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A sensitive chemiluminescence based immunoassay for the detection of cortisol and cortisone as stress biomarkers

Jwan O. Abdulsattar^{1*} and Gillian M. Greenway²

Abstract

An electrochemically based antibody immobilization was used to perform environmentally and clinically relevant immunoassays for stress hormones biomarkers (cortisol and cortisone) using chemiluminescence (CL) detection. To achieve CL detection, the ferrocene tag on the antibodies was first oxidised, and this then acted as a catalyst for the luminol and hydrogen peroxide CL reaction. The conditions were optimised and measurements were made with an incubation time of 30 min. Using this approach limits of detection were obtained of 0.47 pg mL^{-1} and 0.34 pg mL^{-1} also R^2 0.9912 and 0.9902 for cortisol and cortisone respectively with a linear concentration from 0 to 50 ng mL^{-1} . The method was then applied to Zebrafish whole body and artificial saliva samples. For the Zebrafish sample recoveries of 91.0% and 90.0% were obtained with samples spiked with cortisol and cortisone, for artificial saliva the recoveries were 92.59% and 90.73% respectively. Interference studies showed only minor effects on the measurement of the analyte. A comparison between this procedure and the standard enzyme-linked immunosorbent assay (ELISA) procedure gave approximately the same R^2 values.

Keywords: Stress hormones, Biosensors, Immunoassays, Chemiluminescence

Introduction

In this study, the biomarkers of interest are stress hormones, including cortisol and cortisone that belongs to glucocorticoids (GCs) family, which are secreted depending on environmental and behavioral triggers, and follow a circadian rhythm (all day cycle) (Corbalán-Tutau et al. 2014). The relationship between cortisol levels and chronic stress is detected as cortisol aim is to transfer cellular processes from metabolic functions to functions that are necessary for immediate survival (i.e., in the “fight or flight” response). However, the long-term elevation of GCs starves some tissues of necessary resources and inhibits immune action, increasing the tendency to disease (Protopopova 2016; Zainol Abidin et al. 2017). For healthcare monitoring which can be used for understanding human day-night stress hormones cycle secretion, simple low-cost measurement systems are

needed. Environmental applications would include the effect of pollutants on fish (Kaushik et al. 2014).

Various approaches have been taken to determine stress hormones in biological samples. However, these methods either require sophisticated equipment or involves procedures with rigorous control of the experimental conditions (Oßwald et al. 2019; Luo et al. 2019; Sturmer et al. 2018; Miller et al. 2013; Del Corral et al. 2016; Gatti et al. 2009; Yeh et al. 2013; Barcellos et al. 2007; IZAWA et al. 2015; Ashley et al. 2011; Weltring et al. 2012; Russell et al. 2014; Ammann et al. 2014; Erickson et al. 2012; Kartsova and Strel'nikova 2007; Gao et al. 2015; Ceccato et al. 2014; Saracino et al. 2014; Sánchez-guijo et al. 2014; Yeh et al. 2015). Chemiluminescence (CL) is widely used for analytical measurements due to its simplicity, sensitivity and low-cost optical requirements (Adcock et al. 2019; Smith et al. 2019). The CL reaction of luminol with hydrogen peroxide system is frequently utilised with catalysts which include either enzymes such as horseradish peroxidase (Liu and Zhang 2015) or metal catalysts such as ferrocenecarboxaldehyde (Luo et al. 2012) that contain iron.

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The CL reaction used in this work is based a previous method described by Wright et al. for the determination of pregnancy hormones (Wright et al. 2018) in which firstly the antibody was electrochemically immobilised on to an ITO electrode, a method which was found to provide reproducible and reliable results. Secondly, the oxidised form of the redox tag (ferricenium) on the antibody acts as the heterogeneous catalyst for the chemiluminescent reaction between hydrogen peroxide and luminol.

The objective of the research presented in this paper was to provide simple, low cost and quantitative measurement system for the determination of stress hormones that was both selective and sensitive. The method was applied to Zebrafish samples and artificial saliva samples.

Experimental section

Materials and methods

Anti-cortisol antibody, anti-cortisone antibody and cortisol (hydrocortisone) were purchased from Abcam biochemicals, UK. Cortisone, phosphate buffer saline tablet (PBS), ferrocenecarboxyaldehyde (FcCHO), potassium carbonate (K_2CO_3), sodium borohydride ($NaBH_4$), hydrochloric acid (HCl), potassium chloride (KCl), 4-nitrobenzene diazonium tetra-fluoroborate, N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS), Tween 20, bovine serum albumin (BSA), N,N-dimethylformamide (DMF), ammonium nitrate, potassium phosphate, potassium citrate, uric acid sodium salt, urea, lactic acid sodium salt, bovine submaxillary gland mucin type I-S, tricaine methane sulfonate (500 mg ml^{-1} MS-222), luminol and hydrogen peroxide were obtained from Sigma-Aldrich, UK. Acetonitrile (ACN), ethanol (EtOH), sodium hydroxide (NaOH) and tetrabutylammonium perchlorate (TBAP) were sourced from Fisher Scientific, UK. N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) was sourced from Fluka, UK.

Two biological samples were tested:

Zebrafish (whole-body): All experiments were carried following the agreed procedures in the ethical approval; they were carried out approximately the same time of the day to avoid the fluctuations in cortisol occur due to the natural circadian rhythms. Zebrafish were captured and euthanized with tricaine methane sulfonate (500 mg ml^{-1} MS-222). Whole-body cortisol extraction was performed according to the method described by Canavello et al. (2011). The yellowish lipid extract obtained was reconstituted with 1 ml of cortisol standard dissolved in phosphate buffer saline (PBS) and stored at 4°C for 24 h. An alternative method was tested by repeating all the above processes except the extraction method was omitted.

Artificial saliva: the second biological sample was artificial saliva samples. A recipe outlined by West et al.

(2002) was used to prepare an artificial human saliva sample. For all cortisol and cortisone standard, there was a slight difference where they were made in artificial saliva instead of PBS (10 mM). The electrochemical immunoassay was repeated using the above biological samples. All the above procedures were carried out for both cortisol and cortisone.

For all the electrochemical experiments conducted in this paper, a standard three electrodes setup including the Ag/AgCl (reference electrode), nickel wire (counter electrode) and an indium tin oxide (ITO) electrode (CB-50INCUV, sheet resistance $5\text{--}15\ \Omega$, dimensions $7 \times 50 \times 0.7\text{ mm}$, Delta Technologies Ltd., USA) (working electrode), the electrochemical experiments were conducted using a PalmSens Potentiostat (Palm Instruments, Netherlands). Copper tape was placed on one end edge of the conductive side of an ITO electrode to complete the electrical cycle.

