

**DEVELOPMENT OF 17- α -HYDROXY PROGESTERONE
IMMUNOASSAY USING DIFFERENT SPACERS IN
IMMUNOGEN AND ENZYME CONJUGATE**

by

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*Dedicated to my
late friend
Bhupi...*

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Abstract

17- α -hydroxy-progesterone is a clinically important steroid hormone which is mainly secreted by zona fasciculata cells of adrenal glands and is routinely measured to screen the patients of congenital adrenal hyperplasia (CAH), a disorder of the steroid biosynthetic pathway observed due to the malfunctioning of enzymes involved in the conversion of cholesterol to cortisol. The classical form of CAH is characterized by severe salt wasting and presence of pheno-typically altered genitalia by birth, whereas non-classical CAH manifests postnatally and mainly presents symptoms of hirsutism and amenorrhoea. As 17- α -OH-P is an effective indicator for monitoring 21-hydroxylase functioning, its accurate and reliable measurement in serum is thus required in the diagnosis and management of CAH. Radioimmunoassays have been widely used for 17- α -OH-P estimation but they have been replaced by non-isotopic labels due to their inherent radiation hazards, disposable problems and license requirements. Among the non-isotopic assays enzyme immunoassays have been preferred over fluoroimmunoassay, chemiluminescent and bioluminescent immunoassays because of their simplicity and cost-effectiveness. The enzyme immunoassays reported so far for 17- α -OH-P estimation are either not very sensitive which is a requirement for post therapeutic management of CAH or are not very specific showing varying degree of cross-reactions with other analogous steroids

The present study has been taken up to develop a sensitive, specific and rapid ELISA for 17- α -OH-P by modifying the immunogen and enzyme conjugates using various spacer molecules. N-hydroxy succinimide-carbodiimide reaction has been used to prepare the immunogen and enzyme conjugates with various spacer molecules using 17- α -OH-P-3-CMO as hapten and HRP and BSA as enzyme and carrier protein respectively. In the first approach, immunogen and enzyme conjugate were prepared to develop a homologous immunoassay without having any spacer molecule in enzyme conjugate and immunogen. A very good

immunoreactivity of the enzyme conjugate 17- α -OH-P-3-CMO-HRP was observed with anti-17- α -OH-P-3-CMO-BSA antibody. The immunoassay was highly specific with very good sensitivity of 0.18 ng/ml. This assay was further validated for analytical recovery, precision and its correlation with EQUAS scheme and well established RIA kit.

In another approach, six homobifunctional spacers namely, urea(U), carbonylhydrazide (CH), adipic acid dihydrazide (ADH), ethylene diamine (ED), gammaaminobutyricacid-urea (GU) and 6-aminocaproicacid-urea (AU) were used for preparing the enzyme conjugate with an aim to improve the sensitivity of the homologous assay without any significant compromise with the assay specificity. 17- α -OH-P-3-CMO-CH-HRP, 17- α -OH-P-3-CMO-ADH-HRP, 17- α -OH-P-3-CMO-U-HRP, 17- α -OH-P-3-CMO-ED-HRP, 17- α -OH-P-3-CMO-GU-HRP and 17- α -OH-P-3-CMO-6-ACA-HRP enzyme conjugates were characterized by Matrix Assisted Laser Desorption Ionisation–Time of Flight (MALDI-TOF) to know the steroid density and confirm the coupling of 17- α -OH-P-HRP. The immunoreactivity of anti 17- α -OH-P-3-CMO-BSA antibody was evaluated with these 17- α -OH-P-spacer-HRP conjugates. The use of spacer in enzyme conjugates improved the homologous assay sensitivity without any significant compromise on the specificity except in case of 17- α -OH-P-3-CMO-ADH-HRP where cross reaction was observed to be twice of the homologous assay. The assay using 17- α -OH-P-3-CMO-CH-HRP was found to have the best sensitivity of 0.008 ng/ml among the bridge heterologous assays by inserting spacer in the enzyme conjugate whereas with use of 17- α -OH-P-3-CMO-ADH-HRP the assay sensitivity was 0.06ng/ml which was least of all the bridge heterologous assays. The better assay sensitivity with the use of 17- α -OH-P-3-CMO-CH-HRP was mainly attributed to the efficiency of the conjugate in reducing the bridge binding and steric hindrance (in homologous assays) due to the presence of a five atom CH as a spacer molecule which is highly hydrophilic and rigid due to the presence of polar groups and mesomeric effect in the

entire molecular chain length. But the presence of 4 hydrophobic methylene units in ADH renders the enzyme conjugate flexible enough so that steroid may easily fold to get buried either in the methylene units or in the hydrophobic pockets in the enzyme, resulting in decreased immunoreactivity and the ability to counter the steric hindrance posed by the enzyme. These methylene units in ADH also interact with the Ab due to hydrophobic interaction resulting in tighter binding of the enzyme conjugate thereby decreasing the sensitivity of the competitive assay. Moreover, the methylene units interact with the other steroids and solid phase by hydrophobic interactions to give higher cross reactions and non specific binding. All the other enzyme conjugates with hydrophilic spacers showed better assay sensitivity and specificity than with the use of 17- α -OH-P-3-CMO-ADH-HRP. The assay using 17- α -OH-P-3-CMO-CH-HRP was further validated for recovery, precision and correlation studies.

