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Research Article

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High yield extraction of crude HCG hormone from pregnant women

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ABSTRACT

Urine from the pregnant women was collected and then subjected to estimate the protein content which was found to be 243.73 mg with a biological activity of 50IU/mg. Since, purity of a protein has been directly proportional to its biological activity, the obtained protein HCG was subjected to the solvent precipitation method. Subsequently, the purity increased to 150 IU/mg in content of 81.24 mg. After the salt fractionation method, the protein content 48.89 mg exhibited 250 IU/mg of biological activity. This led to the extraction of crude HCG of 24.36 mg with a biological activity of 500 IU/mg.

Key words: HCG, purification, biological activity, 500IU/mg

INTRODUCTION

Human Chorionic Gonadotropin (hCG), is a heterodimer and a glycoprotein is produced by syncytiotrophoblast of the placenta during the implantation [1]. Steroid hormones like progesterone (P4) and estradiol (E2) along with gonadotropins which includes the human chorionic gonadotropin (hCG) are paramount in regulating the menstrual cycle and in the establishment and maintenance of pregnancy [2, 3] by trophoblastic invasions [4] promoting angiogenesis. During the commencement of the first trimester, hCG interacts with its receptor LHCG and thus facilities the maintenance of the corpus luteum, allowing it to secrete the hormone progesterone [5], apparently making hCG responsible for all the early pregnancy symptoms. The levels of hCG in early pregnancy are measured to be doubled for every 2-3 days with an increase of about 60% for every two days [6]. However, it was noted that the rise in the hCG levels is consistent for 10-12 weeks of gestation and thereafter tends to plateau or ever drop off [7]. One of the most vital roles of hCG is to make sure that the fetus is provided with the essential nutrients required for its proper growth by effectively utilizing the fat stored in the body of the pregnant women [8]. There are an additional set of hormones that are produced at different concentration and which exert various functions during the pregnancy period. They are Human Placental Lactogen (hPL), Estrogen and progesterone.

EXPERIMENTAL SECTION

The prime requirement for the experiment is the urine of pregnant women. Urine from various pregnant women is collected by agents. Benzoic acid used for the precipitation of the protein is obtained commercially and is also used at various other steps as the experiment proceeds further. Likewise, methanol, acetone, diethyl ether is also purchased commercially. The dialysis, ultra filtration, ion exchange and affinity chromatographic material used is

also ventured commercially. Urine from pregnant women was collected by agents. Further, the desired concentration of the protein is ensured by the Immunoassay technique.

CALCULATION:

(Test sample/std sample) x std concentration.

This is an assay method in which the concentration of HCG is determined. Bio assays may be assessed qualitatively and quantitatively. In the qualitative bioassay the quality of the protein HCG is analysed while in the quantitative method, the concentration of the potency of a substance is determined by the measurement of its biological response. Used immature male rats of the same strain,19 to 28days old, differing in age by not more than 3days and having body masses such that the difference between the heaviest and the lightest rat is not more than 10grams. The rats were assigned at random to 6 equal groups of at least 5 animals. Sets of litter mates were available, assigned one litter mate from each set to that the each group and marked according to litter. Chosen 3 doses of the reference preparation and 3 doses of the preparation to be examined such that the smallest dose is sufficient to produce a positive response in some of the rats and the largest dose does not produce the maximal response in all the rats. Doses were used in geometric progression and as an initial approximation total doses of 4 IU,8 IU and 16IU were tried although the dose will depend on the sensitivity of the animals used, which may vary widely.

Dissolved separately the total quantities of the preparation to be examined and of the reference. Preparation corresponding to the daily doses were used in sufficiently phosphate-albumin buffered saline pH 7.2 R such that the daily dose is administered in a volume of about 0.5ml.Suitable antimicrobial preservative were added such as 4g/l of phenol or 0.02 g/l of thiomersal. Stored the solutions at 5±3°C . Injected subcutaneously into each rat the daily dose allocated to its group, on 4 consecutive days at the same time each day. On the fifth day, about 24h after the last injection, euthanized the rats and removed the seminal vesicles. Extraneous fluid and tissue were removed and weighed the vesicles immediately. Results were calculated by usual statistical methods, using the mass of the vesicles as the response(The precision of the assay may be improved by a suitable correction of the organ mass with reference to the body mass of the animal from which it was taken; an analysis of covariance may be used). The estimated potency is not less than 80 percent and not more than 156 percent of the stated potency. The confidence limits (P=0.95) of the estimated potency are not less than 64 percent and not more than 156 percent of the stated potency.

