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Low ambient temperature with the onset of winter can disturb radio immuno assay due to increased association constant of antibody

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ABSTRACT

In our laboratory, Radio-immuno assay(RIA) for quantification of estradiol-17β was standardized using anti-oestradiol(Prof. Niswender, Colorado State University, USA) and 2,4,6,7,16,17-^{3}H oestradiol(Amersham Biosciences Ltd., U.K.) as tracer. Antibody titer was determined to give maximum binding of 50% with the tracer. This titer was used for assays between July to November 2004, all of which exhibited a perfect displacement of tracer by increasing concentration of calibrators. With the onset of winter, assay procedure was suddenly deteriorated, resulting into no displacement of tracer by calibrators. Buffers, standards and other chemicals were prepared freshly but yielded no improvement in repeat trials, the observations of the results obtained in repeated trials revealed that there were significant displacement of tracer in only 2-3 of highest concentration calibrators out of total 10 calibrators. For remaining 7 calibrators, displacement was not visible, though the binding of tracer was very high. While facing this problem, we come across to an important aspect of antibody-heptanes interaction, that low temperature increases association constant of antibody. Working on this point, the antibody titer was recalibrated for winter atmospheric conditions. It was found to increase by 10 folds. Successful re-standardization was accomplished with altered antibody titer. It was concluded that low ambient temperature might disturb your RIA due to increased association constant(Ka) of antibody. © 2007 Trade Science Inc. - INDIA

INTRODUCTION

Radio immunoassay(RIA) is a competitive assay based on antigen-antibody bindings for quantification of antigen of interest in biological fluids/ tissue samples. Competitors include the antigen of interest in biological sample and its radiolabeled homologous(tracer) to compete for binding to fixed and limited sites of antibodies. A set of calibrators from zero to substantial range of expected concentration of antigen in biological samples are run in each assay. The binding of tracer would be highest in zero calibrator but in other calibrators, displacement of tracer occurs depending upon the concentration of calibrator. Higher the concentration of the calibrator, lesser will be the binding of tracer. Determination of bound/total fraction of tracer forms the basis for quantification of antigen in the sample. Attributes of RIA like high sensitivity, specificity, ease of performance and wide applicability made it a valuable tool and it has particularly emerged as a major technique for endocrine investigations. Antibody with high specificity and affinity are used in RIA. Antibody titer, which allows maximum binding of 50% tracer, is generally used in assay. For practical purposes antibody titer, which allows 35-50% binding to tracer, can be safely employed. Binding below 25% and above 50% is not desirable as the assay may loose precision and accuracy. Once the assay is standardized, the same quantity of antibody is usually employed for subsequent assays. There may be numerous factors, which affect the performance of RIA and these needed to be taken care of. Recently in our laboratory, we have come across through effect of low ambient temperature with the onset of winters which may disturb your standardized parameters of RIA due

to increased association constant(Ka) of antibody, is reported here.

MATERIALS AND METHODS

Chemicals

1. Antiserum

Anti-oestradiol serum was procured from Professor Niswender of Colorado State University, USA. Lyophillized antiserum was reconstituted to make 1 ml of antiserum. The reconstituted antiserum was kept as aliquots of 10-20 µl at -40^{0}C till used.

2. Radiolabeled oestradiol

2,4,6,7,16,17-^{3}H oestradiol 9.25 MBq was procured from Amersham Bio-Sciences Limited, U.K.

3. Phosphate buffer saline(PBS)

Mono and Di-basic sodium phosphate salts were used as follows:

Solution A: 27.6 gms of di-sodium hydrogen phosphate (Na_2HPO_4)(Merck, India) was dissolved in 1000 ml of distilled water.

Solution B : 31.2 gms of sodium dihydrogen phosphate (NaH_2PO_4. $2H_2O$)(Merck, India) was dissolved in 1000ml of distilled water

M PBS was prepared with below solutions as follows.