ADH, ED and 6-ACA-GABA-ADH spacers were also used for preparing immunogens and resulted in generation of anti-17- α -OH-P-3-CMO-ADH-BSA, anti-17- α -OH-P-3-CMO-ED-BSA and anti-17- α -OH-P-3-CMO-6-ACA-GABA-ADH-BSA BSA antibodies respectively which were evaluated for their tryptophan (Trp) fluorescence to analyze the effect of the spacers in the immunogens on the quality of the resulting antibodies. Anti-17- α -OH-P-3-CMO-BSA antibody which was generated against immunogen without any spacer showed highest immunoreactivity and Trp fluorescence. Though anti-17- α -OH-P-3-CMO-ED-BSA Ab gave good binding with five out of the seven enzyme conjugates used and its Trp fluorescence was comparable to the anti-17- α -OH-P-3-CMO-BSA antibody, it did not give displacement with the enzyme conjugate studied. Anti-17- α -OH-P-3-CMO-6-ACA-GABA-ADH-BSA Ab gave binding and displacement with only two enzyme conjugates, while 17- α -OH-P-3-CMO-ADH-BSA did not give binding with any of the enzyme conjugates used and their Trp fluorescence was low compared to anti-17- α -OH-P-3-CMO-ED-BSA Ab. Both

the immunoassays thus developed using anti-17- α -OH-P-3-CMO-6-ACA-GABA-ADH-BSA Ab were highly non specific showing almost five times cross reaction compared to the homologous assay having no spacer molecule in either of the immunoconjugates. It was concluded that spacers can be used for modifying and preparing the enzyme conjugates for improving the assay sensitivity instead of inserting them in the immunogens.

A colloidal gold based rapid, immunochromatographic strip for 17- α -OH-P was developed by utilizing anti-17- α -OH-P-3-CMO-BSA antibody and 17- α -OH-P-3-CMO-BSA-Gold conjugate. Gold nanoparticles were synthesized and characterized using various techniques. The size of NPs was 36nm with a zeta potential of -44.1mV as determined by Dynamic light scattering. AFM of 17- α -OH-P-3-CMO-BSA-Gold conjugate showed a distinct change in the surface topography as compared to the bare gold NPs. The immunochromatographic strip was prepared by coating anti-17- α -OH-P-3-CMO-BSA antibody at the capture line, anti-BSA antibody at the control line and 17- α -OH-P-3-CMO-ADH-BSA-gold conjugate on reservoir matrix. Competitive inhibition assay was developed on this strip by using 17- α -OH-P standards of different concentrations. The lower detection limit of the developed assay was found to be 1.25 ng/ml by visual observation. The strip was semi-quantitative but took less than 20 min for result development as compared to 80min required in quantitative ELISA.

Contents

Topic	Page No.
Chapter I - Introduction and literature review	
1. Introduction	1
1.1. 17- α -hydroxy-progesterone	2
1.1.1. Biosynthesis and regulation	2
1.1.2. Clinical implications of impaired 17- α -hydroxy-progesterone levels	4
1.2. Analytical techniques for detection of biological compounds	6
1.2.1. Bioassays	6
1.2.2. Physical and chemical methods	6
1.2.3. Binding assays	7
1.2.3.1. Classification of immunoassay	8
1.3. 17- α -hydroxy-progesterone estimation in biological fluids	10
1.3.1. Competitive protein binding assays	10
1.3.2. Physical methods	11
1.3.3. Immunoassay	12
1.3.3.1. Radioimmunoassay	12
1.3.3.2. Enzyme linked immunosorbent assay	13
1.3.3.3. Fluorescence and luminescent immunoassays	14
1.4. Rationale and objectives of the present work	15
References	21

Chapter II - Development of ELISA for 17- α -hydroxy-progesterone without any spacer in immunogen and enzyme conjugate

2.1. Introduction	26
2.2. Experimental	32
2.2.1. Materials	32
2.2.2. Composition of buffers for coating, blocking and preparation of immunoreagents	33
2.2.3. Immunogen preparation	33
2.2.4. Immunogen characterization	34
2.2.4.1. Bradford assay	34
2.2.4.2. UV spectroscopic characterization	34
2.2.5. Immunization and collection of antiserum and normal rabbit serum (NRS)	35
2.2.6. Purification of the IgG fraction from antiserum and NRS	35
2.2.7. Preparation of 17- α -OH-P-3-CMO-HRP enzyme conjugate	36
2.2.8. MALDI-TOF Characterization of 17- α -OH-P-3-CMO-HRP enzyme conjugate	37
2.2.9. Evaluation of antibody and enzyme conjugate by checkerboard assay	37
2.2.10. Evaluation of the assay parameters for competitive ELISA of 17- α -OH-P	38
2.2.11. Validation of immunoassay for 17- α -OH-P	41
2.3. Results and Discussion	42
2.3.1. Immunogen Preparation	42
2.3.2. Immunogen Characterization	42
2.3.2.1. Bradford protein estimation of immunogens	42
2.3.2.2. Hapten density of immunogens by UV spectrophotometry	43
2.3.3. Preparation of 17- α -OH-P-3-CMO-HRP enzyme conjugate	43

