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Immunosensor with fluid control mechanism for salivary cortisol analysis

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ABSTRACT

The purpose of this research is to demonstrate a new design for a cortisol immunosensor for the noninvasive and quantitative analysis of salivary cortisol. We propose a cortisol immunosensor with a fluid control mechanism which has both a vertical flow and a lateral flow. The detected current resulting from a competitive reaction between the sample cortisol and a glucose oxidase (GOD)-labeled cortisol conjugate was found to be inversely related to the concentration of cortisol in the sample solution. A calibration curve using the relative detected current showed a R^2=0.98 and CV=14% for a range of standard cortisol solutions corresponding to the concentrations of native salivary cortisol (0.1–10 ng/ml). The measurement could be accomplished within 35 min and the cortisol immunosensor could be reused. These results show promise for realizing an on-site and easy-to-use biosensor for cortisol. Used for evaluation of human salivary cortisol levels, the cortisol immunosensor measurement corresponded closely with commercially available ELISA method (R^2=0.92). Our results indicate the promise of the new cortisol immunosensor for noninvasive, point of care measurement of human salivary cortisol levels.

1. Introduction

There is consistent evidence that many forms of psychological stress increase the synthesis and release of cortisol (Charney, 2004). Excessive and sustained cortisol secretion can have serious adverse effects (Karlamangla et al., 2002). Chronic elevations of cortisol levels can lead to brain aging (Landfield, 1987), hippocampal atrophy and cognitive impairment (Lupien et al., 1994; McEwen et al., 1999), loss of bone mineral density (Michelson et al., 1996), sarcopenia (Lamberts et al., 1997), or immune dysfunction (Dhabar and McEwen, 1997). It is possible to use the absolute value and circadian variation of the cortisol concentration in individuals as diagnostic indexes of stress-related diseases (Knorr et al., 2010).

Serum cortisol concentration comprises the fraction of free cortisol that is regarded as more biologically active (Mendel, 1989) but also the fraction bound to transport proteins and albumin. Easily accessible saliva is increasingly utilized as an alternate biofluid for assessing levels of cortisol. Saliva primarily manifests the free (unbound) cortisol and salivary cortisol concentrations have been shown to correlate well with the level of free cortisol in serum (Vining and McGinley, 1987; Gozansky et al., 2005).

Serum total cortisol concentrations represent both the fraction of free cortisol thought to be biologically active (Mendel, 1989) as well as the cortisol bound to transport proteins and albumin. Serum total cortisol comprises approximately 80% bond to cortisol-binding globlin and 10% bond to serum albumin (Heyns et al., 1967). The free cortisol index (serum total cortisol/cortisol-binding globlin; FCI) might be better index for hypothalamic–pituitary–adrenal (HPA) status than the serum total cortisol (le Roux et al., 2003). In contrast, salivary cortisol concentrations have been shown to correlate well with the level of serum free cortisol (Vining and McGinley, 1987; Gozansky et al., 2005) thus rendering the measurement of salivary cortisol a useful alternate method for assessing free (unbound) cortisol levels in serum. However, the challenges of salivary cortisol measurement include levels that are up to 100-fold lower than the serum total cortisol (Aardal and Holm, 1995) and a wide range concentrations from 0.1 to 10 ng/ml (0.278–27.8 n mol/l).

Methods for determining the salivary cortisol concentration include radio immunoassay (RIA, Lee and Goeger, 1998), liquid chromatography combined with mass spectrometry (LC–MS,

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Shibasaki et al., 1997; Frerichs and Tornatore, 2004; Nelson et al., 2008), capillary chromatography (Rao et al., 1999), high-performance liquid chromatography (HPLC, Neufeld et al., 1998; Turpeinen and Stenman, 2003), gas chromatography combined with mass spectrometry (GC–MS, Furuta et al., 2000), and surface plasmon resonance (SPR, Stevens et al., 2008; Frasconi et al., 2009). More common methods utilize enzyme-linked immunosorbent assay (ELISA) kits for the analysis of salivary cortisol (e.g., Salimetrics, LLC, PA, USA; Susman et al., 1999).