TABLE 1: BIOASSAY RESULTS – 500IU/MG PURIFICATION PROTOCOLS

Product Name:			**HCG -500 IU/mg (B.P.)**										
Batch No:			***PHD 001***		**Date of analysis:**			02.11.2014					
Reg. No:			**JLSR/11/01001/14**		**Date of completion:**			06.11.2014					
Label claim:			**500 IU /mg**		**Date of report:**			10.11.2014					
Date of Mfg:			**Oct' 2014**										
Protocol No.			**JLS/IAEC-01/01/11/2014**										
Set I (Working Standard)		**2044.11**	**IU**	X	1	vial	50	ml	(40.88 IU/ml)				
Dilution details: Potency 2044.11 IU/vial			10.00 ml→ 51.10 ml (8.00 IU/ml) → 10 ml→ 20 ml (4.00 IU/ml) → 10 ml→ 20 ml (2.00 IU/ml)										
W.Std. B.No: WS/CHG05													
Date of Mfg:		**20/05/2014**	**Date of Expiry:**			**20/05/2015**							
Time of buffer preparation:			10.12 A.M.		**Daily dose of injection:**		0.5 ml						
Time of injection:			10.45 A.M.										
Buffer Lot.No.			**JLS/01/11/2014**										
ANIMAL DISTRIBUTION & DISSECTION DETAILS													
Total Dose	I.D. Mark	Body weight (in gms)		Wt of semival vesicles (in mg)	Total Dose	I.D. Mark	Body weight (in gms)		Wt of seminal vesicles (in mg)	Total Dose	I.D. Mark	Body weight (in gms)		Wt of seminal vesicles (in mg)
		Initial	Final				Initial	Final				Initial	Final	
4.0 IU	H	30	39	26	8.0 IU	H	30	40	38	16.0 IU	H	30	41	51
	B	31	41	31		B	32	42	41		B	30	43	56
	T	32	43	27		T	33	43	35		T	33	43	54
	HB	33	45	32		HB	34	46	39		HB	34	45	57
	BT	36	46	29		BT	35	46	37		BT	35	46	52
	HBT	-	-	-		HBT	-		-		HBT	-	-	-
	HT	-	-	-		HT	-	-	-		HT	-	-	-
	A	-	-	-		A	-	-	-		α	-	-	-
	Total Wt			**145**		Total Wt			**190**		Total Wt			**270**
Cage No				1	Cage No				2	Cage No				3

Set II (Sample Single Assay)	**500**	IU	X	10	mg	100	ml	(50.00IU/ml)
Diln. details: Potency 500IU/mg		10.00 ml→62.5 ml (8.00 IU/ml) → 10 ml→ 20 ml (4.00 IU/ml) → 10 ml→ 20 ml (2.00 IU/ml)						
Time of buffer preparation:		10.12 A.M.		**Daily dose of injection:**		0.5 ml		
Time of injection:		10.51 A.M.						
Buffer Lot.No.		**JLS/01/11/2014**						

ANIMAL DISTRIBUTION & DISSECTION DETAILS

Total Dose	I.D. Mark	Body weight (in gms)		Wt of seminal vesicles (in mg)	Total Dose	I.D. Mark	Body weight (in gms)		Wt of seminal vesicles (in mg)	Total Dose	I.D. Mark	Body weight (in gms)		Wt of seminal vesicles (in mg)
		Initial	Final				Initial	Final				Initial	Final	
4.0 IU	H	30	41	28	8.0 IU	H	30	39	39	16.0 IU	H	30	40	56
	B	33	43	25		B	31	40	36		B	32	42	51
	T	34	43	31		T	34	40	40		T	32	44	55
	HB	35	45	29		HB	35	43	37		HB	34	44	59
	BT	36	45	27		BT	36	45	34		BT	36	46	57
	HBT	-	-	-		HBT	-		-		HBT	-	-	-
	HT	-	-	-		HT	-	-	-		HT	-	-	-
	A	-	-	-		A	-	-	-		α	-	-	-
	Total Wt			**140**		Total Wt			**186**		Total Wt			**278**
Cage No				4	Cage No				5	Cage No				6

❖ *Where H= Head,B= Back,T= Tail,HB= Head Back,BT=Back Tail,HT=Head Tail,α= Without Mark*

