YOUNGSTOWN STATE UNIVERSITY

Graduate School

Thesis

Submitted in Partial Fulfilment of the Requirements

For the Degree of Master of Science

STUDIES OF A CHROMATOGRAPHIC METHOD FOR ASSAY OF HUMAN FOLLICLE STIMULATING HORMONE

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ABSTRACT

STUDIES OF A CHROMATOGRAPHIC METHOD FOR ASSAY OF HUMAN FOLLICLE STIMULATING HORMONE

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In clinical laboratories the determination of Human Follicle Stimulating Hormone (hFSH) can aid the clinician in the diagnosis, treatment, and prognosis during endocrine malfunctions. Only a few years ago such determinations could not have been performed. The development of Radioimmunoassay (RIA) made it possible for the assay of hFSH with high specificity and sensitivity. Presently the clinically acceptable method for the assay of hFSH is RIA.

The object of this study was to design an alternative assay technique that can be compared to RIA. The test method did not determine the immunological activity but rather the hFSH total concentration. The hFSH was separated from the other serum constituents by Sephadex gel filtration. The hFSH was shown to be in the ammonium bicarbonate eluent from the gel filtration by an RIA technique. A concentration step was performed prior to electrophoresis. Cellulose acetate was used for the support medium in the electrophoresis technique. The protein bands obtained

through electrophoresis were stained by Ponceau S. Upon clearing the cellulose acetate plates scanning of the hFSH peaks were done by using a densitometer with an integration unit. This allowed quantitation of the hFSH. The integrator counts were related to the initial hFSH concentration. The test method values were then statisically compared to the comparative method which was RIA. The test method proved to have limited success in the determination of hFSH. The clinical acceptance of the alternative method will be dependent upon futher testing using a larger number of samples.

ACKNOWLEDGEMENTS

I would like to acknowledge my advisor Dr. Inally Mahadeviah to whom I am very indebted for the successful completion of this thesis. I am also very grateful to Dr. F. Smith and Dr. L. Spiegel for reading and making invaluable suggestions.

To St. Elizabeth Hospital Medical Center, who supplied me with serum specimens, I wish to express my appreciation.

I would like to thank the faculty at Youngstown State University, fellow graduate students, friends, and my family. Without their unselfish efforts and support this thesis would not have been possible.

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CHAPTER I

INTRODUCTION

Human Follicle Stimulating Hormone (hFSH) is a glycoprotein secreted by the beta cells of the Anterior Pituitary gland. It has a molecular weight of 35000, an isoelectric point of 5.0[1], and consists of two subunits as described by Parlow and Shome in 1974. The two subunits are hFSH alpha (hFSHα)[2] and hFSH beta (hFSHβ)[3] whose amino acid sequences are shown in figures 1 and 2. hFSHα has 89 amino acid residues and is identical to Human Luteinizing Hormone α-subunit (hLHα) and is virtually identical to Human Chorionic Gonadotrophin α-subunit (hCGα) except for three

```
                                                  10
H-Val-Glx-Asp-Cys-Pro-Glx-Cys-Thr-Leu-Glx-Glx-Asn-Pro-Phe-

                        20
Phe-Ser-Gln-Pro-Gly-Ala-Pro-Ile-Leu-Gln-Cys-Met-Gly-Cys-Cys-

30                                                40
Phe-Ser-Arg-Ala-Tyr-Pro-Thr-Pro-Leu-Arg-Ser-Lys-Lys-Thr-Met-

                        50
Leu-Val-Gln-Lys-Asn(CHO)-Val-Thr-Ser-Glx-Ser-Thr-Cys-Cys-Val-

    60                                                70
Ala-Lys-Ser-Tyr-Asn-Arg-Val-Thr-Val-Met-Gly-Gly-Phe-Lys-Val-

                        80
Glx-Asn(CHO)-His-Thr-Ala-Cys-His-Cys-Ser-Thr-Cys-Tyr-Tyr-His-

    89
Lys-Ser-OH
```

Fig.1. Proposed linear amino acid sequence of the alpha subunit of human follicle stimulating hormone.

10
(Asx,Ser)-Cys-Glu-Leu-Thr-Asn(CHO)-Ile-Thr-Ile-Ala-Ile-Glu-

20
Lys-Glu-Glu-Cys-Arg-Phe-Cys-Ile-Ser-Ile-Asn(CHO)-Thr-Thr-

30 40
(Thr,Asx,Trp)-Glu-Thr-Cys-Ala-Gly-Try-Cys-Tyr-Thr-Arg-Asp-

50
Leu-Val-Tyr-Lys-Asp-Pro-Ala-Lys-Pro-Arg-Ile-Gln-Lys-Thr-Cys-

60 70
Thr-Phe-Lys-Glu-Leu-Val-Tyr-Glu-Thr-Val-Arg-Val-Pro-Gly-Cys-

80
Ala-His-His-Ala-Asp-Ser-Leu-Tyr-Thr-Tyr-Pro-Val-Ala-Thr-Gln-

90 100
Cys-His-Cys-Gly-Lys-Cys-Asp-Ser-Asp-Ser-Thr-Asp-Cys-Thr-Val-

115
Arg-Gly-Leu-Gly-Pro-Ser-Tyr-Cys-Ser-Phe-Gly-Glu-Met-(Glx,Lys)

Fig. 2. Proposed linear amino acid sequence of beta subunit of human follicle stimulating hormone.

extra amino acids on the N-terminus of the hCGα. hFSHβ has 115 amino acid residues of which 49 are in the same sequence Human Thyroid Stimulating Hormone β-subunit (hTSHβ) and 39 amino acid sequence identical to hLHβ. All human gonadotrophins consist of two structually distinct subunits. The dissociation of hFSH into alpha and beta subunits results in the loss of biological activity. Also essential for biological activity, but not immunoreactivity, is Sialic Acid (N-Acetylneuraminic Acid). 16% of the hFSH content is Charbohydrate. Decreased biological activity of FSH is related to the extent of charbohydrate loss. The beta subunit of glycoprotein hormones is the portion responsible for the target tissue specificity [4,5]. The β-subunit binds

with the membranes of its target tissue cells.

In females FSH stimulates the ovarian follicle to increase in size and to mature. In males FSH is associated with the stimulation of spermatogenesis. The production and secretion from the pituitary of FSH is influenced by a neurochemical releasing factor from the hypothalamus [6]. A FSH releasing factor from the hypothalamus (FSH-RF) is thought to exist but to date its presence has not been established. The initiation of this response is influenced by stimuli from the cerebral cortex. The neural regulation appears to have an influence upon it due to a negative feedback mechanism[7]. When FSH concentrations decrease, it stimulates the release of FSH-RF. This is an example of a negative feedback mechanism as illustrated in figure 3.

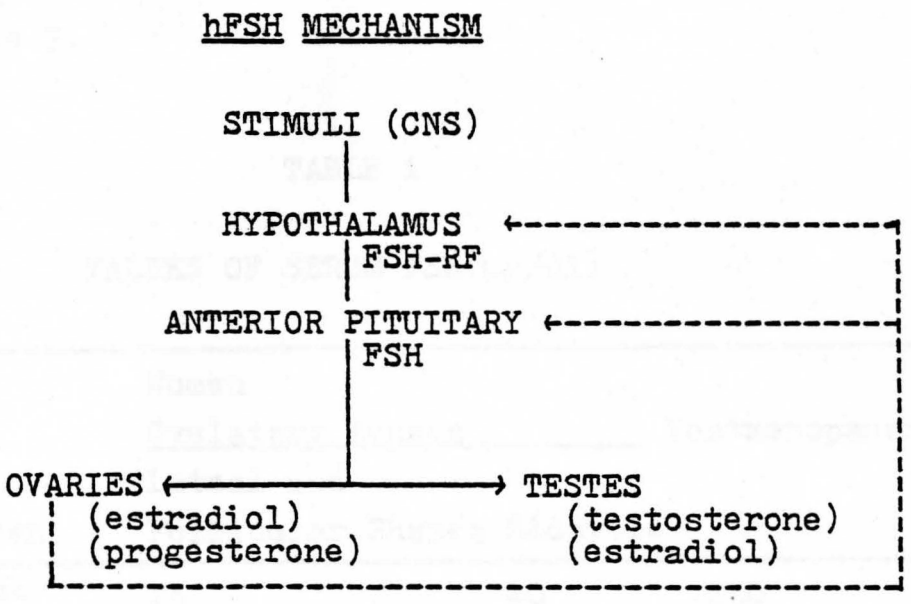


Fig. 3. Proposed hFSH mechamism.

Women during their child bearing period have varying concentration of FSH[8]. FSH secretion increases during the follicular phase then falls prior to its peak at midcycle with the lowest levels occuring in the luteal phase. In the follicular phase the corpus luteum secretes estradiol and progesterone which has an effect on the hypothalamus. In the luteal phase the ovarian follicles also secretes estradiol and progesterone. It is thought that FSH secretions are not constant but rather pulsatile or sporadic[9]. Males have relatively fixed FSH levels dependent on the status of testicular function[10]. The Leydig Cells of the testes secretes testosterone and estradiol. Testosterone does affect the anterior pituitary while the estradiol affects the hypothalamus[11]. Clinically acceptable concentration of FSH are shown in table 1 and acceptable radioimmunoassay values are shown in table 2.

TABLE 1

VALUES OF SERUM FSH(μg/dl)

	Children		Women		
	Prepubertal		Ovulatory Menses		Postmenopausal
	Both sexes	Men	Luteal and Follicular Phases	Midcycle	
Mean	4	25	25	40	250
Range	0-20	10-60	10-50	25-45	150-400

TABLE 2

RIA VALUES OF FSH (mIU/ml*)

Men	Women			
	Normal	Midcycle	Premenopause	Postmenopause
7-24	30-95	2x normal	4-30	40-250

RIA values for FSH determined by Bio Science Laboratory of Van Nuys, California. * denotes that IU is defined as one unit of activity which is that amount which catalyze one micromole of substrate per minute.