CL detection system consists of a QHY6 charge-coupled device (CCD, QHXCCD, USA) fitted with a high-resolution pixel lens (8 mm, Computer, USA).

CL instrumentation shows an ITO electrode alignment under the CCD camera in a dark box, and images were recorded using Image J software and a laptop connected to the camera.

Experimental procedures

ITO electrode modification with $-NH_2$ group

The ITO electrode was cycled voltammetrically from $+0.7\text{ V}$ down to -0.5 V at 0.1 V s^{-1} using the deposition solution consisting of 0.1 M tetrabutylammonium perchlorate (TBAP) and 2 mM 4-nitrobenzene diazonium tetrafluoroborate in acetonitrile. Then the electrode was washed with acetonitrile and placed into aqueous ethanol solution (90:10, v/v) containing 0.1 M potassium chloride, where the electro-formation of a phenylamine was achieved during 3 reduction-oxidation scans using cyclic voltammetry, starting at $+0.4\text{ V}$ with a sweep to -1.25 V at 0.1 V s^{-1} . This was done following Dou et al. (2012) procedure.

Synthesis of antibody tagged with ferrocene

The antibodies were chemically labelled with a redox tag, using the methodology outlined by Dou et al. (2012). This was achieved by the addition of a $10\text{-}\mu\text{l}$ aliquot of (2 mg ml^{-1}) of anti-cortisol antibody to $190\text{ }\mu\text{l}$ of phosphate buffer saline (PBS) (10 mM), and the pH was adjusted to ~ 9 using 5% aqueous potassium carbonate (K_2CO_3) (5% w/v). Then a solution of ferrocenecarboxaldehyde was dissolved in dimethylformamide (DMF) (20 mg in $200\text{ }\mu\text{l}$) and added to the antibody solution. After an incubation of 30 min to permit the formation of an imine product $\text{Ab-N}=\text{CH-Fc}$; a reduction of the resulting imine to secondary amine was carried out by the addition of sodium borohydride (2 mg)

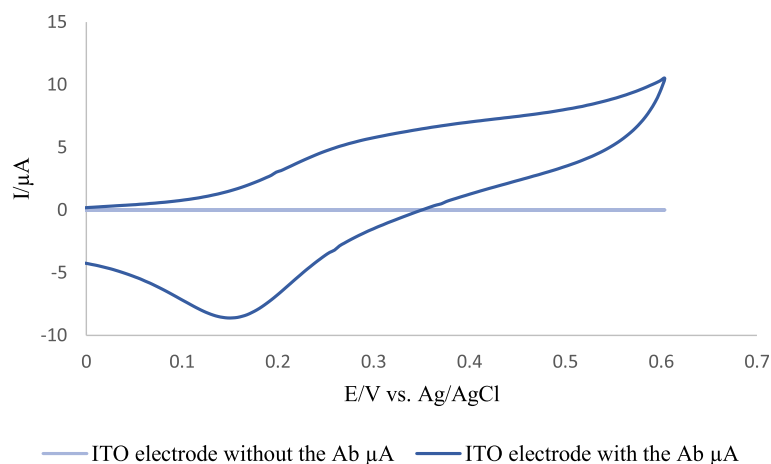


Fig. 1 Cyclic voltammogram of the modified ITO electrode surface with and without the tagged ferrocene antibody. Scan rate 100 mV s^{-1} . Ten millimolar PBS was the solution used to conduct all the experiments

to give Ab-NH-CH₂-Fc. This solution was left for 10 min, afterward, the solution was readjusted to pH ~ 7 using hydrochloric acid (HCl) (0.1 M).

The antibody was purified to remove any ferrocene excess; by centrifugation (12,000 rpm for 20 min), then the supernatant was transferred into a 2 \times viva spin (500, GE Healthcare, Sweden); the solution was then re-centrifuged (12,000 rpm for 10 min), and the purified antibody on the top vial was topped up with 100 μl PBS (10 mM) and centrifuge as a washing step. The final solution was stored in the fridge (4°C) until required.

Immobilization of ferrocene labeled antibody onto the ITO electrode

Coupling of the primary amine group of the modified electrode surface to the carboxylic acid groups of the antibody was carried out according to a previously reported paper by Dou et al. (2012). This was achieved by mixing a 10 μl

aliquot of the ferrocene tagged antibody with an equal volume of an activation buffer consisting of 2 mM (*N*-(3-dimethylaminopropyl)-*N'*-ethyl carbodiimide hydrochloride) (EDC) and 5 mM (*N*-hydroxysulfosuccinimide sodium salt) (sulfo-NHS) in 2 ml PBS (10 mM) for 15 min to activate the -COOH on the antibody to facilitate the bonding to the electrode via an amide linkage. Subsequently, 30 μl of this solution was added to the modified ITO electrode and left covered for 18 h in the fridge at 4°C .

Stress hormones measurements

To measure the hormones, the modified electrode was washed with 0.1% Tween 20 (0.1 v/v) in PBS (10 mM) solution to effectively remove excess material from the electrode, then the electrode was treated with bovine serum albumin (BSA) (1%, w/v) in PBS (10 mM) for 30 min as it considered sufficient time for blocking unoccupied sites on the ITO electrode. Then it was washed with 0.1% Tween 20 in PBS, and an electrochemical oxidation process was carried out, in which a cyclic voltammetry scan was carried out using PBS (10 mM) spotted onto the immobilized antibody circles starting from -0.05 V up to $+0.6 \text{ V}$ at scan rate 10 mV s^{-1} , this involved one scan; this was carried out to convert the ferrocene attached to the antibody immobilized onto the modified ITO electrode to the ferrocenium cation.

Thirty microliters of 50 ng ml^{-1} of antigen was added to the circle on the ITO electrode containing the immobilized antibody with the ferrocenium cation attached. This was left initially for 1 h incubation time. Afterward, a mixture of 30 μl of 20 mM luminol and 10 mM hydrogen peroxide is added. Light intensity was calculated using Image J software after the image was taken by the CCD camera, for each measurement; there was a blank subtraction.