The clinical utility of salivary cortisol measurements has been restricted by the lack of appropriate technology platforms that allow near "real-time" detection and quantification of salivary cortisol. Integrating early identification and risk stratification in patients with stress-related disease will require that care providers have timely access to salivary stress biomarker data. Typically, biological samples collected by human subjects are processed in centralized laboratories which result in extended reporting times and are fraught with several potential quality failure points. Thus, the development of easy-to-use analytical devices for on-site diagnosis of salivary cortisol promises greater integration and impact in the clinical care of patients with stress-induced diseases.

For measurement strategies that depend on the molecular recognition of cortisol, the speed and selectivity of the immunological reaction are special consideration. Immunosensors proposed for cortisol analysis have incorporated piezoelectric (Attili and Suleiman, 1995), used conjugates (Cook, 1997; Mirasoli et al., 2002), ultrasound (Cook, 2002), colloidal gold conjugate (Leung et al., 2003), gold nanowires (Kumar et al., 2007), and graphite electrodes (Goyal et al., 2010) elements. However, these methods typically require a pre-treatment to remove impurities such as proteins and other steroid hormones from the sample in order to improve sensitivity and delete cross-reactivity. This pre-treatment makes difficult to realize real-time detection of salivary cortisol. In particular, the unreacted cortisol in the saliva sample raises the baseline of the detected signal and restricts the sensitivity of these immunosensors.

In this paper, we describe the development of a new immunosensor design that incorporates a fluid control mechanism that will allow point-of-use, quantitative measurement of salivary cortisol without a pre-treatment. The fluid control mechanism incorporates a lateral flow and a vertical flow component. The immune reaction occurs in the vertical flow component. The lateral flow component serves to remove proteins, other steroid hormones, and unreacted enzyme-labeled conjugate in the sample for a short time. The sensitivity and accuracy of the cortisol immunosensor are reflected through the detected current characteristics.

2. Materials and methods

2.1. Chemicals

A monoclonal anti-cortisol antibody (2330-4879, host: Mouse, AbD Serotec, UK) was used for the immunoassay of the sensor. Cortisol-21-hemisuccinate (086-05583, Wako Pure Chemical Industries, Ltd., Japan) and glucose oxidase (GOD; EC 1.1.3.4, Amano Enzyme Co., Ltd., Japan) were used to synthesize an enzyme labeled cortisol conjugate. Sulfuric acid (H_2SO_4; Cas no. 7664-93-9, Wako Pure Chemical Industries, Ltd., Japan) and hydrogen peroxide (30% H_2O_2; Cas no. 8007-30-5, Wako Pure Chemical Industries, Ltd., Japan) were used for preparing piranha solution. A 5-carboxy-1-pentanethiol (C387, Dojin Chemistry Laboratory, Ltd., Japan) was used to form a self-assembled monolayer (SAM) membrane. *N*-hydroxysulfosuccinimide (Cas no. 6066-82-6, Thermo Scientific Pierce Protein Research Products, IL) and 1-ethy1-3-carbodiimide (Cas no. 1892-57-5, Wako Pure Chemical Industries, Ltd., Japan) were used to activate the SAM membrane, and ethanolamine (Wako Pure Chemical Industries, Ltd., Japan) was used to deactivate it. Bovine serum albumin (BSA; Cas no. 9048-46-8, Wako Pure Chemical Industries, Ltd., Japan) was used for blocking, and phosphoric acid buffer solution (PBS; pH 7.3, 1 mM, Dulbecco A, Oxoid Ltd., UK) was used as a buffer solution. Cortisol standard solution (free cortisol, C008-125UL, Luminos LLC, MI) was used to determine a calibration curve. In order to prevent retention of the sample solution, a surfactant (0.1% sucrose aliphatic acid ester, Daiichi Industrial Pharmaceutical Co., Ltd., Japan) was added to the PBS. Glycine (Cas no. 56-40-6, 0.1 M, Wako Pure Chemical Industries, Ltd., Japan) and sodium chloride (Cas no. 7647-01-0, 0.1 M, Wako Pure Chemical Industries, Ltd., Japan) were used to prepare a glycine–HCl buffer solution (pH 3.1).

2.2. Cortisol immunosensor with fluid control mechanism

Cortisol is a monovalent antigen with a low molecular weight (362.47). An approach involving an immunoassay, we had previously synthesized a GOD-labeled cortisol conjugate (MW: 30 kDa) dissolved in PBS (0.05 mg/ml and 1.67 nM) for use in competitive binding assays for qualitative analyses which could be judged visibly by the appearance of lines (Tahara et al., 2010).

