is applied. Further, in the latter case, the starch gel plate should preferably be prepared a day before labelling for firm setting of the gel. As Fig. 2 shows, the autoradiogram of the starch gel plate in the separation of FSH-^{125}I is quite similar in pattern to that obtained in insullin-^{125}I separation[4]. Anyway, the technique can be used for labelled FSH separation when the Sephadex gel is not available.

As Fig. I shows, the Sephadex filtration gives a distinct separation in a relatively short time. The maximum binding fraction of FSH-^{125}I to its antibody was obtained at the first to the filtrate(which are in the peak raising region) to show the B/F (%) of around 60. This is quite different from that reported by Saxena et al [3]. According to the Saxena's data, the maximum binging fraction is located in the peak lowering region. As Fig. 3 shows, the first to the second fraction of the eluate from the starch gel (which are also located in the peak raising region) show a maximum binding. Such a difference may be attributable to the variation factors such as the specific radioactivity, conditioning the gel column, and the eluting rate etc.

The most important thing in the separation of labelled FSH is that the B/F (%) of the maximum binding FSH-^{125}I fraction obtained by the starch gel electrophoresis, The main reason, we propose, is that the detereoration of the labelled hormone during the starch gel electrophoresis is severer than longer purification time at the ambient temperature, to the freezing and thawing for squeezing out the hormone from the frozen gel as described in the procedure.

The radiochemical purity of the labelled FSH after the purification by either method was more than 95 %, which was confirmed by a paper electrophoresis 0.05M veronal buffer as the electrolyte(Fig. 4). The conventional standard method for cheking the radiochemical purity of the radiodinated human serume albumin could not be applid as shown in Fig. 5. As the results show that more than 60% is ^{125}-I, it is far from the value of only 3% obtained by the electrophoresis. In the paper chromatography. FSH might be detereorated by the methanol at the room temperature and thus ^{125}-I might be released. This fact may be able to account the conformation between the radioidinated human serum albumin and the FSH-^{125}I even though they are both a labelled protein with the radioiodine labelled at the tyrosine rings in the molecules.

The results obtained in the determination of the antiserum dilution ratio indicated that the B/F ratio is only 0.8 when the antiserum dilution is 1 : 25 (Fig. 6). Since the amount of the antiserum was

limited it was diluted to 1 : 50 rather than 1 : 25 in the preparation of the incubation mixture for the plot of a standard dose response curve. The severe detereoration of the antiserum and the consequent lower binding potency to the antigen might be due to the long term transportation in the procurement procedure. For the separation of B and F, a paper chromato-electrophoresis technique was applied using Whatman No. 1 paper and the electrolyte of 0.05M barbital buffer according to the method of Saxena et al[3],[7], but it was failed. A shift of the equilibrium between the antigen and the antigen-antibody complex under the circumstances of a high voltage and a relatively high temperature is attributed to the main reason why it couldn's be successful. In both of the experiments of selection of the welllabelled fractions and the plot of the standard doseresponse curve, the separation of B and F was well established by a second antibody technique. Even though the obtained standard dose-response curve is not so steep (Fig. 7), it could be obtained with a good reproducibility. The steeper dose-response curve ranging the B/F from 0.1 to 1.0 is expected if a fresh antiserum with an adeqate titer is used. The data obtained in present work indicate that the poor steepness is not due to the labelled FSH and other technique but only due to the antiserum. A standardization by using the 2nd international reference preparation of a human menopausal (2nd IRP HMG) is further anticipated.

4. Conclusion

Among the pituitary hormones, a FSH radioimmunoassay procedure has been established. The labelling and the separation methods are suitable for a FSH radioimmunoassay. However, the gel filtration technique is superior to the starch gel electrophoresis sn view of the simplicity, short separation time and the bindability of the purified labelled antign to its antibody. For the separation of the free antigen from the antibody bound, the double antibody techique could be successfully applied

Acknowledgement: The authors were indebted to the financial assistance from IAEA under the research contract 1540/RB, which had made this work possible.

REFERENCES

(1) Ratti S. and Davis W.T., Obstet *Gynec. Surv.*, ***24***, *200 (1969)*

(2) Jaffe R. and Midgley A.R. *Jr.,ibid.*, ***24***, *289 (1969)*

(3) B.B. Saxena et al., *J. Clin. Endocr.*, ***28***, *519 (1968)*

(4) J.Kim, T.H.Kim, and Y.S. Kim, *Unpublished work (1974)*

(5) Plummer, Introd. Prac. *Biochem., McGraw-Hill (1971) p.63*

(6) Greenwood, F.C. et al. *Biochem. J.* ***89***, *114 (1963)*

(7) B.B. Saxena et al., *Acta Endocrinol.* ***63***, *(Suppl. 142) 185 (1969)*