The absence of a negative feedback mechanism will result in the following abnormal conditions. Elevated levels of FSH are then found in:

1) Primary Hypogonadism

a. Turner's Syndrome- ovarian failure.

b. Klinefelter's Syndrome- testicular malfunction or deficiency.

c. Irradiation of the gonads.

d. Castration

e. Precocious puberty

Decrease levels of FSH are found in the following conditions:

1) Hypopituitarism

2) Hypothalamic disturbance- due to central nervous system malfunction or by such drugs as phenothiazine[11].

Purpose of Study

hFSH is important for proper sexual development in both males and females. In males it is essential for normal spermatogenesis and testosterone secretion. In females FSH is essential for growth and development of the primary ovarian follicles. It is therefore clinically significant to determine concentrations of FSH in order to arrive at a proper diagnosis and treatment by a physician.

Radioimmunoassay (RIA) has become the accepted method for the determination of glycoprotein hormones as in the case of FSH. These methods are very sensitive and specific but involve the usual problems associated with radioactive materials. The RIA procedures require sophisticated instruments and highly skilled technical personnel. Many smaller or rural laboratories do not have the capacity to perform such assays. Therefore it is necessary to find an alternative assay for FSH. The goal of this study was to develop an alternative test method of FSH determination using RIA as the comparative method.

The alternative method has an initial separation by Sephadex gel filtration which allows FSH to be separated from the other serum proteins. A portion of the eluted fraction was used for electrophoresis. Following electrophoresis the bands were stained with Ponceau S and then quantitated using a densitometer.

CHAPTER II

THEORY OF THE METHODS

A. Radioimmunoassay

RIA is a hybrid of radiochemistry and immunology. The radiochemical technique employed is quantitative, highly sensitive, and is a variant of the isotope dilution technique which is dependent on knowing the specific activity of a radioactive tracer before and after mixing in a chemical medium. The immunological technique employed is a variant of the reaction between an antigen and antibody. RIA combines the sensitivity of radiochemical techniques with a dimension of greater specificity. RIA has been increasing our knowledge by elucidation of many biochemical pathways which were unknown only a few years ago. The technique RIA has improved diagnosis and treatment of many disease states which would have been impossible before its development.

The basic principle of radioimmunoassay is shown in figure 4. Labelled (Ag*) and unlabelled (Ag) antigen compete

$$\begin{aligned} Ag^* + Ab &\rightleftharpoons Ag^*\text{-}Ab \\ Ag^*\text{-}Ab + Ab' &\rightleftharpoons Ag^*\text{-}Ab\text{-}Ab' \\ Ag + Ab &\rightleftharpoons Ag\text{-}Ab \\ Ag\text{-}Ab + Ab' &\rightleftharpoons Ag\text{-}Ab\text{-}Ab' \end{aligned}$$

Fig. 4. The basic principle of RIA using a second antibody technique. * indicates radio labelled antigen.

for the same binding sites on the first antibody (Ab). This is in accordance with the law of mass action. The concentration of labelled antigen (Ag*-Ab) is inversely related to the concentration of unlabelled antigen. After an equilibrium has been established the second antibody is added. The second antibody (Ab*) has specificity for the antibody of the labelled or unlabelled antibody-antigen complex. The second antibody attaches to the antibody-antigen complex to form a mixed or double antibody-antigen complex. The mixed antibody-antigen complex is then precipitated with ammonium sulfate. The precipitate is settled by centrifugation and the supernatant is discarded. By measuring the portion of double antibody-antigen which is labelled along with standard solution containing varying concentrations of antigen, a standard curve can be constructed. The unknown antigen value can then be interpolated from such a standard curve as shown in figure 5.

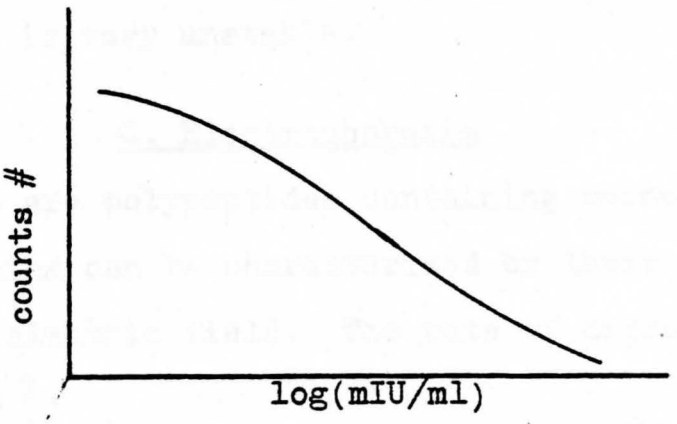


Fig. 5. Typical standard curve for RIA.

B. Gel Filtration

Gel filtration using Sephadex during the late 1950's became an established technique for separation of substances of different molecular weights[13]. Sephadex is a polymer of dextran cross-linked with epichlorohydrin. Dextran has a large number of hydroxyl groups which causes the Sephadex to be strongly hydrophilic and is responsible for its ability to swell in an electrolyte solution. Sephadex has the capacity to separate substances according to their molecular size. Molecules larger than the exclusion limit cannot penetrate the largest pores of the swollen Sephadex bead and is therefore contained and passed in the mobile liquid phase. Smaller molecules depending on their size and weight will penetrate to varying degrees the Sephadex bead pores. These substances will be eluted from the Sephadex beads in order of decreasing molecular size. Gel filtration is a powerful tool for chromatographic separation of biological substances according to molecular size even if the substance is very unstable.

C. Electrophoresis

Proteins are polypeptides containing amino acid residues. Proteins can be characterized by their rate of migration in an electric field. The rate of migration is dependent on the [7]:

1) net electrical charge per molecule and molecular weight.
2) the spatial configuration of the molecule.
3) the magnitude of the applied current.
4) temperature, and
5) support medium.

Proteins have electrical charges depending on their extent of ionization. In other words they are ampholytic, which means that they can be either positively or negatively charged depending on their extent of ionization. When the solution is more basic than the isoelectric point of the solute, that solute is in an anionic form and thus migrates toward the anode. Besides the pH of the solution, its ionic strength can be significant in its effect on the speed and direction of the molecule. The size of the ionic cloud surrounding a given charged molecule determines the rate of migration and zone sharpness. The ionic strength of the solution must not exceed a critical concentration or it will be counterproductive or the sharper zone resolution is then decreased by the Joule effect. Many of the solutions used are monovalent basic buffers so as to minimize any association between buffer and the protein. The support medium is cellulose acetate. Ponceau S, a protein dye, was used to stain the FSH bands following electrophoresis. A densitometer can then quantitate the bands to give integration counts. The integration counts can be used to determine

the FSH concentration in each sample.

It can be assumed that for the most part only hFSH is in the concentrated eluent. This is supported by figure 6 which shows the absorbance of human serum at 280 nm being eluted through a Sephadex gel filtration column.

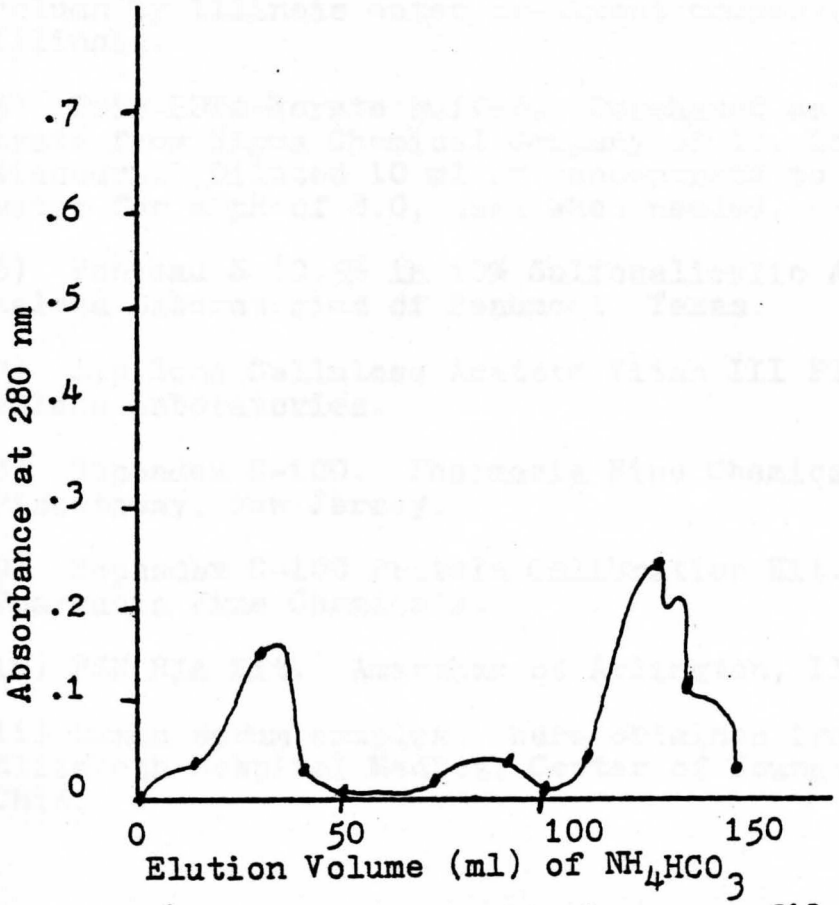


Fig. 6 . Human serum absorbance profile at 280 nm. Volume of sample was 1.0ml. Data points are shown every 10 ml.

CHAPTER III

MATERIALS AND APPARATUS

A. Materials

The chemicals required for this study were all Analytical Reagent grade and used without futher purification.

1) Ammonium bicarbonate, General Chemical Company, New York, N.Y.

2) Glacial acetic acid, Mallinckrodt, St. Louis. Missouri.

3) Methanol, Mallinckrodt.

4) Distilled deionized water. Distilled water from Corning Meg-Pure was passed through an ion-exchanger column by Illinois water treatment company, Rockford Illinois.

5) Tris-EDTA-Borate buffer. Purchased as a concentrate from Sigma Chemical Company of St. Louis, Missouri. Diluted 10 ml of concentrate to 27 ml of water for a pH of 8.0, used when needed.

