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Research Article

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Affinity chromatography mediated purification of hCG hormone

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ABSTRACT

Affinity chromatography by Carboxy methyl blue Sepharose was used to increase the purity and further the biological activity to more than 12000 IU/mg from 1.05mg of protein. The glycoprotein was then assessed for its conformational and functional ability in rats and could efficiently pronounce itself has a protein with increased purity

Key words: Affinity chromatography, CM-sepharose, biological activity

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INTRODUCTION

The first crystal structure of hCG was elucidated by Lapthorn *et al* [1]. Biochemistry of hCG identifies it to be a glycoprotein, composed of non- covalently linked α and β subunits. This is a heterodimer consisting of 92 amino acid residues in α subunit and the β subunit consists of 121 amino acids [2] . Both the subunits possess high content of mannose moiety and are held together by non – covalent hydrophobic and ionic interactions. The molecular weight of hCG is approximately around 36,000 [3]. However, it is noted that 25-41% of the molecular weight is attributed by the sugar side chain [4]. The subunits are folded in a much similar manner forming two hairpin loops, at one end and a single loop at the other end [5]. The stability of the heterodimer is achieved when the carboxyl terminal peptide (CTP) of hCG β is in contact with hCG α, thus, forming a seat belt around the hCG α which includes the residues from 93-110 amino acids from the β subunit. The final closing of the β 26-110 a bridge lock the seat belt and secures the αβ dimer preventing its disassembly [6]. It is the carbohydrate moiety and its specific location on the peptide chain that decides the folding and the assembly of the subunits. The normal secretion of hCG is ensured by the oligosaccharide branches from Asn 52 and Asn 78 and the removal of hCG α reduces the correct α-β dimerization [7,8]. Disulphide bonds are required for hCG stable conformation [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. Only after the assembly of the α-β subunits, the disulphide bond β 26-110 is evolved [10]. Human Choronic Gonadotropin (hCG), belongs to the cysteine knot family and exists along with the luteinizing hormone, follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). hCG is a heterodimer with α and β subunits. It is initially secreted by the pre- implantation embryo and subsequently by the trophoblast. In continuation of our earlier work [11] we are reporting further purification of hCG with more biological activity

EXPERIMENTAL SECTION

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. Affinity chromatography by Carboxy methyl blue Sepharose was used to increase the purity. Further, the desired concentration of the protein is ensured by the Immunoassay technique. 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 analyzed while in the quantitative method, the concentration of the potency of a substance is determined by the measurement of its biological response.

RESULTS AND DISCUSSION

Bioassay results – 13000IU/mg purification

The amount of HCG obtained from the earlier reported [11] was 4.23 mg of protein with a biological activity of 3000 IU/mg. To attain increased purity of HCG, the above product was subjected to and allowed to pass through the affinity column chromatography using Carboxy methyl blue Sepharose. This led to the formation of the highly purified HCG with >12000IU/mg of biological activity from 1.05 mg of protein content. The measurement of the activity at every step was performed in the rats with 4 set experimental setup (Table 1). Set-1 was the working standard with dilution details, potency 2044.11 IU/Vial --->50ml (40.88IU/ml) --->10.00ml--->51.10ml (8.00 IU/ml)--->10ml--->20ml (4.00 IU/ml)--->10ml--->20ml (2.00 IU/ml). However, the rest of the experiment remained the same as above. Set-2 dilution details for sample, single assay were same as in the previous experiments but had different potency (Table 2). 10.00 ml→100 ml (130.00 IU/ml), 10 ml→ 162.5 ml (8.00 IU/ml), 10 ml→ 20 ml (4.00 IU/ml) 10 ml→ 20 ml (2.00 IU/ml) was observed. The rest of the parameters, however, remained the same. Set-3 was the sample duplicate assay had a similar parameter to set-2 and set-4 was blank assay with phosphate buffer. It can be noted that as the protein content decreases in order to attain high purity levels, the biological activity of the hCG gradually increased.