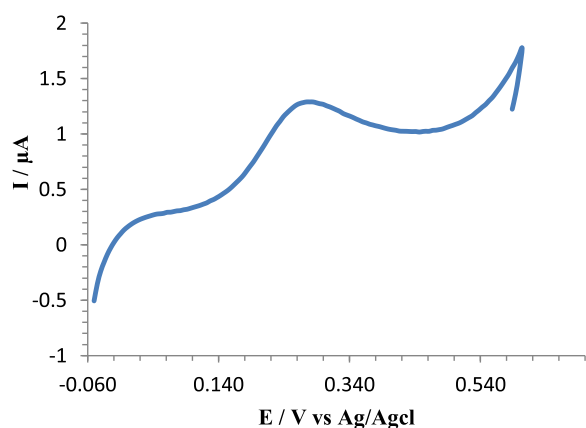
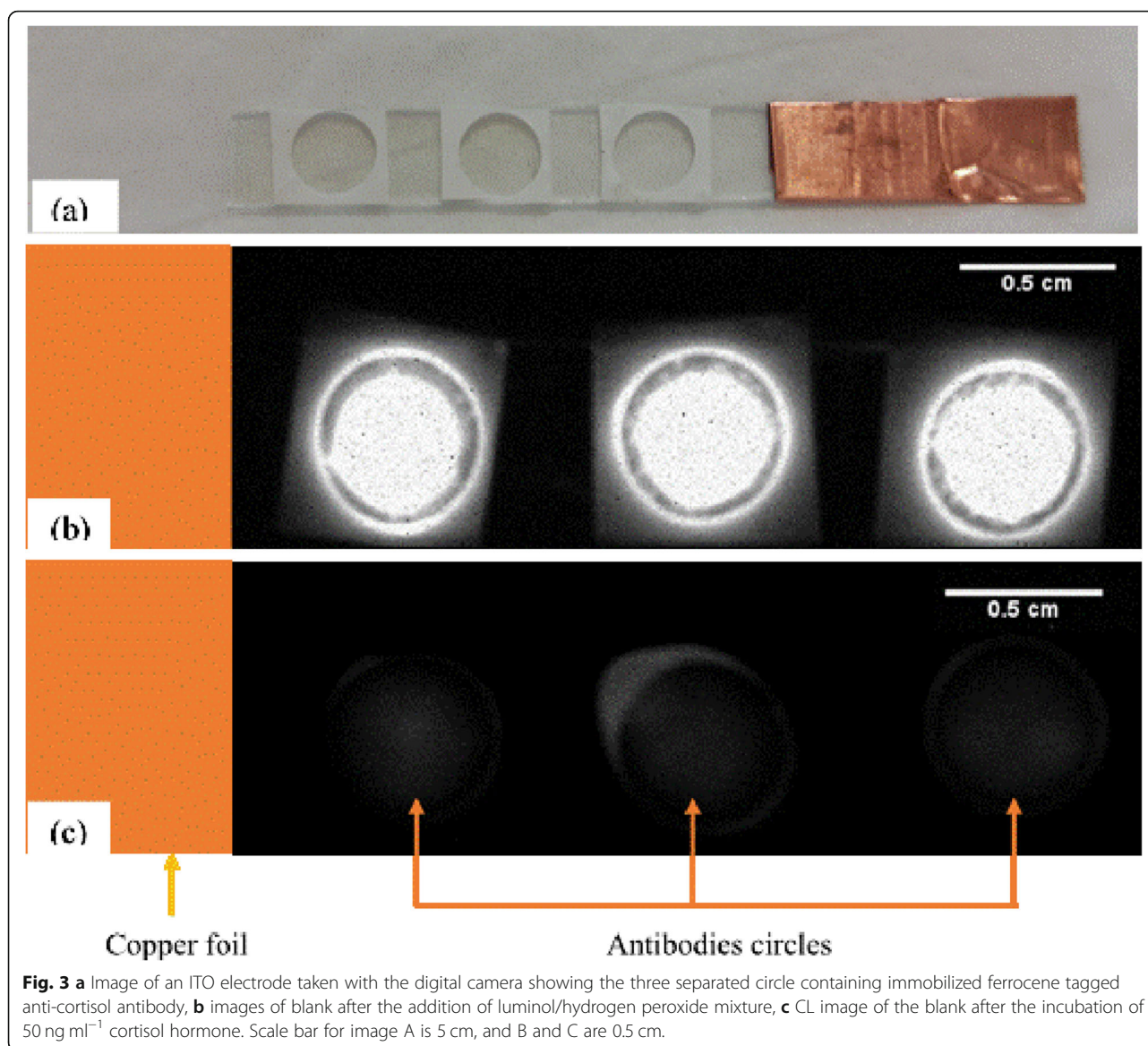


Fig. 2 Cyclic voltammogram for the oxidation of ferrocene tagged antibody to ferrocenium cation that catalyses luminol/hydrogen peroxide showing an oxidation peak at $+0.25 \text{ V}$. Scan rate 10 mV s^{-1}



Stability

The stability of the modified ITO electrode tagged with ferrocene after oxidation was investigated. CL measurements were taken immediately, after 1 day and after 1 week to compare the results. The ITO

electrodes were stored in the PBS until it was needed at 4 °C.

Optimization of CL variables

CL experiments were conducted to evaluate the influence of luminol concentration, hydrogen peroxide concentration, incubation time and exposure time in order to achieve the maximum CL immunoassay emission signal. The luminol and hydrogen peroxide mixture volume for all conducted experiments was 30 µl.

Various concentrations (1, 5, 10, 20, 30 and 40 mM) of luminol were added to a fixed concentration 10 mM of hydrogen peroxide and the mixture was spotted onto the circle on the ITO electrode to determine the highest signal. After fixing the luminol concentration that gives the maximum signal, different hydrogen peroxide

Table 1 The precision of immobilisation ferrocene tagged antibody on the ITO electrode surface

Within electrode	Circle 1 RLU	Circle 2 RLU	Circle 3 RLU	Average ± STD	RSD%
Electrode 1	253.04	252.97	249.69	251.90 ± 1.91	0.75
Electrode 2	237.33	218.81	233.49	237.30 ± 3.79	1.61
Electrode 3	213.02	199.19	191.11	200.13 ± 9.52	4.76
Electrode 4	225.69	219.34	215.08	220.03 ± 5.33	2.42
Final results				227.34 ± 5.14	2.38

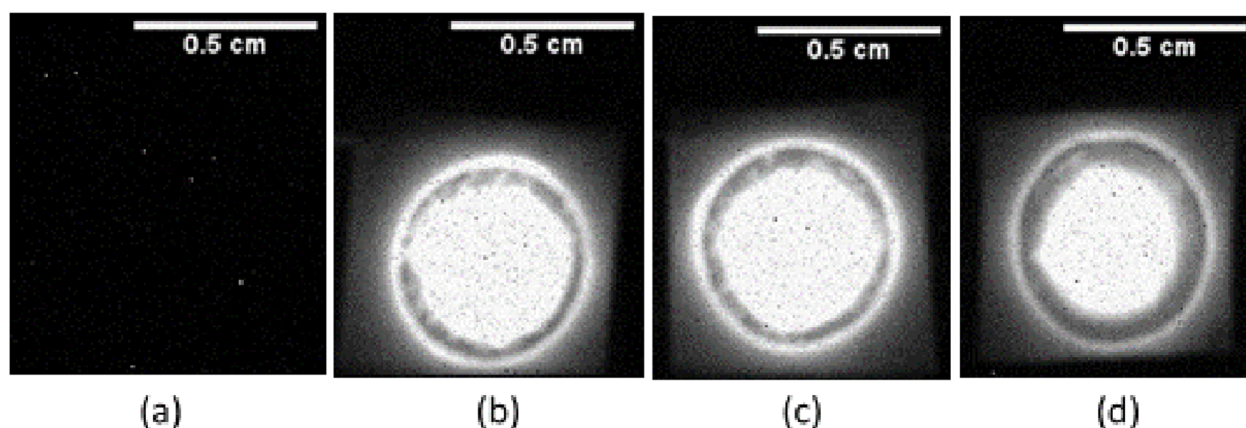


Fig. 4 Images of CL signal related to ferrocene tagged antibody, (a) characterise the ferrocene tagged antibody before ferrocene oxidation, (b) immediately after the oxidation, (c) 1-day oxidation (d) after 1-week oxidation. The scale bar is 0.5 cm.