Solution A -305ml
Solution B -195ml
Sodium chloride(Merck, India)- 9gm
Distilled water ad to -1000ml

4. Assay buffer

Assay buffer was prepared as follows. 0.1M PBS-1000 ml ; Egg white(Sigma, USA)-1Gm

5. Charcoal suspension

Prepared fresh for use at every assay. 500 mg of Norit(Activated charcoal)(S.D. fine chemicals, India) was suspended in 200 ml of assay buffer and kept for stirring on melting ice at least for one hour before use.

6. Scintillation fluid

Scintillation fluid was prepared as follows:

1,4-di(2-(5-Phenyloxazolyl) benzene(POPOP) (CDH, India)-250 mg; 2,5-diphenyloxazole(PPO) (CDH, India) -10 gm; Toluene(CDH, India)-2.5 L.

7. Calibrators

Stock calibrator A

10 mg of oestradiol-17β(CDH, India) was dissolved in 50 ml of ethyl alcohol(West Bengal Chemicals, India) to constitute stock calibrator A of 0.2 mg per ml or 0.02 mg per 100 µl or 20 µg per 100 µl.

Stock calibrator B

100 µl of stock calibrator A was further diluted in 50 ml of ethyl alcohol to constitute stock calibrator B of 0.4 µg per ml or 0.04 µg per 100 µl or 40 µg per 100 µl

Working stock calibrator

500 µl of stock calibrator B was taken into a 50 ml vial, the alcohol was evaporated under nitrogen vapor. On drying 40 ml of assay buffer was added and mixed on vortex machine. This was then heated to 60^0 celcius in a water bath for 10 minutes to constitute a working calibrator of 500 pg per 100 µl.

Lower calibrators of 250 pg. 125 pg, 62.5 pg, 31.25 pg, 15.62 pg, 7.82 pg and 3.91 pg were prepared by serial dilutions in assay buffer from working calibrator prepared as above.

Antibody titer curve

Serial dilutions of antiserum were prepared in assay buffer as 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800 and 1:25600etc. These dilutions were incubated with fixed amount of tracer (10000cpm) overnight at 4^0 celcius temperature. After incubation, the free and bound fractions were separated by Charcoal method. 500 µl of charcoal suspension kept on stirring under melting ice was added to each tube and mixed on vortex machine. Centrifugation was then accomplished in refrigerated centrifuge(C 30-Remi make, India) at 4000 rpm for 15 minutes. Pellet of charcoal absorbing the loose fraction of tracer gets settled in bottom. Separation of bound fraction was accomplished by decanting supernatant in scintillation vial. 5 ml of scintillation fluid was then added to each scintillation vial. Mixing was accomplished on vortex machine. Settling period of 24 hrs was observed before counting for disintegrations. Counting was accom-

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plished in liquid scintillation counter LS 6500(Beckman coulter, USA). Bound/ total fraction was calculated for each antibody dilution and a curve was plotted using bound/total fraction on vertical axis and antibody dilution on horizontal axis. The antibody titer, which allows binding of 50% of tracer was determined and used for assay standardization.

Assay standardization

Different calibrators were incubated with antibody and tracer for overnight incubation at 4^0 celcius followed by separation of bound and loose fractions by charcoal suspension, decanting supernatant into scintillation vials, addition of scintillation fluid, mixing and counting in counter after settling period of 24 hrs exactly in similar fashion as described previously. B/Bo for each calibrator were determined and a curve was plotted on logit-log paper using B/Bo on vertical axis and concentration of calibrator on horizontal axis. B refers to binding of respective calibrator, whereas Bo refers to binding in zero calibrator.