6) Ponceau S (0.5% in 10% Sulfosalicylic Acid). Helena Laboratories of Beaumont, Texas.

7) Zip Zone Cellulose Acetate Titan III Plates. Helena Laboratories.

8) Sephadex G-100. Pharmacia Fine Chemicals of Piscataway, New Jersey.

9) Sephadex G-100 Protein Calibration Kit. Pharmacia Fine Chemicals.

10) FSH RIA Kit. Amersham of Arlington, Illinois.

11) Human serum samples. Were obtained from St. Elizabeth Hospital Medical Center of Youngstown, Ohio.

B. Apparatus

1) K 26/70 Gel Filtration Column. This column was purchased from Pharmacia Fine Chemicals.

2) Automatic Fraction Collector. The 3 ml fractions were collected by a Fractomat Automatic Fraction Collector with a photoelectric volumetric dispensing head from Buchler Instruments of Fort Lee, New Jersey.

3) Electrophoresis. This was done utilizing a Titan power supply with electrophoresis chamber. The densitometric scans were made by a Quick Scan Flur-Vis of which all of these instruments are made by Helena Laboratories.

4) Gamma Counter. The gamma counts for the RIA were performed on a Clinical Spectrometer Model 1000 which has a sample changer Model 1200 form Nucleus of Oak Ridge, Tennessee.

5) Flash-Evaporator. Concentration was performed by a Buchi Rotovapor of Switzerland.

6) Spectrophotometer. Beckman 26 reading spectrophotometer was used for relative absorbance measurements from Beckman Instruments of Fullerton, California.

7) Centrifuge. All techniques requiring centrifugation were performed on a GLC-1 centrifuge from Sorvall Company of Newton, Ct.

8) pH Meter. All pH measurements were performed on a combination pH electrode from Senorex of Irvine, California, on a Metrion IV pH meter from Coleman Instruments of Maywood, Illinois.

9) Pipettes. Micropipettes with disposable tips were used to do the required pipetting techniques, they were purchased from Centaur Chemical Company of Stomford, Conn.

10) Drying Oven. A Stabil-Therm Constant Temperature Cabinet was used in the electrophoresis technique, was from Blue M Electric Company of Blue Island, Illinois.

11) Vortexing. The apparatus for vortexing was from the Vortec Corporation of Cincinnati, Ohio.

CHAPTER IV

EXPERIMENTAL

A. Sample Preparation

The serum samples from St. Elizabeth Hospital Medical Center of Youngstown, Ohio, were selected at random with respect to race, age and sex. The majority of the specimens studied were from women. The specimens were initially collected as whole blood in vacutainer tubes. They were allowed to clot, then they were spun at 3000 g for 10 minutes. The serum was then removed in 2-3 ml aliquots and placed in sterile disposable 10 ml test tubes. Parafilm was placed over the top of the tubes prior to freezing. The specimens remained frozen until needed. Prior to use the serum was allowed to thaw at room temperature. It was then mixed gently to ensure a homogeneous sample.

B. Gel Filtration

Sephadex G-100 lot # 4202 was prepared for a Pharmacia Column K 26/70[13]. The Sephadex G-100 gel particle size was 40-120 μ with a bed volume per gram dry gel of 15-20 ml. The gel was heated in a boiling water bath for 5 hours in several volumes of 0.05 M ammonium bicarbonate at pH 8.0. After heating, the gel was then allowed to sit overnight in this solvent to get optimal swelling of the Sephadex beads. The buffer was then removed leaving a thick

slurry of the gel. The slurry was then poured slowly down the wall of the vertically mounted K 26/70 column. The gel was applied in a single step. Air bubbles were avoided to ensure even packing. Too fast an application of the gel to the column, or a thin slurry, would cause air pockets which could result in uneven flow and cause zone broadening or overlapping of what usually would be distinct zones. The flow of eluent was started immediately upon filling the gel into the column. The operational pressure, the difference between free surface of eluent in the reservoir and the outlet, was between 40-70 mm Hg. This is shown in figure 7 . Four column volumes of eluent were passed through the column to equilibrate the gel bed at a higher flow rate than used in the study. The column at this point was checked for homogeneity, and then it was calibrated.

Pharmacia calibration kit for Sephadex G-100 lot # 6819 was used for checking homogeneity and for calibration. The proteins used for calibration have their pertinent data presented in table 3 which was obtained from the kit supplier.

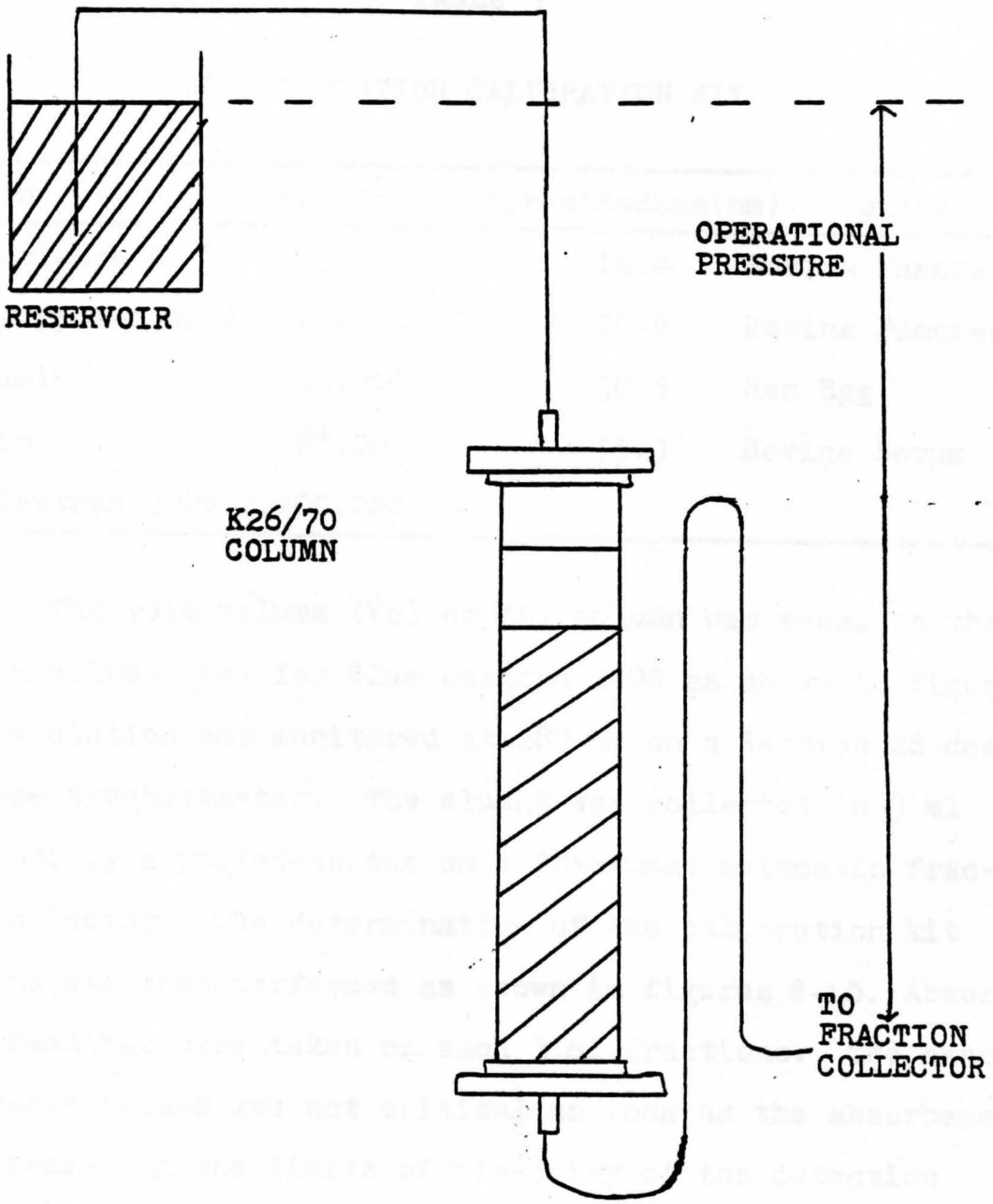


Fig. 7. Operational pressure diagram of the K26/70 column.