concentrations (1, 5, 10, 20, 30 and 40 mM) was tested. The incubation time for the antigen was carried out varying incubation times (5, 10, 15, 20, 30, 45 and 60 min). The optimization of exposure time was carried out by taking measurements of a time interval from 50 to 700 s.

Calibration procedures

Cortisol and cortisone stock solutions were prepared to give a solution of 100 ppm for each stock solution, from which the standards were prepared in PBS (10 mM) to give working standards ($0.001\text{--}50\text{ ng ml}^{-1}$) for cortisol and cortisone solution carried out.

Stress hormones determination in biological sample

In order to determine stress hormones in a biological sample including Zebrafish whole-body which is physiologically homologous to humans and other mammalian species, permitting scientists to obtain new insights into

the pathways and mechanisms relevant to human clinical treatments and pathogenesis (Egan et al. 2009) and artificial saliva samples, the procedure in materials and methods were repeated for both samples individually and for both hormones (cortisol and cortisone).

Interferences

The samples selected for analysis were Zebrafish whole-body and artificial saliva samples; these have complex matrices which despite the high selectivity of the antibody-antigen reaction may have constituents that would interfere with the results. The species selected to investigate were cortisol, cortisone, prednisolone, 11-deoxycortisol, progesterone, corticosterone and testosterone (all purchase from Sigma-Aldrich, UK) with a standard concentration of 50 ng ml^{-1} which was chosen to be greater than the higher concentration of each compound expected to exist naturally within these samples (Yeh et al. 2013; Wood 2009), and the procedures

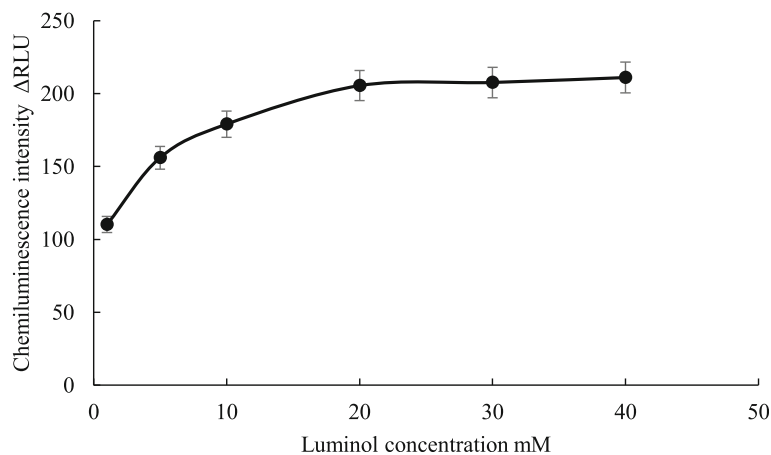


Fig. 5 A calibration curve showing the influence of luminol concentrations while keeping the concentration of hydrogen peroxide constant (10 mM) on the CL emission signal

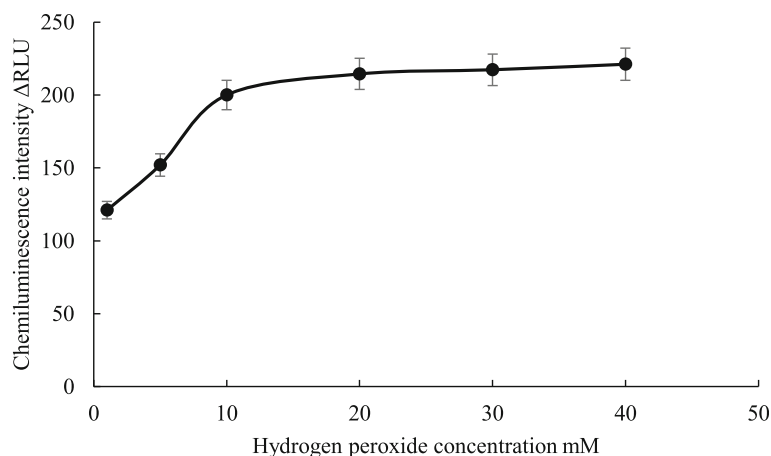


Fig. 6 Graph showing the influence of hydrogen peroxide concentration while keeping the concentration of luminol constant (20 mM) on the CL emission signal

outlined in experimental procedures was used for each carried out different antigen.

For each compound, a stock solution was prepared by dissolving 5 mg in ethanol and making the volume up to 50 ml in PBS (10 mM) to give a concentration of 100 ppm, then serial dilutions were made to prepare 50 ng ml^{-1} working solution.

Comparison study with standard Enzyme-Linked Immunosorbent Assay (ELISA)

For comparison, a standard ELISA method was performed using a human salivary cortisol assay kit (ab 154996- cortisol ELISA kit, Abcam, UK). The ELISA assay was carried out as per the instructions provided. The absorbance of the sample was measured at 450 nm within 30 min of stop solution addition; a Labtech International plate reader was used to make the measurements.

Results and discussion

Characterization of ferrocene tagged anti-cortisol immobilized onto ITO electrode surface

To confirm the success of the immobilization process, cyclic voltammetry measurements were conducted for the modified ITO electrode surface without the antibody (blank) and with the tagged ferrocene antibody. This step was done following Kevin et al. characterization of ferrocene tagged antibodies (Wright et al. 2018). Figure 1 shows the comparison between blank ITO electrode surface and modified ITO electrode surface with the tagged ferrocene antibody using cyclic voltammetry.

An oxidation peak at + 0.25 V from Fig. 1 was seen for the ITO electrode with the tagged ferrocene antibody. This confirms the oxidation of the ferrocene to the ferrocenium cation on the ITO electrode surface with a reduction peak at + 0.25 V, as would expect no peak was seen for the blank ITO electrode.