RESULTS AND DISCUSSION

Between July to November 2004, more than 50 assays for quantitation of oestradiol-17β were run in our laboratory using 100µl of antibody of 1:1500 titer, which allowed maximum binding of 50% tracer. All assays were satisfactorily accomplished in the sense that different calibrators used displaced tracer from binding to antibodies in respective proportions. As presented in TABLE 1, the different calibrators employed showed bindings inversely proportional to their concentration. With the onset of winters, the assay got disturbed in the sense that binding exhibited by different calibrators did not differ(TABLE 2). This could not be instantly understood. Replacement of buffers, standards and all other chemical measures did not help to resolve the problem. To restandardize the assay, the effect of low temperature on antibody affinity[1] was considered. Low temperature is associated with increase in values of association constant and antibody affinity. Working on these lines the antibody titer was recalibrated and it was observed to have increased by 10 folds(1:15000). The recalibrated titer was when used it gave perfect displacement between different calibrators(TABLE 3).

TABLE 1: Average CPM, B/Bo ratio with different calibrators used for quantitation of estradiol-17β originally with 1:1500 antibody titer during July to November 2004

Particular	Average cpm	B/B_0 percent
Zero Std.	5076	100
3.32Pg/ml	4578	90.1
6.65Pg/ml	4265	84
13.12Pg/ml	4022	79.2
26.25Pg/ml	3513	69.2
52.5Pg/ml	2778	54.7
105Pg/ml	1978	38.9
210Pg/ml	1118	22.0

TABLE 2: Average CPM, B/Bo ratio with different calibrators used for quantitation of estradiol-17β originally with 1:1500 antibody titer during December 2004, when it got disturbed.

Particular	Average cpm	B/B_0 percent
ZeroStd.	5136	100
3.32Pg/ml	5269	102.5
6.65Pg/ml	4848	94.3
13.12Pg/ml	5048	98.2
26.25Pg/ml	5019	97.7
52.5Pg/ml	5545	107.9
105Pg/ml	5145	100.1
210Pg/ml	4986	97.0

TABLE 3: Average CPM, B/Bo ratio with different calibrators used for quantitation of estradiol-17β originally with 1:15000 antibody titer during winter

Particular	Average CPM	B/B_0 Percent
Zero Std.	5325	100
3.32Pg/ml	4429	83.2
6.65Pg/ml	4062	76.3
13.12Pg/ml	2759	51.8
26.25Pg/ml	1853	34.8
52.5Pg/ml	1347	25.3
105Pg/ml	919	17.2
210Pg/ml	645	12.1

Low temperature is associated with increase in association constant(Ka) of antibody. If the antibody-haptan reaction is represented by following equation,

$$Ab + H \leftrightarrow Ab\text{-}H$$

Then, the association constant(Ka) can be given by following equation,

$$Ka = [Ab\text{-}H]/[Ab][H]$$

The relationship between Ka and temperature can be described mathematically by equation $\Delta F^0 = \Delta H^0 - T\Delta S^0$

Here ΔF^0-refers to standard free energy of binding,

ΔH^0 - refers to Enthalpy contributions to binding

ΔS^0 - refers to Entropy contributions to binding

Larger the negative value of ΔF^0, greater is the

chemical affinity of antibody -haptan. At low temperature the value of ΔF^0 is reduced for antibody-haptan reaction, which increases chemical affinity for this reaction.

Reduced values of ΔF^0 are due to ΔH^0, it is usually negative, a reflection of the decreased binding at increased temperature ΔS^0 varies widely with the antibody from relatively large to essentially negligible values.

From the results observed, it was concluded that low ambient temperature with the onset of winter can disturb RIA with previously calibrated antibody titer during summer, due to effect of low temperature on association constant(Ka) of antibody. The problem could be resolved by recalibration of antibody titer curve.

REFERENCE

[1] C.W.Parker; The Immunoassay, Thermodynamic and Kinetic Considerations(Chapter no.6). Immunolgic specificity and cross reactivity (Chapter no.4). 'In Text book on Radioimmunoassay of Biologically active compounds', Prentice-Hall, INC. Englewood Cliffs, New Jersey, **(1976)**.