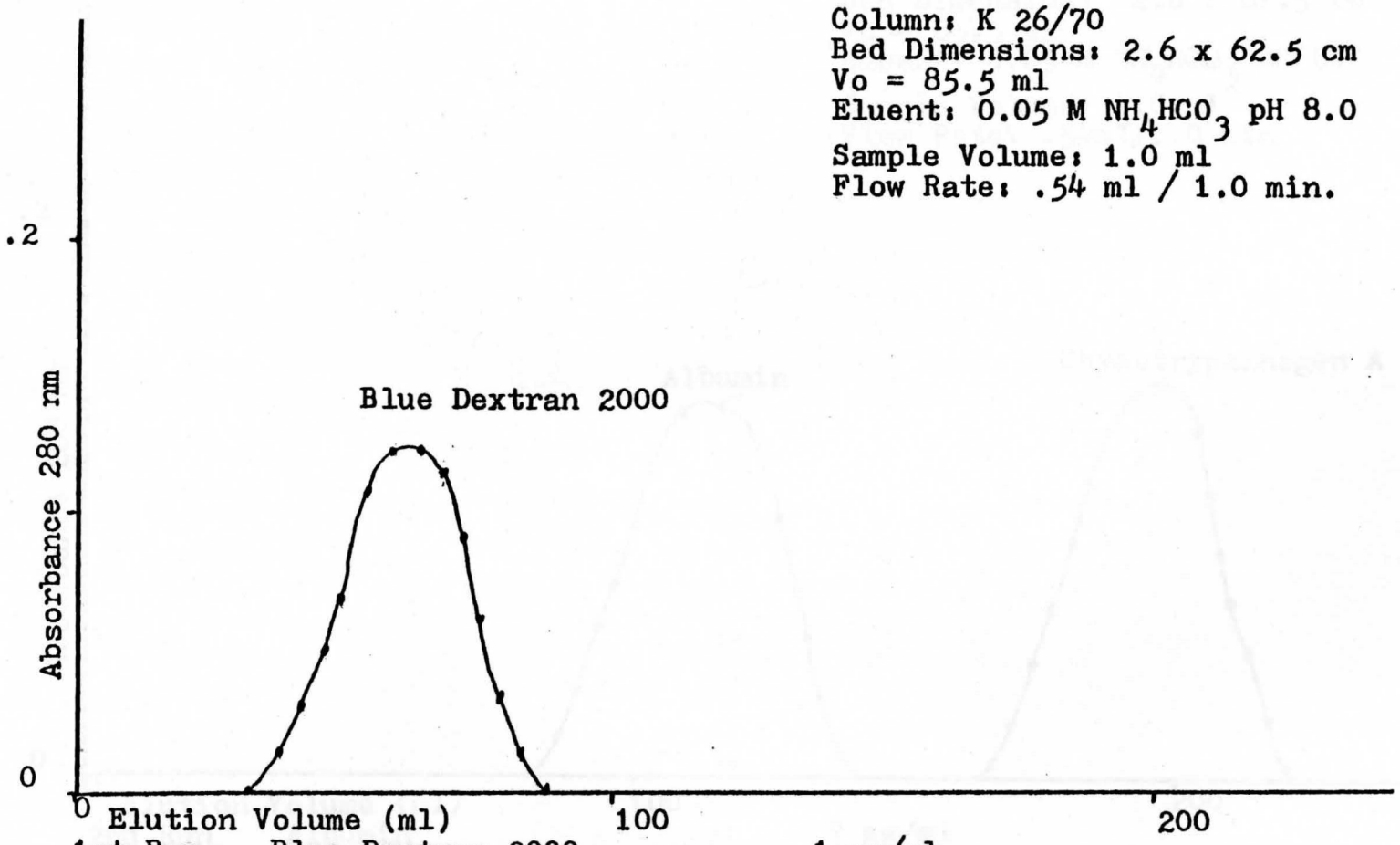
TABLE 3

GEL FILTRATION CALIBRATION KIT

Protein	Mol.Wt.	Stokes'Radius(nm)	Source
Ribonuclease A	13,700	16.4	Bovine Pancreas
Chymotrypsinogen A	25,000	20.9	Bovine Pancreas
Ovalbumin	43,000	30.5	Hen Egg
Albumin	67,000	35.5	Bovine Serum
Blue Dextran-2000	2,000,000		

The void volume (Vo) of the column was equal to the elution volume (Ve) for Blue Dextran 2000 as shown in figure 8 . The elution was monitored at 280 nm on a Beckman 26 dual beam spectrophotometer. The eluent was collected in 3 ml fractions by a photodetector on a Fractomat automatic fraction collector. The determination of the calibration kit proteins was then performed as shown in figures 8-10. Absorbance readings were taken on each 3 ml fractions. The exact absorbance values are not critical as long as the absorbance peaks remain in the limits of linearity of the detection instrument. The elution profiles show how much eluent does it take to get the protein of interest off of the column. The elution volumes from figures 8-10 were measured by the center of the elution peak as determined by the intersection of the two tangents drawn through the sides of the peak. This is shown in figure 11 .

ELUTION PROFILE OF CALIBRATION KIT PROTEINS



1st Run Blue Dextran 2000 1 mg/ml

Fig. 8. Blue Dextran 2000 Elution Profile. One out of four data points are shown on the graph.

ELUTION PROFILE OF CALIBRATION KIT PROTEINS

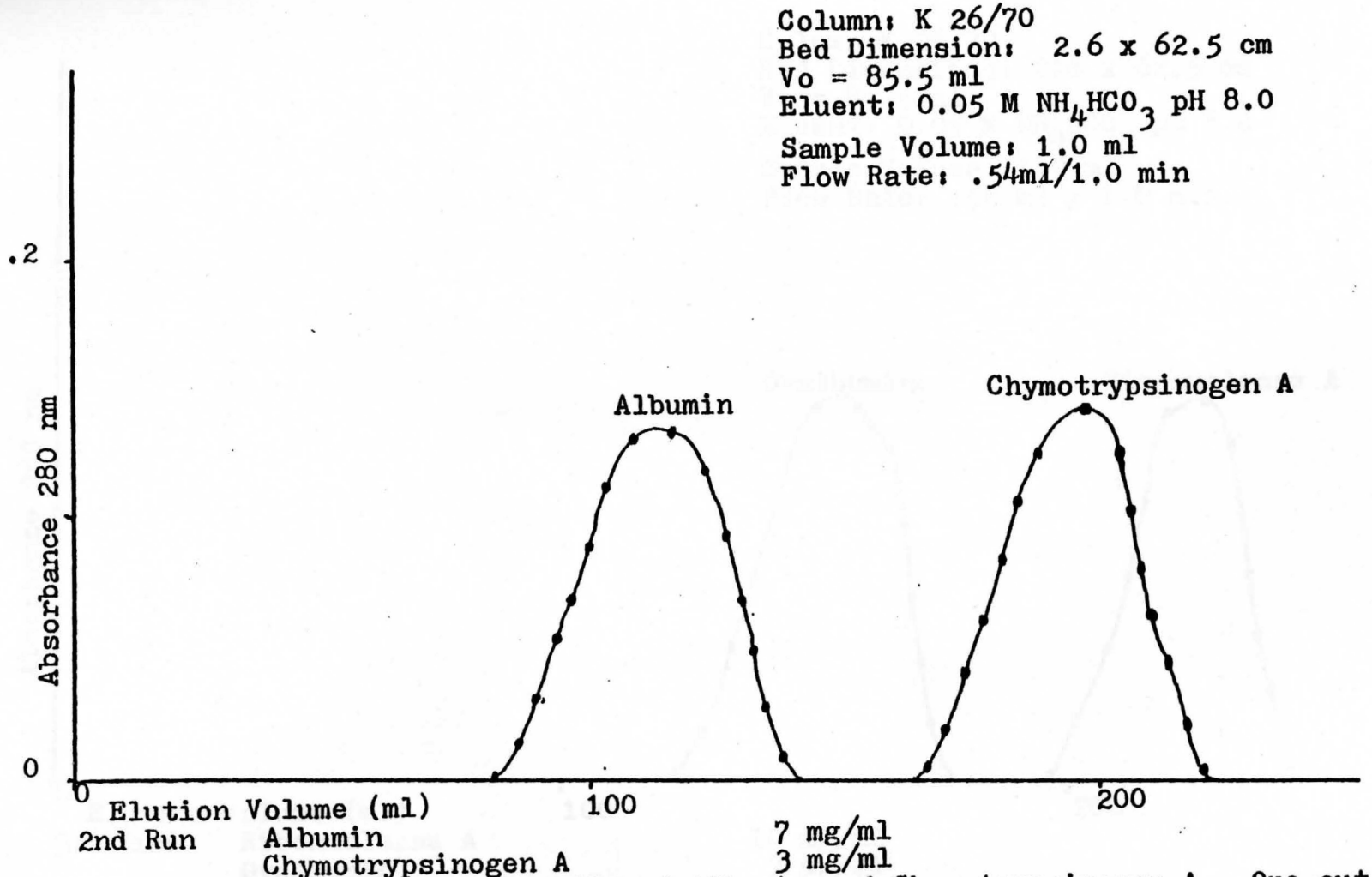


Fig. 9. Elution Profile of Albumin and Chymotrypsinogen A. One out of four data points are shown on the graph.