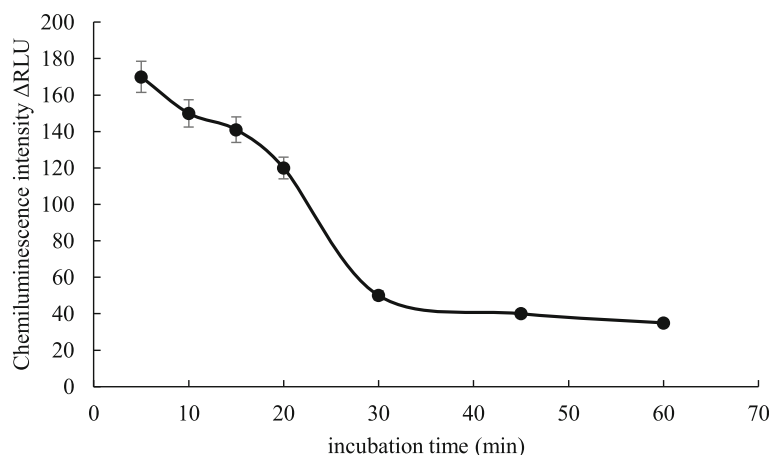


Fig. 7 The graph shows the effect of different incubation time on the CL signal after the addition of 30 μl of 50 ng ml^{-1} of cortisol standard solution.

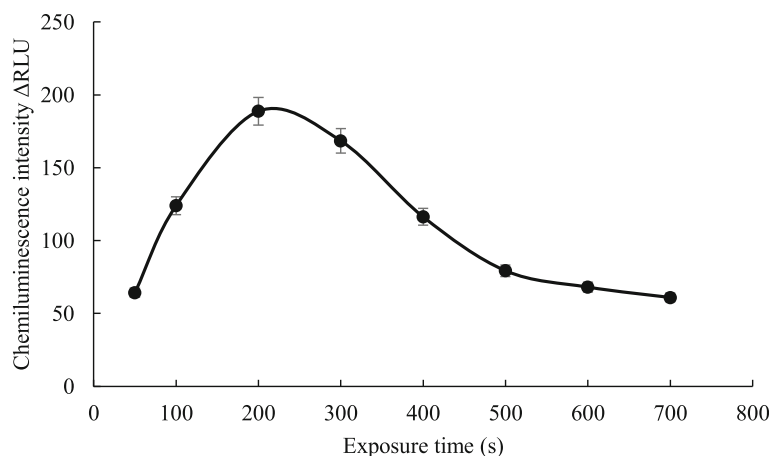


Fig. 8 The graph for exposure time optimisation after the addition of luminol (20 mM) and hydrogen peroxide (10 mM) mixture over the time interval from 50 to 700 s showing the highest CL signal at 200 s.

Oxidation of Ab-Fc on the surface

The first step to carry the CL procedure was the oxidation of the ferrocene to ferrocenium cation using cyclic voltammetry as can be seen in Fig. 2. The scan rate was 10 mV s^{-1} to enable more time at a set potential which would kinetically favor the ferrocene oxidation (Partington et al. 2018).

The oxidation peak at $+0.25 \text{ V}$ shown in Fig. 2 represents the ferrocene oxidation to ferrocenium cation. This is essential for the CL reaction to occur with the iron located in the ferrocene carboxaldehyde acting as a catalyst for luminol/hydrogen peroxide (Wilson and Schiffrin 1998). The next step was to investigate the ferrocenium cation ability as a catalyst to luminol and hydrogen peroxide mixture to produce the CL emission signal.

Investigation of CL emission

To investigate the CL emission before and after the addition of an antigen, a preliminary experiment was

conducted by adding $30 \mu\text{l}$ of luminol and hydrogen peroxide mixture to the ferrocene tagged antibody before and after the incubation with 50 ng ml^{-1} cortisol hormone which was chosen because it has the highest concentration, so it would clear CL signal (Fig. 3).

Figure 3 demonstrated the applicability of ferrocene to be used as a catalyst in the luminol/hydrogen peroxide reaction (Hassan et al. 2014). As can be seen, a decrease in the CL signal from 227.3 RLU for the blank (Fig. 3b) to 21.5 RLU after the addition of the antigen (50 ng ml^{-1} cortisol standard solution) (Fig. 3c) was observed.

As would be expected the CL emission signal was highest without the addition of the antigen. The signal then decreased when the antigen was added because it bound to the antibody thus reducing the electron transfer at the surface (blocking effect) (Dou et al. 2012) yielding a lower CL signal.

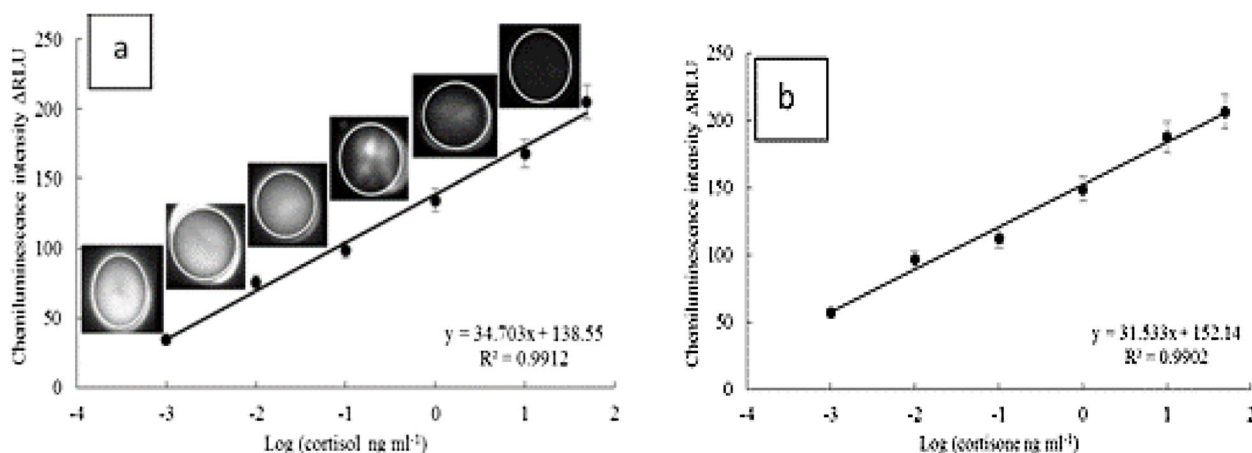


Fig. 9 Calibration curve of **a** cortisol and **b** cortisone standard solutions with concentrations from 0 to 50 ng ml^{-1} . Standards plotted against the ΔRLU (relative light unit) emission response, insert in the figure **a** CL image of each standard cortisol solution.

