Notification No.: 540/2023

Date of Notification: 23-06-2023

Date of Viva-voce: 05-06-2023

Name of Scholar : Divya Verma

Name of Supervisor : Dr. Sonu Chand Thakur

Name of Co-Supervisor : Dr. T.G. Shrivastav

Name of Department : Centre for Interdisciplinary Research in Basic Sciences

Topic of Research : Development of immunoassay for 17- alpha methyl testosterone using aromatic spacers in immunogen and enzyme conjugate

FINDINGS

In this research work, direct competitive ELISA of 17α-methyl testosterone using antibody against immunogens (with spacers and with no spacer) and enzyme conjugates (with spacers and with no spacer) has been developed to quantify 17 α MT in the serum directly. The immunogens and enzyme conjugates were prepared by NHS mediated Carbodiimide reaction. Aromatic spacers were integrated among 17α-methyl testosterone-3-carboxymethyloxime (17 α MT-3-CMO) and Bovine serum albumin (BSA) or Horseradish peroxidase (HRP) for immunogens and enzyme conjugates preparation. The immunogens 17α MT-3-CMO-DPS-BSA, 17α MT-3-CMO-ODA-BSA, 17α MT-3-CMO-B-BSA, 17α MT-3-CMO-PPD-BSA and 17α MT-3-CMO-D-BSA were prepared using the five aromatic spacers, namely; 4,4'-Diaminodiphenyl sulphide (DPS), 4,4'-Oxydianiline (ODA), Benzidine (B), p-Phenylenediamine (PPD), and Dapson (D). They were then characterized through UV spectroscopy method to confirm the attachment of spacers and further antibodies against them were generated in rabbits.

Similarly, using these aromatic spacers the preparation of E.Cs 17α MT-3-CMO-DPS-HRP, 17α MT-3-CMO-ODA-HRP, 17α MT-3-CMO-B-HRP, 17α MT-3-CMO-PPD-HRP and 17α MT-3-CMO-D-HRP were done, and then

checked for immunoreactivity and binding studies with above antibodies. Subsequently, all the combinations were analyzed for displacement assay, sensitivity, and specificity. Out of all the thirty six combinations, 17 α MT-3-CMO-ODA-BSA antiserum with 17 α MT-3-CMO-DPS-HRP E.C displayed enhanced sensitivity i.e. 0.002 ng/mL, improved ED_{50} i.e. 1.54 ng/mL, and better specificity in terms of percentage cross-reaction that is found with only one steroid (Testosterone-5.6%) out of fifty-nine structurally similar steroids. The above mentioned combination was used to analytically validate parameters such as recovery (97.42% to 108.61%), precision (CV% less than 10) and correlation coefficient calculation (0.94). Our ELISA technique which is developed in this work was also validated by estimating 17 α MT levels in the rat serum after giving doses. It can be concluded that the use of aromatic spacer ODA (4,4'-Oxydianiline) in immunogen enhanced the assay sensitivity and specificity. Reason could be the resonance effect amid -C=O and $-NH_2$ groups alongside the molecule and presence of double bonds in ring structure which in turn provided rigidity.

ABSTRACT

The direct competitive ELISA of 17α-methyl testosterone using antibody against immunogens (with spacers and without any spacer) and enzyme conjugates (with spacers and without any spacer) has been developed to quantify 17 α MT in the serum directly. The best antibody and enzyme conjugate combination in terms of sensitivity and specificity was used for developing the ELISA for 17α MT by further validation studies like recovery, precision and correlation with available kit. The best combination was also used for the estimation of 17α MT levels in rat serum at different concentrations and time durations. In this study, we have developed homologous ELISA of 17 alpha methyltestosterone using 17 α methyltestosterone-3-Carboxymethyloxime-Bovine serum albumin antiserum and 17 α methyltestosterone-3-Carboxymethyloxime-Horseradish peroxidase enzyme conjugate. The sensitivity was found to be 0.11 ng/mL, ED_{50} was 5.78 ng/mL, and affinity of the assay calculated was $0.02x10^{-8}$ L/mol. The cross-reaction was observed with six steroids (Testosterone- 38.3%, 6-hydrotestosterone- 43.7%, androstenediol- 19.16%, androstenedione- 3.52%, Danazol- 25.14% and Nandrolone-19%) out of fifty nine structurally related steroids for this assay combination. The analytical variables of the assay were studied i.e. Recovery ranged from 94.8% to 111.6%, Intra and Inter-assay CV% was found to be less than 10%, Correlation coefficient calculated was 0.96 (R^2) by comparing our kit with commercially available kit. The ELISA was further validated by estimating 17α methyltestosterone levels in rat serum after giving them injections of 17 α MT intramuscularly. Then, we conjugated spacers (bridge) between enzyme and 17 α Methyl testosterone and their impact on the analytical parameters such as sensitivity, ED_{50}, and specificity of the assay was studied. We have developed bridge heterologous ELISA for the detection of 17 α Methyl testosterone by incorporating aromatic spacers between 17α Methyl testosterone-3-Carboxymethyloxime and Horseradish peroxidase label through N-hydroxysuccinimide mediated carbodiimide reaction method. Out of these five

combinations, the combination 17α MT-3-CMO-BSA and 17α MT-3-CMO-Benzidine-HRP was the best. The sensitivity calculated was 0.02 ng/mL, ED_{50} was 2.95 ng/mL, and affinity of the assay calculated was $0.086x10^{-8}$ L/mol. The cross-reaction was observed with four steroids (Testosterone-5.14%, 6-hydrotestosterone- 6%, Danazol-0.9% and Nandrolone-0.85%) out of fifty nine structurally related steroids for this assay combination. The analytical variables of the assay were studied i.e. Recovery ranged from 97.4% to 108.6%, Intra and Inter-assay CV% were found to be less than 10%, Correlation coefficient calculated was 0.96 (R^2) by comparing our kit with commercially available kit. The ELISA was further validated by estimating 17α methyltestosterone levels in rat serum after giving them injections of 17 α MT intramuscularly. After that, incorporation of aromatic spacers (bridge) between 17α methyltestosterone and BSA (immunogen) and 17α methyltestosterone and HRP (enzyme conjugate) was done and studied their effect on sensitivity, $ED_{50,}$ and specificity of the ELISA. Out of all the thirty six combinations including homologous, bridge homologous and bridge heterologous combinations, 17 α MT-3-CMO-ODA-BSA antiserum with 17 α MT-3-CMO-DPS-HRP E.C displayed enhanced sensitivity i.e. 0.002 ng/mL, improved ED_{50} i.e. 1.54 ng/mL, and better specificity in terms of percentage cross-reaction that is found with only one steroid (Testosterone-5.6%) out of fifty-nine structurally similar steroids. The above mentioned combination was used to analytically validate parameters such as recovery (97.42% to 108.61%), precision (CV% less than 10) and correlation coefficient calculation (0.94). Our ELISA technique which is developed in this work was also validated by estimating 17 α MT levels in the rat serum after giving doses. The current statistics of the experiment signifies/concludes that the use of aromatic spacer ODA (4,4'-Oxydianiline) in immunogen enhanced the assay sensitivity and specificity. Reason could be the resonance effect amid -C=O and $-NH_2$ groups alongside the molecule and presence of double bonds in ring structure which in turn provided rigidity.