ELUTION PROFILE OF CALIBRATION KIT PROTEINS

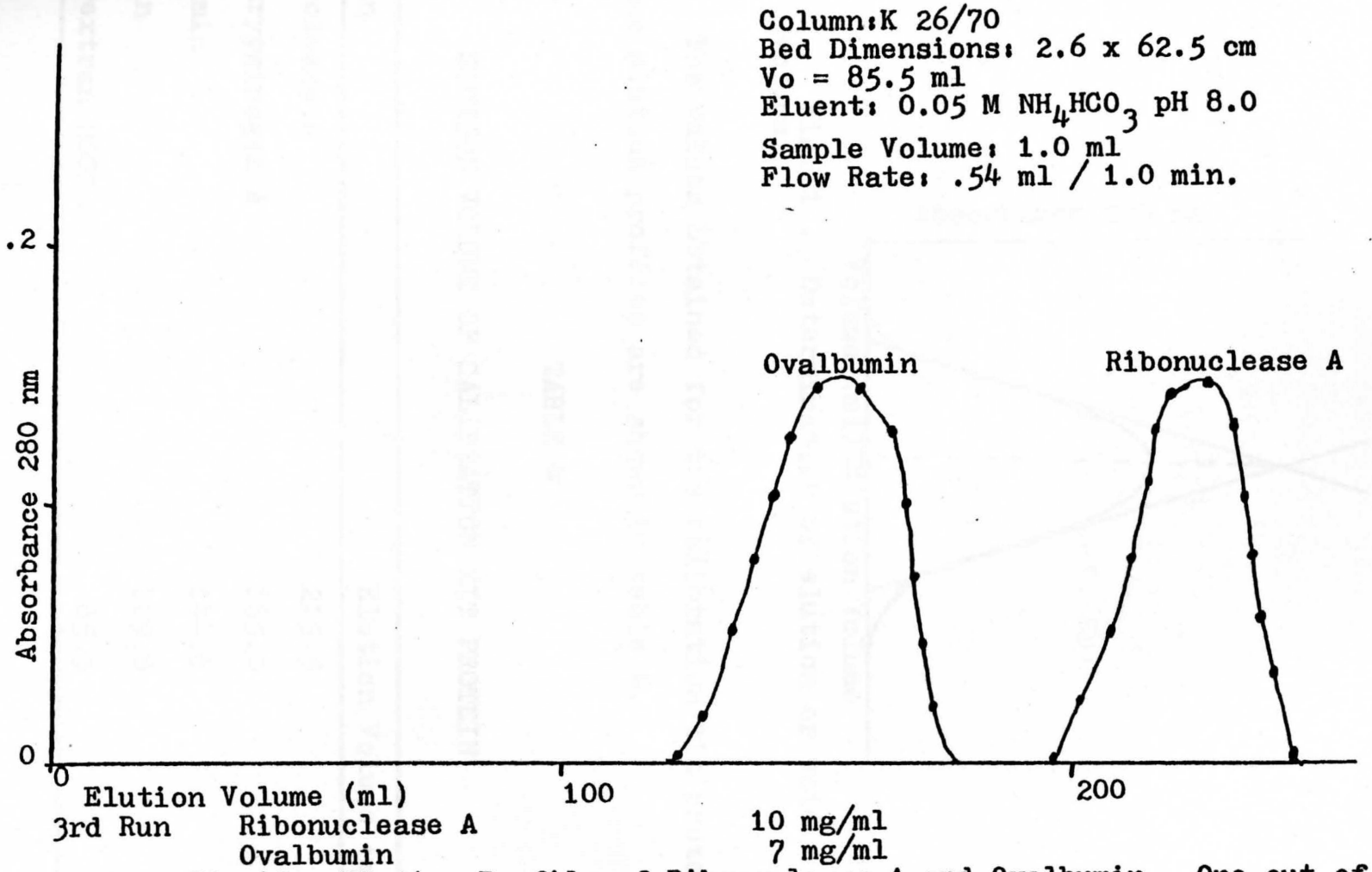


Fig.10. Elution Profile of Ribonuclease A and Ovalbumin. One out of four data points are shown on the graph.

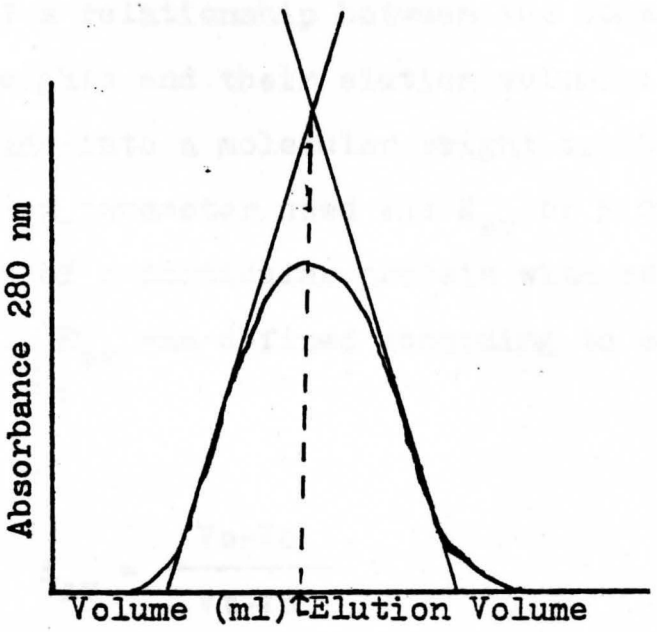


Fig.11 . Determination of elution or void volume.

The values obtained for the calibration kit proteins from the elution profiles are shown in table 4.

TABLE 4

ELUTION VOLUME OF CALIBRATION KIT PROTEIN

Protein	Elution Volume (ml)
Ribonuclease A	215.5
Chymotrypsinogen A	185.0
Ovalbumin	144.0
Albumin	119.0
Blue Dextran 2000	85.5

There exist a relationship between the logarithm of the molecular weights and their elution volumes. This relationship was made into a molecular weight calibration curve[11]. The elution parameter used was K_{av} or K average. K_{av} is the constant of a particular protein with respect to its elution volume. K_{av} was defined according to equation 1.

$$K_{av} = \frac{Ve-Vo}{Vt-Vo} \qquad (1)$$

Where: Ve = elution volume for that protein
Vo = column void volume
Vt = total bed volume

For each protein of the calibration kit a K_{av} was determined which is shown in table 5.

TABLE 5

VALUES FOR K_{av}

Protein	Experimental	Literature
Ribonuclease A	.53	---
Chymotrypsinogen A	.41	.45
Ovalbumin	.24	.29
Albumin	.14	.19

Literature values from reference 13.

K_{av} was used because it is less sensitive to variation in column preparation and it does not require the questionable determination of internal volume. K_{av} was then plotted on a linear scale versus molecular weight on a logarithmic scale as shown in figure 11. The experimental results are linear with the same slope as that of data from the literature[13].

The K_{av} of FSH was then determined by taking its molecular weight and obtaining the corresponding K_{av} from figure 11. The K_{av} was then placed in equation 1 and the elution volume for FSH was determined. The elution volume for FSH was determined to be 159 ml. This means that the peak of the FSH in this study was in the 54th fraction. Fraction 53 and 55 were also collected. These three fractions gave a molecular weight range for protein recovery form 33,000 to 35,500.

C. Concentration and Confirmation

The fractions from gel filtration were refrigerated until the concentration step could be performed. The 9 ml from fractions 53-55 were evaporated to dryness in a Buchi Rotovapor while warmed in a water bath at 60°C. The residues were then resuspended in 1 ml of 0.05 M NH_4HCO_3 pH 8.0. A FSH RIA determination with I^{125} was then done on these samples.

Amersham FSH RIA kit lot # 118 was used for the initial serum determinations on samples 10-14 and also the

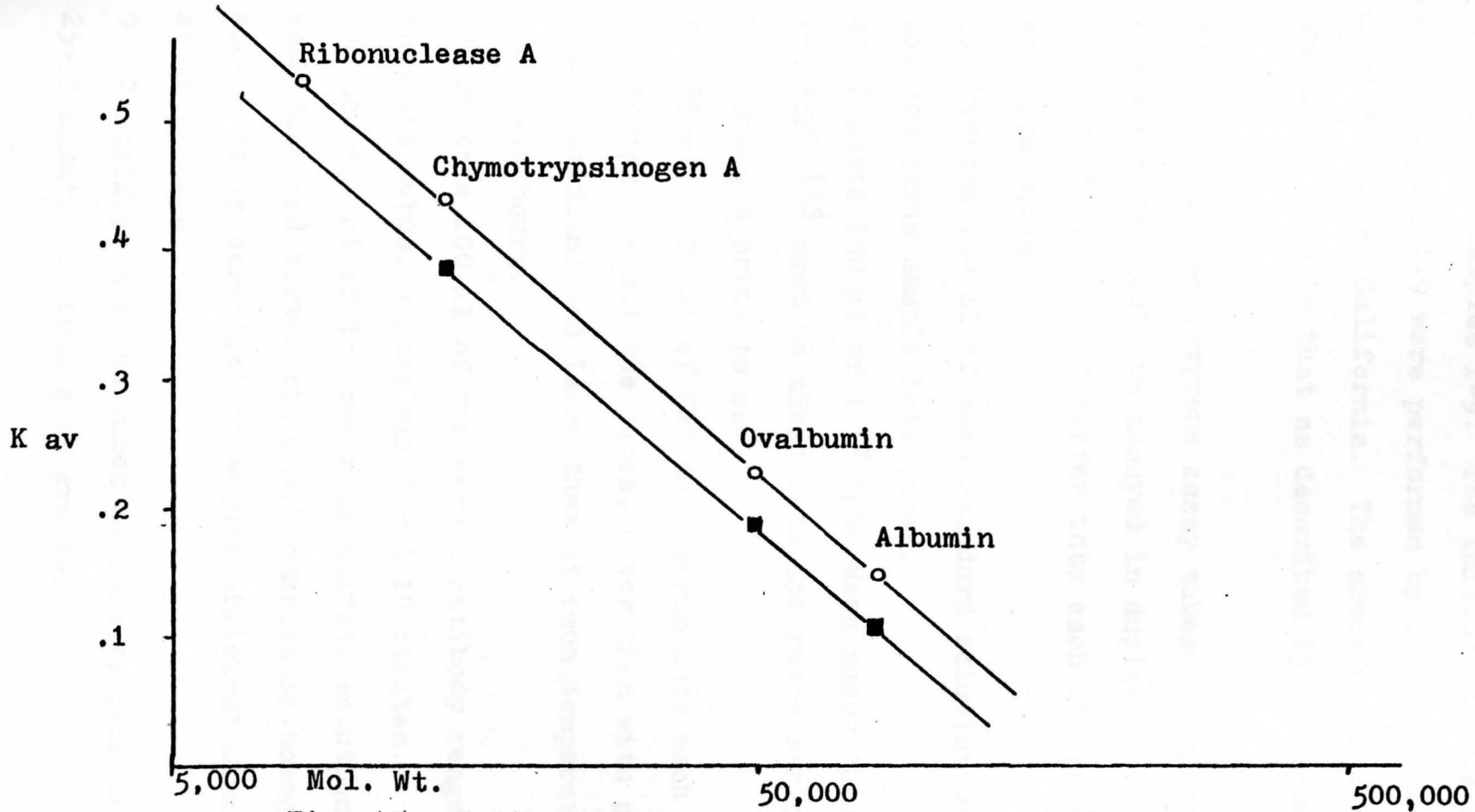


Fig. 12. Calibration curve with literature (■) and experimental (o) values.

RIA confirmation on samples 1-9. The initial FSH RIA determinations of samples 1-9 were performed by Bio Science Laboratories of Van Nuys, California. The procedure for FSH RIA determination used is that as described by Amersham follows:

1) Label the polystyrene assay tubes. Standards and unknowns should be assayed in duplicate.

2) Pipette 100 µl of buffer into each of the zero standard tubes.

3) Pipette 100 µl of each standard solution or patient serum sample into tubes.

4) Pipette 100 µl of I^{125} into each assay tube. The FSH I^{125} must be mixed with the resin strip for 1 to 3 hours prior to use.

5) Pipette 100 µl of FSH anti-serum into each tube.

6) Vortex mix all the tubes, cover then with plastic film (parafilm) and leave them at room temperature for 16-24 hours.

7) Pipette 100 µl of the second antibody reagent into the tubes, vortex mix for 5-10 minutes.