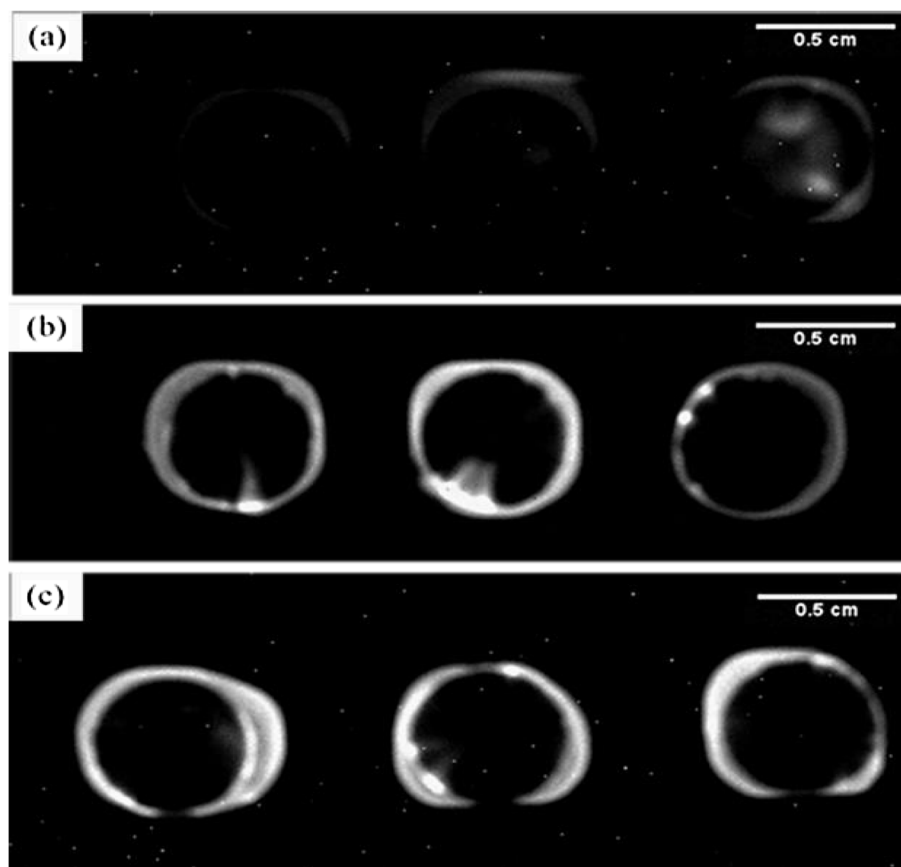


Fig. 10 CL image of Zebrafish whole-body sample spiked standard cortisol solution (50 ng ml^{-1}) added to ferrocene anti-cortisol antibody immobilized onto modified ITO electrode **a** without extraction, **b** with extraction and **c** fish sample without the addition of standard and without extraction. Scale bar is 0.5 cm

Experiments were carried out to investigate the reproducibility and effectiveness of the ferrocene tagged antibody immobilization on the ITO electrode surface. The previously described experiment was repeated on three circles within the same electrode (intra-assay) and different circles on different electrodes (inter-assay) individually for four electrodes. The results are summarized in Table 1.

The data shown in Table 1 for the RSD % for three circles within the same electrode was between 0.75 and 4.76%, whereas the RSD% between different circles of different electrodes was 2.38%. This shows that there is no significant difference in CL signal between circles on the same electrode and circles on different electrodes.

Table 2 The Recovery and RSD values of cortisol and cortisone standard solution spiked in the Zebrafish whole-body sample ($n = 3$)

Analyte	Conc. added (ng ml^{-1})	Rec (%)	RSD %
Cortisol	50	91.0	1.25
Cortisone	10	90.0	2.00

The stability of ferrocenium cation

Simplicity is the key point in constructing a point of care system; therefore, the stability of ferrocene tagged antibody after oxidation is important so that it does not need to be oxidized just before measurement. To investigate the stability images were captured for the three circles containing the ferrocene tagged anti-cortisol antibody before oxidation, directly after the ferrocene oxidation by luminol/hydrogen peroxide mixture, 1 day and 1 week after the ferrocene oxidation after being kept in 10 mM PBS solution for the whole period of experiment at 4°C .

Figure 4 shows promising results for ferrocene stability, where (a) no light emission was observed because the non-oxidized ferrocene does not catalyse the luminol/hydrogen peroxide reaction (b) immediately after oxidation which shows an increase in the signal up to 224.01 RLU as expected because of the ferrocenium cation action as a catalyst (Jain 2013) where the ferrocenium cation breaks down the hydrogen peroxide creating hydrogen radical which reacts with luminol and emitting the CL signal (c) and (d) shows a minor

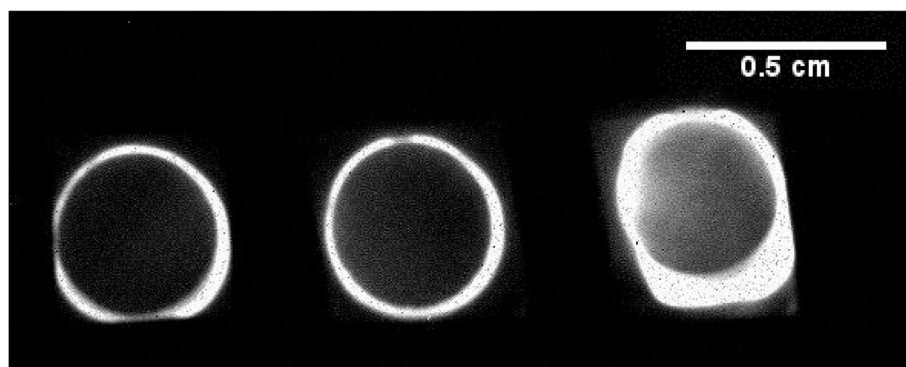


Fig. 11 Represent the signal after the addition of artificial saliva spiked with cortisol standard solution 10 ng mL^{-1} . The scale bar is 0.5 cm

decrease in the CL measurement giving an RLU of 220.12 and 196.65 for the ferrocene tagged antibody after oxidation and storage in 10 mM PBS for 1 day and 1 week respectively which does not impact the analysis.

Optimization of operating variables

The effect of different operating conditions (luminol concentration, hydrogen peroxide concentration, incubation time and exposure time) on the CL emission signal were investigated to achieve the maximum CL signal.

Influence of luminol concentration

Experiments were carried out to evaluate the influence of different luminol concentrations ranging from 1 to 40 mM on the CL signal.

CL experiments were carried out at different luminol concentrations ranging from 1 to 40 mM) as cited in Fig. 5, where increasing the concentration of luminol leads to an increase in the CL signal between 1 and 20 mM where 20 mM luminol concentration reaches the optimum signal of 205.4 RLU. Self-absorption was seen for higher concentrations (Chen et al. 2012). And therefore, 20 mM luminol was used for all experiments within this paper.