Table 1: Bioassay results – 13000iu/mg purification protocols

Product Name:	**HCG -13000 IU/mg (B.P.)**		
Batch No:	***PHD 004***	**Date of analysis:**	02.11.2014
Reg. No:	**TLR/11/01001/14**	**Date of completion:**	06.11.2014
Label claim:	**13000 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														
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	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	-	-	-
	α	-	-	-		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)	**13000**	IU	X	10	mg	→	100	ml	(1300.00IU/ml)
Diln. details: Potency 13000 IU/mg		10.00 ml→100 ml (130.00 IU/ml) 10 ml→ 162.5 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

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	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	-	-	-
	α	-	-	-		A	-	-	-		α	-	-	-
	Total Wt			**140**		Total Wt			**186**		Total Wt			**278**
Cage No				4	Cage No				5	Cage No				6

DUPLICATE ASSAY →

Set III (Sample Duplicate Assay)	**13000**	IU	X	10	mg		100	ml	(1300.00IU/ml)
Diln. details: Potency 13000 IU/mg	10.00 ml→100 ml (130.00 IU/ml) 10 ml→ 162.5 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	-	-	-
	α	-	-	-		A	-	-	-		α	-	-	-
	Total Wt			**149**		Total Wt			**195**		Total Wt			**266**
Cage No				7	Cage No				8	Cage No				9

BLANK ASSAY

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

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	
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	-	-	-
	α	-	-	-		A	-	-	-		α	-	-	-
	Total Wt			**58**		Total Wt			**0**		Total Wt			**0**
Cage No				10	Cage No				11	Cage No				12

Table 2: Calculation of potency

Ta = Ts – Tstd	T1 = Std low
Tstd = T1+T2+T3	T2 = Std medium
Ts = T4+T5+T6	T3 = Std high
Tb = (T3+T6) - (T1+T4)	T4 = Sample low
i* = Interval between successive log doses of std preparation &spl preparation	T5 = Sample medium
M= 4i* X Ta/3Tb + log R Anti log M=	T6 = Sample high
R = Vstd / Vs	Std – Standard
Potency = Anti log M	S – Sample
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 =4.11242
Anti log M=12954.40
Potency =12954.40 IU/vial
Set II (Duplicate Assay)
Ta =5
Tb =242
Tstd=605
Ts =610
I =0.30103
M =4.12224
Anti log M=13250.62
Potency =13250.62 IU/vial
Avg. Potency = 13102.51IU/mg
Assay (%) =100.8% of L.A

REFERENCES

[1] A.J. Lapthorn et al. *Nature*. **1994**; 9;369(6480):455-61.
[2] Morgan FJ. *The Journal of Biological Chemistry*; **1975**;250(13),5247-5258.
[3] Lay-Harn Gam, Aishah Latiff . *Int J Biol Sci* ; **2005**; 1(3):103-109. doi:10.7150/ijbs.1.103
[4] LA cole. *Reprod Biol Endocrinol*. **2010**; 8: 102.
[5] Hao Wu1, Joyce W Lustbader 2, Yee Liu1, Robert E Canfield2 and Wayne A. Hendricksonl *Current Biology* , **1994**, 2:545-558
[6] Gamier, J., Osguthorpe, DJ., Robson, B. *J Mol. Biol;***1978**; 120, 97-120.
[7] S.F. de Medeiros and R.J. Norman. *Human Reproduction*;**2009**;15(1) 69–95.
[8] http://shodhganga.inflibnet.ac.in:8080/jspui/bitstream/10603/8173/3/03_chapter%201.pdf
[9] E Bedows et al. *Indian journal of experimental biology*; **2002**; 40;467-476
[10] Mishra AK, Mahale SD, Iyer KS. *Biochim Biophys Acta,* **2003**;1645:49 – 55
[11] Uttam Kumar Neerudu , Ravi Burgula , Ramavath Redyanaik , Ramchander Merugu and Ch. Venkata Ramana Devi . *Journal of Chemical and Pharmaceutical Research*, **2016**, 8(4):1308-1312