8) Add 500 µl of the ammonium sulfate solution to each tube and vortex mix until completely homogeneous. It is essential to ensure efficient mixing at this stage.

9) Centrifuge all the tubes at room temperature for 25-35 minutes at 1000 g or greater.

10) As soon as the centrifuge has stopped, remove the tubes carefully and decant and discard the supernatant solution. Then, keeping the tubes inverted place them on a pad of absorbent tissues to drain for 5-10 minutes. Before turning the tubes upright, press the mouth of each tube against the pad of tissues to remove any droplets adhering to the rim.

11) Count all the tubes in a gamma-counter for the time required to accumulate about 10,000 counts in the zero standard tubes. This will vary from approximately 2-6 minutes depending of the efficiency of the counter and the age of the kit.

The results of the determinations are shown in table 6 and 7. All of the counts in table 6 have had the background counts subtracted from them. The calibration curve of the data from table 6 and 7 are shown in figure 13.

The counts in table 6 and 7 were obtained on a Nucleus clinical spectrometer model 100 with a sample changer model 1200. Sample 1 had one ml and samples 9,10,11, and 12 had two ml for their sample volumes. All other samples had 3 ml sample volumes. In table 6 the background counts was 252 counts per 120 seconds. All the samples were counted for 120 seconds. All samples showed FSH activity. The samples were then evaporated to dryness as previously described. Then they were resuspended in 100 µl of 0.05 M NH_4HCO_3(pH 8.0) The confirmation by RIA of FSH in samples

TABLE 6

FSH BY RIA

Sample	Counts	Mean	FSH(mIU/ml) Eluent	FSH(mIU/ml) Serum Sample
1*	11328 11347	11338	.2	12
2	10589 10547	10568	1.2	5
3	9812 9803	9808	2.8	12
4	9611 9610	9611	4.4	15
5	8054 8054	8054	8.0	6
6	10168 10172	10170	2.4	2
7	10240 10264	10252	2.0	6
8	10539 10586	10563	1.4	11
9	9801 9830	9816	3.0	10
FSH Standards				
0.0	12063 12120	12092		
2.2	10221 10135	10178		
7.4	8219 8155	8187		
19	5446 5420	5433		
62	2788 2813	2801		
300	1525 1543	1534		

Background count was 252 cts/ 120 sec. All counts were for 120 seconds. * denotes male. FSH standards are in mIU/ml.

TABLE 7

FSH BY RIA

Sample	Counts	Mean	FSH(mIU/ml) Serum Sample	Sample Volume (ml)
10	4990 5018	5004	25	2.0
11	3238 3251	3245	47	2.0
12	2837 2806	2822	61	2.0
13	6778 6817	6848	12	3.0
14	2410 2377	2393	85	3.0
FSH Standards				
0.0	11655 11599	11627		
2.2	10821 10662	10742		
7.4	8478 8416	8447		
19	5593 5645	5619		
62	2766 2823	2795		
300	1683 1678	1681		

Background count was 295 cts/ 180 seconds. All counts were for 180 seconds. FSH standards are in mIU/ml.

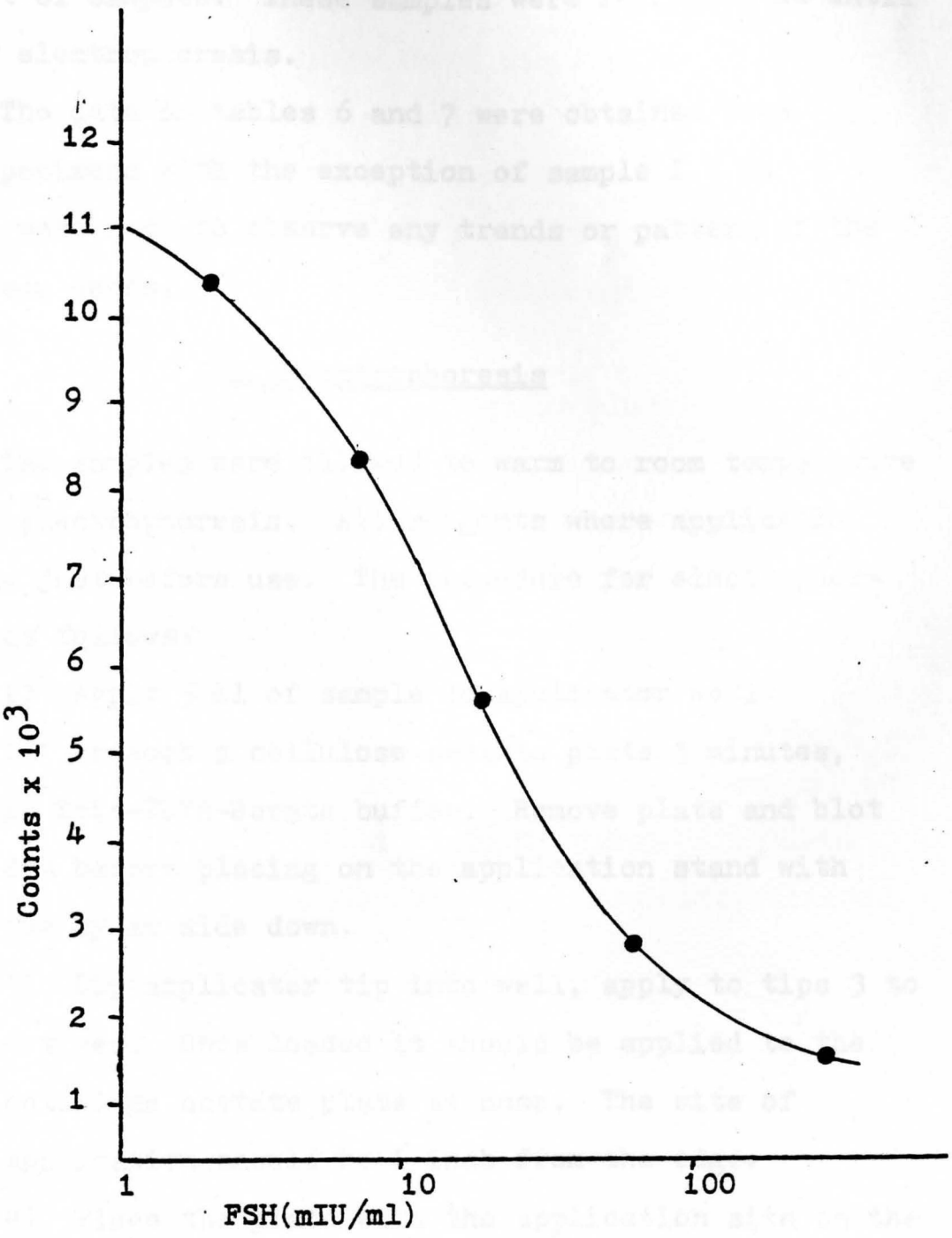


Fig. 13. RIA data from tables 6 and 7. ●denotes FSH standards from table 6 and 7.

10-14 were not necessary for FSH was previously shown in the first set of samples. These samples were refrigerated until used for electrophoresis.

The data in tables 6 and 7 were obtained from female specimens with the exception of sample 1. No attempts were made to observe any trends or pattern of the FSH between sexes.

D. Electrophoresis

The samples were allowed to warm to room temperature prior to electrophoresis. All reagents where applicable were made just before use. The procedure for electrophoresis is as follows:

1) Apply 5 µl of sample to applicator well.

2) Presoak a cellulose acetate plate 5 minutes, in Tris-EDTA-Borate buffer. Remove plate and blot dry before placing on the application stand with the mylar side down.

3) Dip applicator tip into well, apply to tips 3 to 4 times. Once loaded it should be applied to the cellulose acetate plate at once. The site of application should be 1 inch from the edge.

4) Place the plate with the application site on the cathode side of the chamber. The chamber should have been filled with the Tris-EDTA-Borate buffer.

5) Electrophorese for 25 minutes at 200 volts and 2 milliamps per plate.

6) Remove at once upon completion and place in Ponceau S for 10 minutes.

7) Rinse three times for 5 minutes each in 5% v/v glacial acetic acid / water.

8) Then two 5 minutes washes in methanol.

9) Place for 3 minutes in clearing agent. The clearing agent is glacial acetic acid-methanol in a 1:4 v/v ratio.

10) Place plate on a thick glass plate mylar side down and place in a 80°C drying oven.

11) Remove plate after 3 minutes and allow to cool to room temperature. It is now ready to be scanned by a densitometer.

The Helena application device could hold up to eight samples and all samples were applied with such a device. The prepared cellulose acetate plate is illustrated in figure 14. This also shows representative how the

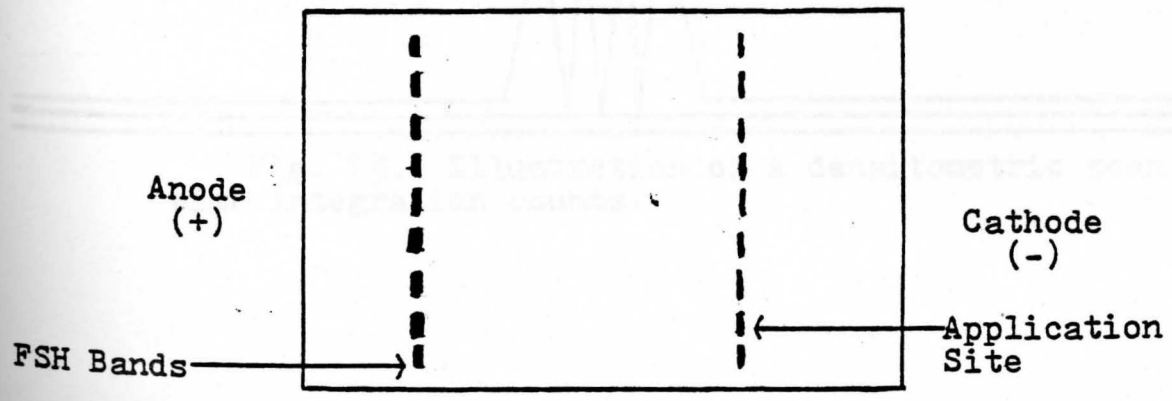


Fig. 14. Illustration of a cellulose acetate plate prior to densitometer scanning.