Influence of hydrogen peroxide concentration

To study the effect of hydrogen peroxide concentrations on CL emission signal different concentrations in the range of (1–40 mM) were tested.

A similar trend to changing concentrations for luminol was observed for hydrogen peroxide (as shown in Fig. 6). The CL emission signal increased with the increasing

concentrations up to 10 mM. Above that concentration, the CL emission signal was seen to stabilise up to 40 mM; therefore, 10 mM hydrogen peroxide was chosen as an optimum concentration.

Influence of incubation time

The incubation time of antibody-antigen interaction was tested. As expected, the incubation time of antibody-antigen interaction was 30 min as shown in Fig. 7 where the CL RLU increased to 168 RLU at 5 min; this may be due to insufficient time to complete the interaction between the antibody and the antigen. Then the signal reached the lowest signal between 30 and 60 min with no significant difference; therefore, 30 min was chosen for further experiments.

Influence of exposure time

Exposure time is a fundamental parameter for an economical point of care system. It can be defined as the length of time that an image required to be exposed to the CCD camera to acquire the highest CL signal. Consequently, it is important to study its influence on the CL signal of over time intervals from 50 to 700 s.

Figure 8 shows that the CL emission signal initially increased with exposure time up to 200 s, and after this time, the CL signal decreases. Therefore, 200 s was deemed more than sufficient to establish the optimum CL signal and used in all subsequent studies.

Calibration curve

To determine cortisol and cortisone, calibration curves were obtained by preparing a set of standards solutions at concentrations ranging from 0 to 50 ng mL^{-1} , and the CL immunoassay was performed.

As expected, the Δ RLU increased with as the concentration of the standard solutions increased (Fig. 9a, b) giving linearity over the range required to analyse the target analyte in the real sample and with correlation coefficients of 0.9912 and 0.9902 for cortisol and cortisone.

Table 3 The Recovery and RSD values of cortisol and cortisone standard solution spiked in the artificial saliva sample for three different electrodes

Analyte	Conc added (ng mL^{-1})	Rec (%)	RSD %
Cortisol	10	92.59	2.04 %
Cortisone	25	90.73	1.98%

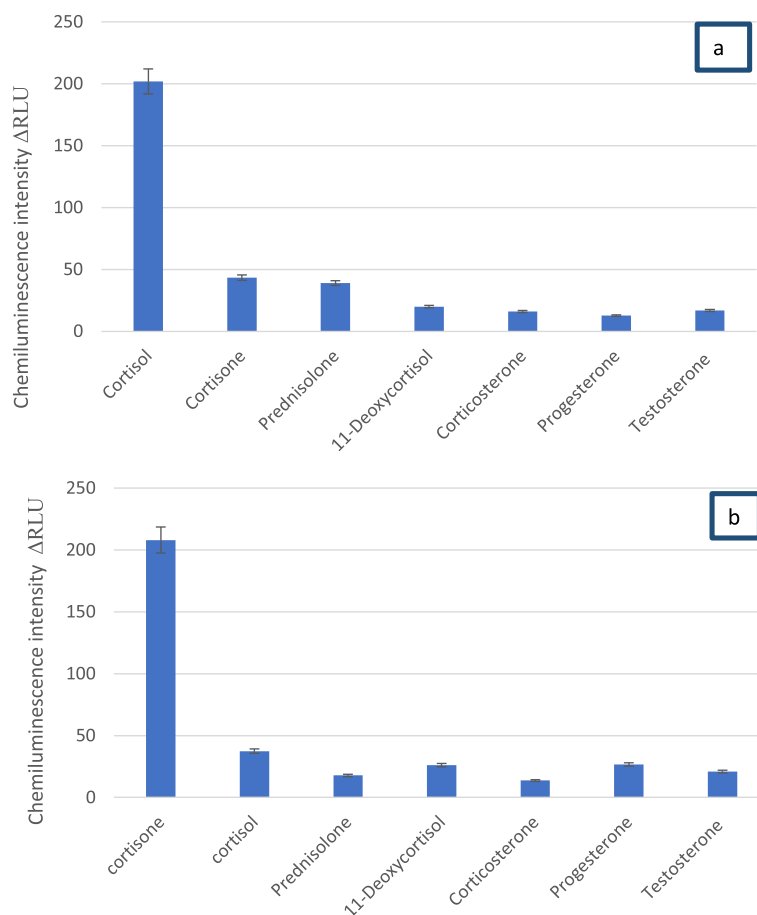


Fig. 12 Column chart specifies the similar structure compounds potential interference with **a** ferrocene tagged anti-cortisol antibody and **b** ferrocene tagged anti-cortisone antibody

Also, the limit of detection for cortisol and cortisone were 0.47 and 0.34 pg ml^{-1} , respectively showing excellent analytical data for stress hormones determination.

Determination of target analyte in Zebrafish whole-body and artificial saliva samples

Further experiments were carried out to determine the concentration of stress hormones in Zebrafish whole-body and artificial saliva samples:

Zebrafish whole-body sample

Measurements were carried out with and without the extraction procedure Fig. 10 shows CL emission for (a) a real sample spiked with 1 ml of 50 ng ml^{-1} of cortisol standard solution and the extraction method was carried out giving an RLU of 22.4, (b) a real sample spiked with 1 ml of 50 ng ml^{-1} without extraction giving an RLU of 29.0. A clear observation was that there was a decrease in the light intensity for the real sample with extraction and without extraction compared with the standard solution of cortisol that gave

an RLU of 21.5. As the difference in the CL signal was small, the use of the sample without the extraction was acceptable with calibration.

An observation of a white round edge for the reaction cycle can be explained by “the coffee ring effect” because the solution evaporate more quickly at the edges than at the centre, and the remaining solution at the center moved outward this movement created concentrated layers forming a dense ring (Shimoni et al. 2014).

The data obtained was used to calculate the recovery and RSD for both cortisol and cortisone as summarized in Table 2.

The data obtained from Table 2 indicates the good recovery for both hormones where cortisol recovery was 91.0% and cortisone was 90.0%. Also the values of the RSD ranged between 1.25% and 2.0% for cortisol and cortisone respectively. These results show the applicability of the immunoassay protocol followed by CL detection for the determination of stress hormones in Zebrafish whole-body sample.