TABLE 2: DUPLICATE ASSAY-500IU/MG PURIFICATION PROTOCOLS

Set III (Sample Duplicate Assay)	**500**	IU	X	10	mg		100	Ml	(50.00 IU/ml)
Diln. details: Potency 500 IU/mg		10.00 ml→ 62.5 ml (8.00 IU/ml) → 10 ml→ 20 ml (4.00 IU/ml) → 10 ml→ 20 ml (2.00 IU/ml)							
Time of buffer preparation:		10.12 A.M.		**Daily dose of injection:**			0.5 ml		
Time of injection:		10.56 A.M.							
Buffer Lot.No.		**JLS/01/11/2014**							

ANIMAL DISTRIBUTION & DISSECTION DETAILS

TotalDose	I.D. Mark	Body weight (in gms)		Wt of seminal vesicles (in mg)	Total Dose	I.D. Mark	Body weight (in gms)		Wt of seminal vesicles (in mg)	Total Dose	I.D. Mark	Body weight (in gms)		Wt of seminal vesicles (in mg)
		Initial	Final				Initial	Final				Initial	Final	
4.0 IU	H	31	40	26	8.0 IU	H	30	42	41	16.0 IU	H	30	39	51
	B	32	42	33		B	30	42	36		B	33	41	55
	T	33	43	30		T	34	43	39		T	33	46	57
	HB	35	46	32		HB	35	46	42		HB	35	46	53
	BT	35	46	28		BT	36	46	37		BT	35	46	50
	HBT	-	-	-		HBT	-		-		HBT	-	-	-
	HT	-	-	-		HT	-	-	-		HT	-	-	-
	A	-	-	-		A	-	-	-		A	-	-	-
	Total Wt			**149**		Total Wt			**195**		Total Wt			**266**
Cage No				7	Cage No				8	Cage No				9

Table 3: BLANK ASSAY-500IU/MG PURIFICATION PROTOCOLS

Set IV (Blank)														
Phosphate buffer														
Time of buffer preparation:				10.12 A.M.		**Daily dose of injection:**			0.5 ml					
Time of injection:				11.06 A.M.										
Buffer Lot.No.				**JLS/01/11/2014**										

ANIMAL DISTRIBUTION & DISSECTION DETAILS

Total Dose	I.D. Mark	Body weight (in gms)		Wt of seminal vesicles (in mg)	Total Dose	I.D. Mark	Body weight (in gms)		Wt of seminal vesicles (in mg)	Total Dose	I.D. Mark	Body weight (in gms)		Wt of seminal vesicles (in mg)
		Initial	Final				Initial	Final				Initial	Final	
2.0 ml	H	32	41	14		H					H			
	B	33	41	9		B					B			
	T	34	43	13		T					T			
	HB	35	45	10		HB					HB			
	BT	36	46	12		BT					BT			
	HBT	-	-	-		HBT	-	-			HBT	-	-	-

	HT	-	-	-		HT	-	-	-		HT	-	-	-
	A	-	-	-		A	-	-	-		A	-	-	-
	Total Wt			**58**		Total Wt			**0**		Total Wt			**0**
Cage No				10	Cage No					Cage No				

RESULTS AND DISCUSSION

The hCG glycoprotein requires the disulphide bonds to attain the final stable conformation, nevertheless, all the non-cysteine that exists within the hCG cysteine knot are required for the dimer formation and assembly [9]. Mishra *et al* [10] in the year 2003 reported that the disulphide bonds β 9-57, β 34-88 and β 38-90 of the hCG β are essential for the formation of the heterodimer while the disulphide bonds α 7-31, α 59-87 and α 10-32 are not required for hCG α to combine with hCG β. It was noted that the cysteine knot with 3 disulphide bonds renders a ring that includes the intervening polypeptide backbone [11]. Structurally, hCG exists in multiple variants in serum and urine samples, but is limited to free hCG α and free hCG β in the serum of pregnant women [12]. It is a general phenomenon that the regular hCG is produced during all the conditions of pregnancies, normal, abnormal, abortions. Moreover, the presence of normal HCG is not only reported in women with pregnancies comprising solely trophoblast tissues or hydatid form [13, 14] but also during the elevated levels of LH. In addition to these, it is also seen in menopausal women [15, 16]. In contrast to the above, during the ectopic pregnancy and spontaneous abortions, the levels of HCG is at reduced levels, while are present to the double normal levels during Down Syndrome pregnancy [17].