FSH bands appeared prior to a densitometric reading. All bands seen were consistent with respect to migration thus demostrating a single protein was present at pH 9.0.

The cleared cellulose acetate plates were then subjected to densitometric scans. A 505 nm filter was used corresponding to the absorption maximum of Ponceau S. A single peak did appear for each of the FSH bands. Shown in figure 15 is an illustration of a typical densitometric scan pattern of FSH.

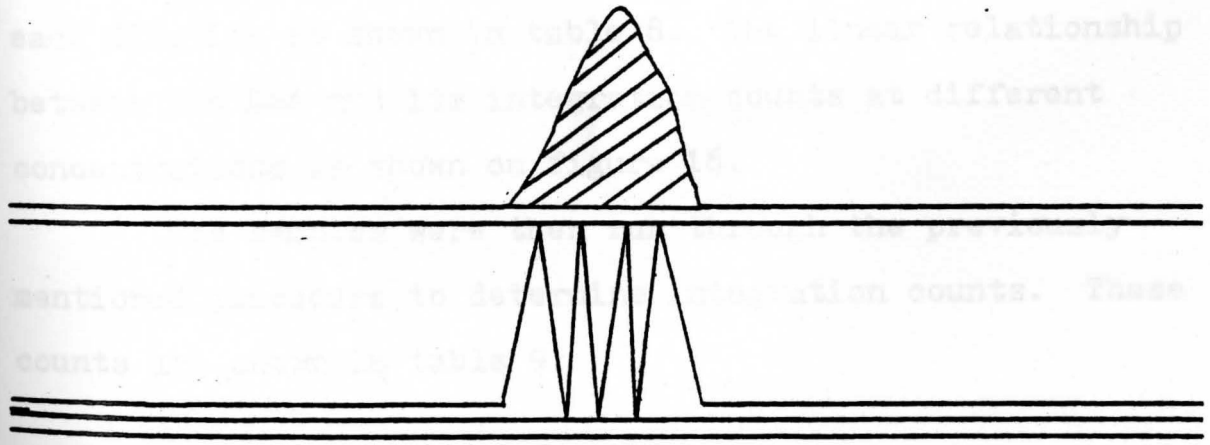


Fig. 15. Illustration of a densitometric scan with integration counts.

Integration counts made by the integration unit on the densitometer are seen under the peak. These counts are representative of the area under the peaks which is shaded as shown in figure 15. The integrator counts can then be related to concentration of the FSH. The scan pattern indicated that in all of the samples studied that a single protein was present due to the gaussian nature of the peak.

The integration counts of the FSH were compared to known concentrations of BSA which was run for integration counts. Bovine serum albumin (BSA) was used for this purpose. A series of dilutions were made from an initial solution of 0.1 gram per ml BSA. BSA was chosen because of the ease of access and it also has constant physical specifications. The serial dilutions were from 1:1 to 1:16. The value for each dilution is shown in table 8. The linear relationship between the BSA and its integration counts at different concentrations is shown on figure 16.

The samples were then run through the previously mentioned procedure to determine integration counts. These counts are shown in table 9.

TABLE 8

INTEGRATION COUNTS OF BSA

Stock Solution Number	Concentration Stock Solution (g/ml)	Volume of Stock Used	# µg BSA	Counts
1	0.1	5µl	500.0	29.4
2	0.5	5µl	250.0	27.4
3	0.025	5µl	125.0	12.6
4	0.0125	5µl	62.5	8.8
5	0.00625	5µl	31.3	2.0

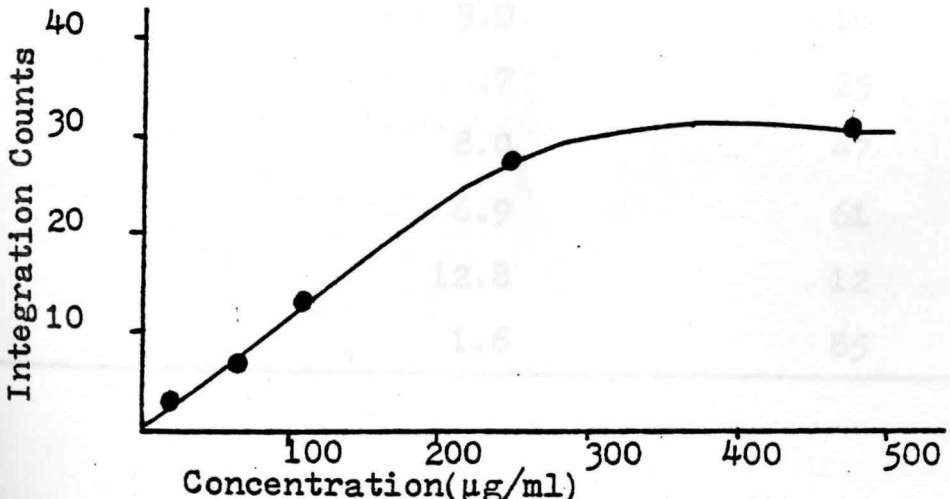


Fig. 16. Linear Relationship between BSA Concentration and Integration counts. Data taken from table 8.

TABLE 9

INTEGRATION COUNTS FOR SAMPLES

Sample #	Counts	RIA (mIU/ml) Serum Samples
1	1.5	12
2	2.6	5
3	1.4	12
4	4.3	15
5	.9	6
6	1.0	2
7	4.8	6
8	3.1	11
9	3.0	10
10	.7	25
11	8.0	47
12	6.9	61
13	12.8	12
14	1.6	85

E. Quantitation of FSH

The integration counts from the densitometric scans were used to predict the initial FSH concentration. Concentration of FSH in the sample was determined by comparing the sample counts to that of a standard. The standard used was the 0.00625 g/ml or the 1:16 of the 0.1 g/ml BSA. By assuming the relationship in equation (2), the concentrations of the samples were computed. A2-3 ml of the serum samples

$$C_s = \frac{I_s \, C_{std}}{I_{std}} \qquad (2)$$

Where: C_{std} = concentration of the standard

C_s = concentration of the sample

I_{std} = integration counts of the standard

I_s = integration counts of the sample

were added to the column initially. Fractions 53-55 were collected for each sample and reduced to 0.1 ml. The integration counts of the samples then represents this 0.1 ml concentrate. The concentration of the 9 ml eluent to 0.1 ml must be taken into consideration. This is done by dividing 90 into the representative value of the 0.1 ml of the concentrated sample. The column effect will be assumed to be

constant for every sample thus it can be ignored in the calculations for every sample. Next the sample size must be taken into account since the initial volumes were different. The number of milliliters of the sample volume were divided into the representative value for FSH. This value was then multiplied by an appropriate conversion factor to change the units to μg/dl. These values have the same units as listed previously in table 1. The results of such calculations are tabulated in table 10.

TABLE 10

EXPERIMENTAL FSH VALUES

Sample #	Counts	Sample Volume (ml) For Serum	Experimental Values(μg/dl)
1	1.5	1.0	26.0
2	2.6	3.0	15.0
3	1.4	3.0	8.1
4	4.3	3.0	24.9
5	.9	3.0	5.2
6	1.0	3.0	5.8
7	4.8	3.0	27.8
8	3.1	3.0	17.9
9	3.0	2.0	26.0
10	.7	2.0	6.1
11	8.0	2.0	69.4
12	6.9	2.0	59.9
13	12.8	3.0	74.1
14	1.6	3.0	9.3

F. Statistical Comparison of Methods

The central tendency of a set of data is defined as the mean ($\bar{x}$). The measurement of dispersion of a set of data about the mean is called the standard deviation (s). The standard deviation expressed as a percentage of the mean is called the coefficient of variation (cv). The equations for the mean, standard deviation, and the coefficient of variation are shown in 3, 4, and 5 respectively. Σ stands

$$\bar{x} = \frac{x_i}{N} \tag{3}$$

$$s = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{N-1}} \tag{4}$$

$$cv = \frac{s(100\%)}{\bar{x}} \tag{5}$$

for the summation of the following terms and x_i refers to integer values of the x value. The study will define x belonging to the comparative method while y will refer to the test method. N stands for the number of samples while N-1 stands for the degrees of freedom. In this study, the data are assumed to fit a parametric statistics or a gaussian distribution. The test method mean, standard deviation and coefficient of variation are shown in table 11 and the comparative method values are in table 12. The variance (v) represents how much variation between values.

TABLE 11

TEST METHOD: STATISTICAL VALUES

Sample #	y (µg/dl)	$y-\bar{y}$	$(y-\bar{y})^2$
1	26.0	- .80	.64
2	15.0	-11.80	139.24
3	8.1	-18.70	349.69
4	24.9	- 1.90	3.61
5	5.2	-21.60	466.56
6	5.8	-33.64	441.00
7	27.8	1.00	1.00
8	17.9	- 8.90	79.21
9	26.0	- .80	.64
10	6.1	-20.70	428.49
11	69.4	42.60	1814.75
12	59.9	33.10	1095.61
13	74.1	47.30	2237.29
14	9.3	-17.50	306.25

$\bar{y}$ = 26.8 s = 23.8 cv = 88.8% v = 4.88

TABLE 12

COMPARATIVE METHOD: STATISTICAL VALUES

Sample #	x (mIU/ml)	$x-\bar{x}$	$(x-\bar{x})^2$
1	12.0	-21.1	445.21
2	5.0	-17.1	292.41
3	12.0	-21.1	445.21
4	15.0	- 7.1	50.41
5	6.0	-16.1	259.21
6	2.0	-20.1	404.01
7	6.0	-16.1	259.21
8	11.0	-11.1	123.21
9	10.0	-12.1	146.41
10	25.0	2.9	8.41
11	47.0	24.9	620.01
12	61.0	38.9	1513.21
13	12.0	-10.1	102.01
14	85.0	62.9	3956.41

$\bar{x}$ = 22.1 s = 25.8 cv = 116.6% v = 5.08

The standard error of the mean ($S\bar{x}$) is representative of the dispersion of the mean value. The standard error of the mean is defined in equation (6). For the test

$$S\bar{x} = \frac{s}{\sqrt{N}} \tag{6}$$

method the $S\bar{y}$ was 6.4 and the $S\bar{x}$ of the comparative method was 6.9. The true mean (μ) of the measurements are representative of the condidence interval around the experimental mean as shown in equation (7). Where t is a multipler

$$\mu = \bar{x} \pm t \frac{s}{\sqrt{N}} \tag{7}$$

appropriate for the degrees of freedom and desired confidence level which shall be throughout this study to be $\pm$ 3 standard deviations or 99.7% confidence level. For the test method the μ is 9.2. Samples 11, 12, and 13 fall outside these limits. On the comparative method the μ is 20.8. Samples 11, 12, and 14 fall outside these limits. A conclusion could be made that systematic error may exist in both methods because the confidence intervals determined does not overlap the ideal interval of 100%.