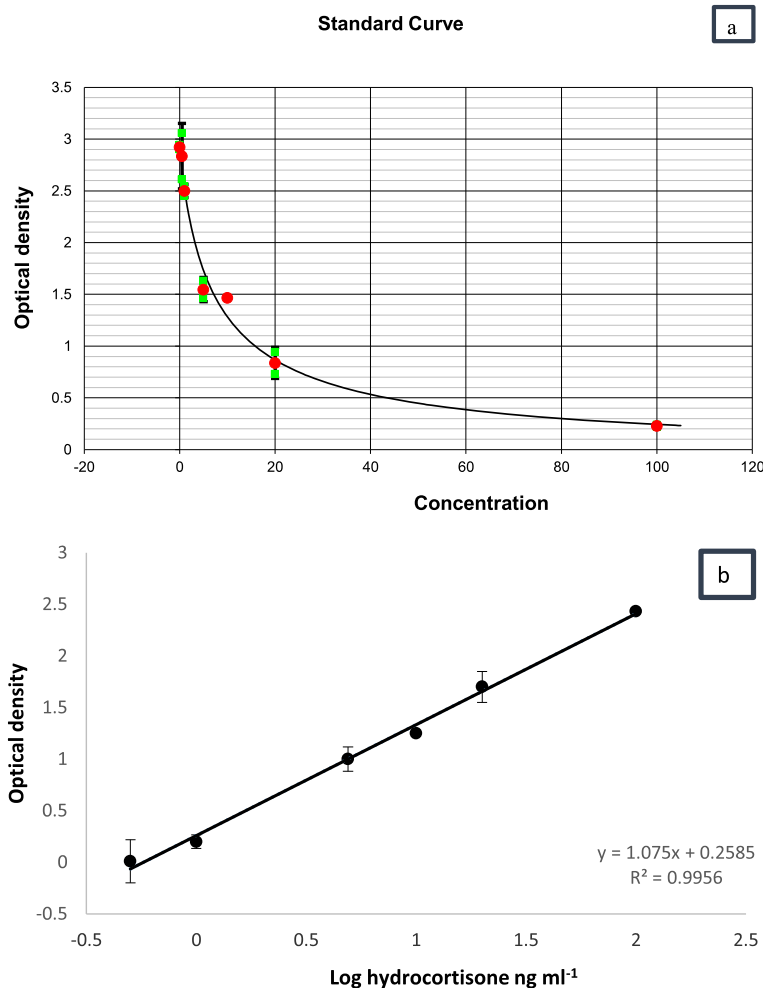


Fig. 13 Calibration curves showing **a** the change in the optical density with the increase of cortisol concentration where the red points indicate the cortisol concentrations, and the green points are the error bar for each concentration, **b** the increase in optical density with a linear trend after subtracting the blank signal from the sample signal

Artificial saliva

The prepared saliva was spiked with 10 ng ml⁻¹ cortisol and 25 ng ml⁻¹ cortisone standard solutions, and the resulting solutions were incubated for 30 min before measuring the CL emission (Fig. 11.)

As can be seen from Fig. 11, the signal decreased due to the reaction between the antibody and antigen. The data obtained from cortisol and cortisone were summarized in Table 3.

From Fig. 11 and Table 3, the recovery was calculated giving a recovery of 92.59% and 90.73% for cortisol and cortisone respectively. Another statistical parameter was calculated which was the RSD % which was 2.04% of cortisol and less value for cortisone giving a value of 1.98%.

Interferences

The antibody-antigen interaction is very selective, and the main interferences were likely to have similar structures

which could interact with the anti-cortisol and anti-cortisone antibodies immobilized onto the modified ITO electrode resulting in false results. A range of similar compound was selected, and CL images were acquired using optimum conditions for cortisol and cortisone.

As can be seen from Fig. 12a, cortisone, prednisolone, 11-deoxycortisol, corticosterone, progesterone and testosterone had very little effect on the anti-cortisol antibody where the RLU for these compounds ranged from 12 to 38. There was slightly more of an effect for cortisone where the RLU was 43, but the CL emission for cortisol was 205 RLU. It can be said that the presence of these compounds does not impact on the adopted immunoassay in this study. Figure 12b shows also the effect of similar compounds on the ferrocene tagged anti-cortisone and as expected there was a minor interference effect from these compounds with slightly more effect from cortisol with 37 RLU, but this inference can be

considered negligible compared with cortisone RLU of 206.42.

Commercial ELISA kit

To evaluate the immunoassay protocol used in this paper, a comparison with a reference method was carried out. Stress hormone is usually tested with a traditional ELISA assay which is performed in the majority of hospital service laboratories. Therefore, it was decided to test the stress hormones using a commercial ELISA and compare the results with the immunoassay protocol.

Figure 13 a and b give the relationship between the optical intensity signal and the standardized concentrations of cortisol. It can be observed that the optical intensity signal increase with the increase of the concentration of the cortisol to give a linear calibration curve with R^2 of 0.995. The Zebrafish whole-body sample was tested giving a concentration of 44.56 ng ml⁻¹ (with extraction) and 46.78 ng ml⁻¹ (without extraction) which confirms that the extraction procedure can be omitted removing additional steps.

There was a good agreement with the proposed method where R^2 for cortisol was 0.991 compared with R^2 for cortisol using ELISA kit was 0.995 with a LOD of 0.12 ng ml⁻¹ (from the data sheet provided by the Abcam company). This applied also for the concentration obtained by the standard method (ELISA) mentioned above compared with the CL immunoassay concentration (47.86 ng ml⁻¹). As a result, it can be concluded from the results obtained from the conventional tests that the development of a sensitive CL immunoassay for stress hormone was achieved.

Conclusion

A new inexpensive rapid CL immunoassay method has been developed to detect stress hormones with reproducible electrochemical immobilization of the antibody in which ferrocene was used as a catalyst for CL detection. The ferrocene was electrochemically converted to the ferrocenium cation. The stability of the oxidized antibody with the ferrocene moiety was investigated, and the results showed high stability after oxidation over 1 week which simplified the proposed protocol.

Calibration curves for both cortisol and cortisone were constructed and give LOD at 0.47 and 0.34 pg ml⁻¹ for cortisol and cortisone with correlation coefficients of 0.9912 and 0.9902 which indicates a high sensitivity of this method. The new method had much lower limits of detection compared to the standard ELISA test (0.12 ng ml⁻¹). Potential interferences were examined, and the results confirmed the selectivity of this protocol.

Abbreviation

BSA: Bovine serum albumin; CAN: Acetonitrile; CCD: Charge-coupled device; CL: Chemiluminescence; EDC: N-(3-Dimethylaminopropyl)-N'

ethylcarbodiimide hydrochloride; ELISA: Enzyme-linked immunosorbent assay; FcCHO: Ferrocenecarboxylaldehyde; GCs: Glucocorticoids; ITO: Indium tin oxide (ITO); PBS: Phosphate buffer saline; RLU: Relative light unit; Sulfo-NHS: N-Hydroxysulfosuccinimide sodium salt; TBAP: Tetrabutylammonium perchlorate

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All raw data used in this manuscript are available and could be supplied upon request

Competing interests

The authors declare that they have no competing interests.

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