2.3.4.	MALDI-TOF characterization of enzyme conjugate	44
2.3.5.	Evaluation of antibody and enzyme conjugate by checkerboard	45
2.3.6.	Evaluation of the assay parameters for competitive ELISA of 17- α -OH-P	48
2.3.7.	Assay validation	52
2.4.	Conclusion	55
	References	56
Chapter III - Development of ELISA for 17-α-hydroxy progesterone using different spacers in enzyme conjugate		
3.1.	Introduction	58
3.2.	Experimental	63
3.2.1.	Materials	63
3.2.2.	Composition of buffers for coating, blocking and preparation of immunoreagents	63
3.2.3.	Preparation of enzyme conjugates using different spacers	63
3.2.4.	Characterization of spacer containing enzyme conjugates	65
3.2.5.	Evaluation of the spacer containing enzyme conjugates with anti-17- α -OH-P-3-CMO-BSA Ab by checkerboard assay	67
3.2.6.	Evaluation of the assay parameters for competitive ELISAs developed for 17- α -OH-P with spacer containing enzyme conjugates	68
3.2.7.	Validation of immunoassay for 17- α -OH-P with spacer in enzyme conjugate	69
3.3.	Results and Discussion	70
3.3.1.	Preparation of enzyme conjugates using different spacers	70
3.3.2.	Hapten density of enzyme conjugates by MALDI-TOF spectrometry	71
3.3.3.	Evaluation of six spacer containing enzyme conjugates with anti-17- α -OH-P-3-CMO-BSA Ab by checkerboard assay	79

3.3.4.	Evaluation of the assay parameters for competitive ELISAs developed for 17- α -OH-P with spacer containing enzyme conjugates	82
3.3.5.	Assay validation	87
3.4.	Conclusion	90
	References	91
Chapter IV - Development of ELISA for 17-α-hydroxy progesterone using different spacers in immunogen		
4.1.	Introduction	92
4.2.	Experimental	93
4.2.1.	Materials	93
4.2.2.	Composition of buffers for coating, blocking and preparation of immunoreagents	93
4.2.3.	Preparation of immunogen with spacers	94
4.2.4.	Immunogen characterization	97
4.2.5.	Immunization and collection of antiserum against spacer containing immunogens	98
4.2.6.	Purification of the IgG from antiserum against spacer containing immunogens	99
4.2.7.	Evaluation of antibodies by checkerboard assays with different enzyme conjugates	99
4.2.8.	Evaluation of the assay parameters for competitive ELISAs of 17- α -OH-P	100
4.2.9.	Trp fluorescence measurement of all anti 17- α -OH-P antibodies	101
4.3.	Results and Discussion	102
4.3.1.	Immunogen preparation using different spacers	102
4.3.2.	Immunogen characterization	102
4.3.2.1.	Bradford protein estimation of immunogens	102
4.3.3.2.	Determination of hapten density of immunogens by UV Spectrophotometry	103

4.3.3. Evaluation of various antibody and enzyme conjugate combinations by checkerboard assay	103
4.3.4. Evaluation of the assay parameters for competitive ELISA of 17- α -OH-P	107
References	113

Chapter V - Development of rapid lateral flow immunochromatographic strip assay for 17- α -hydroxy progesterone using colloidal gold

5.1. Introduction	115
5.2. Experimental	121
5.2.1. Materials	121
5.2.2. Buffers for preparation and storage of gold nanoparticles and gold conjugates	122
5.2.3. Preparation of colloidal gold nanoparticles	122
5.2.4. Preparation of protein conjugate of the colloidal gold	123
5.2.4.1. Stabilization of colloidal gold with the 17- α -OH-P-3-CMO-BSA	123
5.2.4.2. Optimization of pH for conjugation	124
5.2.4.3. Formulation of 17- α -OH-P-BSA-gold conjugate	124
5.2.5. Characterization of colloidal gold and its protein conjugate	125
5.2.6. Preparation and immobilization of immunoreagents	126
5.2.7. Assay principle and procedure	127
5.3. Results and Discussion	127
5.3.1. Preparation of gold nanoparticles	127
5.3.2. Preparation of protein conjugate of the colloidal gold	129
5.3.2.1. Stabilization of colloidal gold with the 17- α -OH-P-3-CMO-BSA	129
5.3.2.2. Optimization of pH for bioconjugation	129
5.3.3. Characterization of colloidal gold and its protein conjugate	130

5.3.4. Preparation and immobilization of Immunoreagents	134
5.3.5. Assay principle and procedure	135
5.4. Conclusion	136
References	137
Chapter VI - Summary and scope for future work	
6.1 Summary	138
6.2 Scope for future work	142