G. Paired t-Test

The paired t-test method was used because the pair of measurements are on one set of samples. The bias of the two methods or the difference between the two means is shown in equation (8). $\bar{y}$ is the mean of the test method

$$\text{bias} = \bar{y}-\bar{x} \tag{8}$$

and $\bar{x}$ is the mean of the comparative method. The bias was determined to be 4.7. The standard deviation of the differences (Sd) is defined in equation (9). The Sd value was

$$Sd = \sqrt{\frac{\Sigma[(y_i-x_i) - \text{bias}]^2}{N-1}} \tag{9}$$

calculated to be 0.194. The ratio of two terms, one of which represents systematic difference or bias, with the other term representing a random error (Sd/N). This ratio is called the t-value (t). The relationship is shown by equation (10). The t-value was calculated to be 90.6.

$$t = \frac{\text{bias}}{Sd/\sqrt{N}} \tag{10}$$

This means the systematic error is 90.6 times larger than the random error term. This could be due to the uncertainty of the test method measurements. This uncertainty might be

caused by the random error of each of the test method measurements. The critical t-value for 14 samples with 99.7% confidence is 2.98. The calculated t-value is much greater than the critical value. This might mean that there may be systematic error present in the test method. Acceptability of performance of the test method can not be solely based on t-values results. This value only indicates that there may exist a random or systematic error. The t-values can take on a variety of values as the bias and Sd approches their ideal value of zero.

H. F Test

By subjecting the data to a F test a comparison of the variance of the test method with the variance of the comparative method. The variance is the square of the standard deviation. The F test aids in the determination of random error or precision. The previous t-test is used more for the systematic error or accuracy. The F test value (F) is defined in equation (11). Where s_1 is the standard

$$F = \frac{(s_1)^2}{(s_2)^2} \tag{11}$$

deviation of the test method and s_2 is the standard deviation for the comparative method. From the data previously stated in tables 11 and 12 a calculated F value of .85 was

determined. The difference in variances of the study may be significant. There exist no differences as shown between the variances or in the random error of the two methods.

I. Linear Regression

A qualitative assessment could be made by just graphing data as shown in figure 17. A quantitative assessment can be made by a linear regression analysis of the analytical error. The slope (b) of the linear regression line is defined in equation (12). Using the data from

$$b = \frac{N\Sigma x_i y_i - \Sigma x_i y_i}{N\Sigma x_i^2 - (\Sigma x_i)^2} \qquad (12)$$

tables 11 and 12 the calculated slope was determined to be 17.1. The y intercept (a) was then determined by equation (13). The y intercept was calculated to be -351.1. The

$$a = \bar{y} - b\bar{x} \qquad (13)$$

standard deviation about the regression line (Sy/x) is defined in equation (14). Yi is calculated from equation (15). The calculation of Sy/x was found to be 3.09×10^6.

$$Sy/x = \sqrt{\frac{\Sigma(y_i - Yi)^2}{N-2}} \qquad (14)$$

$$Yi = a + bx \qquad (15)$$

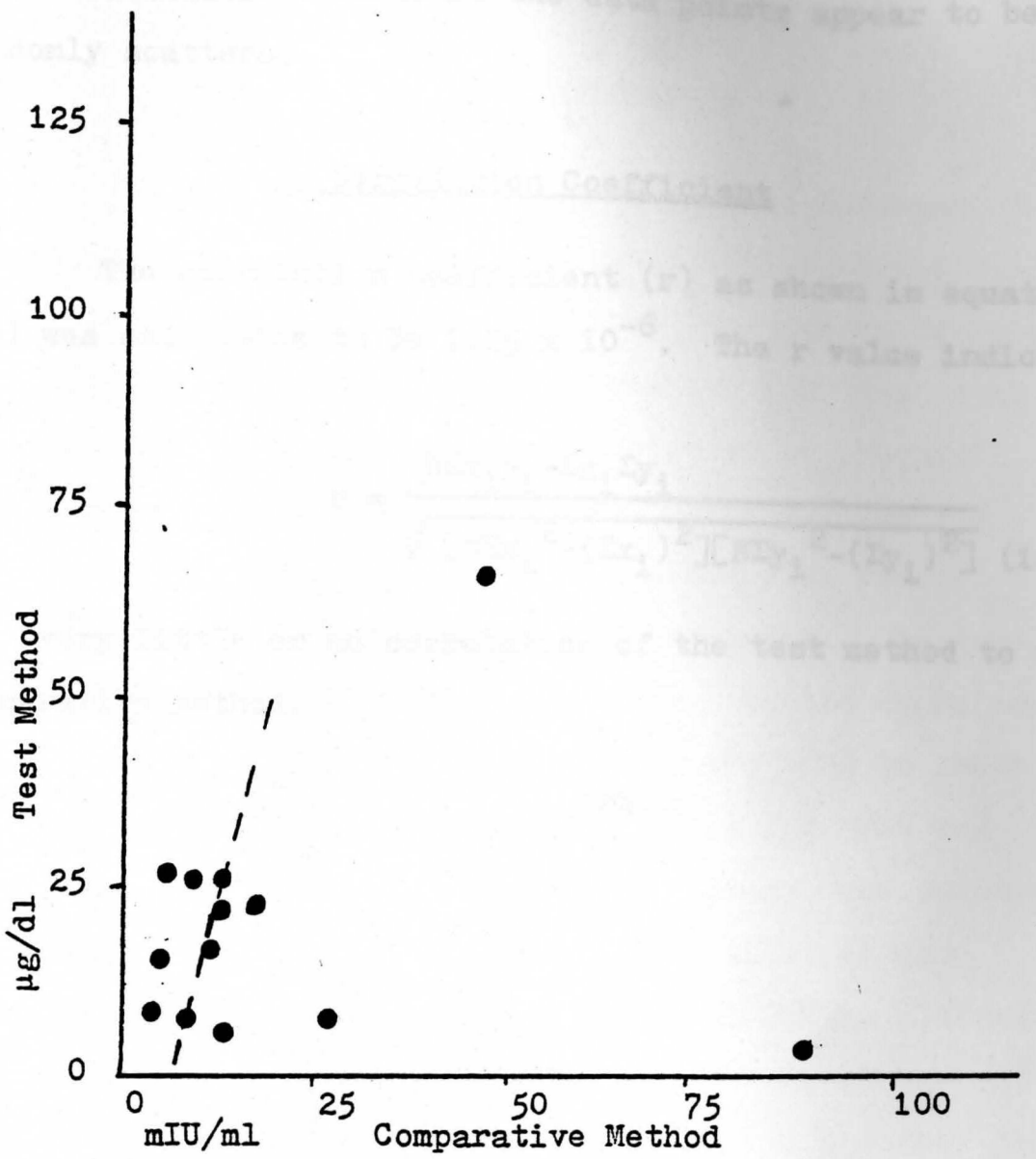


Fig. 17. Semi-quantitative assessment of data by linear regression.

As the calculation indicated the data points appear to be randomly scattered.

J. Correlation Coefficient

The correlation coefficient (r) as shown in equation (16) was calculated to be 1.25×10^{-6}. The r value indicates

$$r = \frac{N\Sigma x_i y_i - \Sigma x_i \Sigma y_i}{\sqrt{[N\Sigma x_i^2 - (\Sigma x_i)^2][N\Sigma y_i^2 - (\Sigma y_i)^2]}} \quad (16)$$

very little or no correlation of the test method to the comparative method.

CHAPTER V

SUMMARY AND CONCLUSION

This study was cumulative toward the development of a chromatographic method for the quantitative determination of hFSH. The separation of hFSH from human serum by Sephadex gel filtration was followed by a concentration step. The concentrated eluent was then subjected to electrophoresis on cellulose acetate plates using a Tris-EDTA-Borate buffer. The proteins were stained with Ponceau S and then processed for densitometric scanning. The scans allowed the proteins to be quantitated. By the comparision of the number of integration counts of the hFSH to those of proteins of known concentrations, a value for hFSH was obtained. The values obtained in this study were compared with those of RIA using the usual statistical methods.

The goal of this study was to develop an alternate method for the determination of hFSH. This goal was accomplished, however, futher experimentation with a rather large number of sample determinations will be required to draw meaningful conclusions. With this in mind the following limited conclusions can be drawn.

First of all the technique or method developed works. It allows the separation and quantitation of hFSH. However the results obtained lack consistency. This the

author believes may be due to a number of reasons. Possibly there may be a reaction occurring in the column matrix. Another reason may be due to a rather small sample size used for the statistical analysis which contains a large systematic and random error component. Greater sensitivity may have been obtained in the staining of the protein bands if other dyes such as Coomassie Brilliant Blue G-250 were used in place of Ponceau S.

It appears that there is varying immunological activity in individuals with respect to total hFSH concentrations. The immunological activity has not been the purpose of this study, but it may be considered in future studies.

The method proposed in this study requires futher development before it could be considered clinically acceptable. Until that time it is the view of the author that RIA be used in hFSH assays.

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