Structurally hCG exists in multiple variants in serum and urine samples, but are limited to free HCGα and hCG β in the serum of pregnant women. Their presence is not only confined to pregnant women, but also seen in serum and urine samples of cancer patients and cancer cell line culture fluids. During 1970 and 1980's much of the structural elucidation of hCG has been done. The aminoacid sequence of hCG subunits was determined in 1970 establishing the presence of 4 N-linked and 4-O linked oligosaccharides [18]. In the corresponding couple of decades, the hCG gene structure of the subunits were delineated. Moreover, the structural determination of N-linked and O- linked oligosaccharides that are secreted in gestational trophobalstic disease [19]. It was thus determined that the polypeptides constitute the formation of hCG, are crucial in making a set of biologically active molecules that include regular hCG, hyper glycosylated hCG and hypergylcosylated hCG β subunit.

To achieve high purity levels is further to increase the activity of the hCG. In this pursuit the experiment proceeds by the injection of the purified hCG into rats in which 10 different groups are preferred and are tagged as cage numbers. The whole experiment is divided into 4 sets. The set consists of working standard, 2044.11 with the dilution details potency 2044.11 IU/Vial includes 50ml (40.88IU/ml) --->10.00ml--->51.10ml (8.00 IU/ml) --> 10ml---> 20ml, (4.00 IU/ml) 10ml--> 20ml (2.00 IU/ml). 0.5ml of volume is injected daily. Set-1 has cage, no.1, cage no.2 and cage no.3. The volume of daily doses of hCG injected into them are 4.0IU, 8.0IU, 16.0IU respectively. The initial body weight and the final body weight of the rats are noted in grams and the weight of seminal vesicles is recorded in mg. However, the total dose varied in the three cages, and in cage-1 4.0 IU, cage-2 8.0 IU & in cage-3 16.0 IU. Set-2 had the testing sample or Compound, since sample has 500IU. Dilution details are different but have same final potency as working standard. Potency 500 IU/mg×10mg --->100ml (50IU/ml) ---> 10.00ml-->62.5ml (800 IU/ml) --> 10ml--->20ml (4.00 IU/ml) --->10ml--->20ml (2.00IU/ml). The cages were cage-4, cage-5 cage-6. Set-3 a sample duplicate assay has same testing sample as in set-2 and was similar to set-2, however, were maintained in cages, cage-7, cage-8 and cage-9 respectively. Set-4 was a blank assay to analyze the results and was maintained in cage-10. The daily dose injected was 0.5ml and the total dose was 2.0 ml. The buffer used for the blank assay was the phosphate buffer. Different purification steps involved to attain 500 IU/mg were as follows. Urine from the pregnant women was collected by the agents. It was then subjected to estimate the protein content and was found to be 243.73 mg with a biological activity of 50IU/mg. Since, purity of a protein has been directly proportional to its biological activity, the obtained protein; HCG was subjected to the solvent precipitation method. Subsequently the purity increases to 150 IU/mg in content of 81.24 mg. After the salt fractionation method the protein content 48.89 mg exhibited 250 IU/mg of biological activity (Tables 1 to 4). This led to the extraction of crude HCG of 24.36 mg with a biological activity of 500 IU/mg.

Table 4: CALCULATION OF POTENCY-500IU/MG PURIFICATION PROTOCOLS

Ta = Ts – Tstd
Tstd = T1+T2+T3
Ts = T4+T5+T6
Tb = (T3+T6) - (T1+T4)
i* = Interval between successive log doses of std preparation & spl preparation
M= 4i* X Ta/3Tb + log R
R = Vstd / Vs
Potency = Anti log M
Average potency = potency of single assay + potency of duplicate assay / 2
Assay % = Average potency X 100 / Label claim potency
Set I (Single Assay)
Ta =-1
Tb =263
Tstd=605
Ts =604
I =0.30103
M =2.69744
Anti log M=498.25
Potency =498.25 IU/vial
Set II (Duplicate Assay)
Ta =5
Tb =242
Tstd=605
Ts =610
I =0.30103
M =2.70726
Anti log M=509.64
Potency =509.64 IU/vial
Avg. Potency = 503.94 IU/mg
Assay (%) =100.8 % of L.A

T1 = Std low
T2 = Std medium
T3 = Std high
T4 = Sample low
T5 = Sample medium
Anti log M= T6 = Sample high
Std – Standard
S – Sample

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