A cortisol immunosensor with a fluid control mechanism was fabricated (Fig. 1a). The cortisol immunosensor has a fluid control mechanism which has a vertical flow and a lateral flow. The vertical flow is through an inlet, a spacer, and onto a flat electrode (Fig. 1b). The flat electrode consists of three platinum electrodes; a working electrode (WE), a counter electrode (CE),

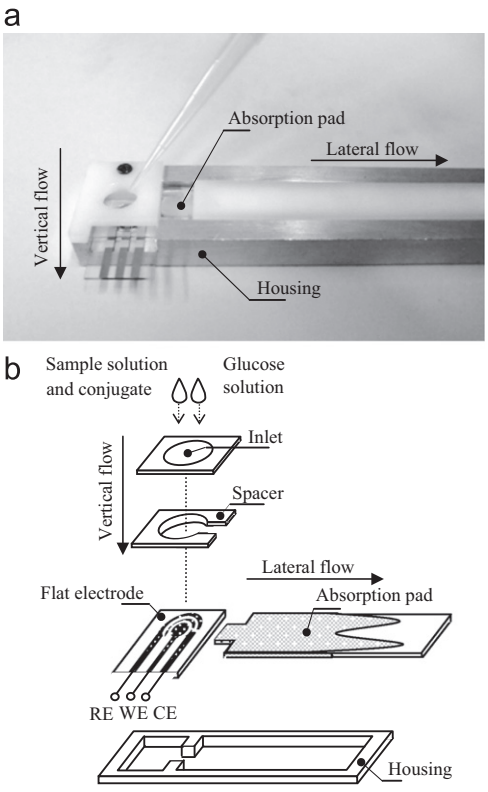

Fig.1. External view and the structure of the sensor. (a) Close-up of salivary cortisol sensor cartridge. (b) Exploded view of sensor cartridge and fluid control mechanism (WE: working electrode, CE: counter electrode, and RE: reference electrode).

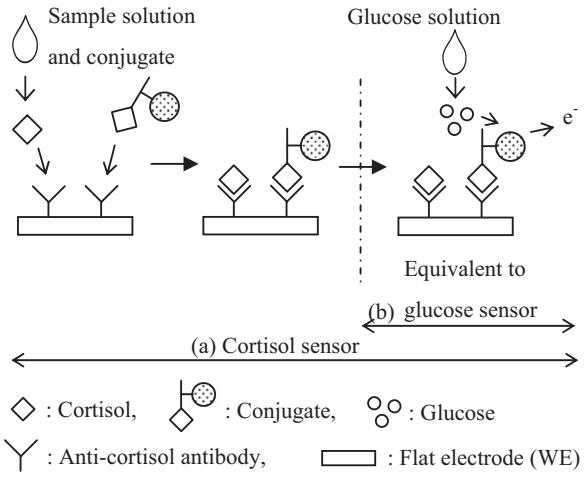

Fig. 2. Electrochemical reaction underlying the biosensor recognition element (WE: working electrode).

and a reference electrode (RE). The lateral flow is through an absorption pad within a housing.

The immune reaction occurs in the vertical flow. Briefly, both the sample solution and PBS including the GOD-labeled cortisol conjugate (the conjugate solution) are dropped into the inlet at the same time, the target molecule (cortisol and antigen) in the sample solution and the GOD-labeled cortisol conjugate react competitively with the anti-cortisol antibody on the flat electrode (Fig. 2a).

When the absorption pad is attached to the surface of the flat electrode, the unreacted GOD-labeled cortisol conjugate moves in a lateral direction through the absorption pad by capillary action. The surface of flat electrode is washed by dropping 100 μl of PBS into the inlet, then the absorption pad is separated from the surface. Surfactant with 40 μl of PBS is dropped into the inlet until the detected current stabilizes at a baseline level.

After that, glucose solution is dropped into the inlet, and an electrochemical reaction takes place at the flat electrode as in a glucose sensor (Fig. 2b). The activity of the GOD-labeled cortisol conjugate is measured by the detected current according to the following reactions:

GOD

$$C_6H_{12}O_6 + O_2 \rightarrow C_6H_{10}O_6 + H_2O_2 \quad (1)$$

$$H_2O_2 \rightarrow + O_2 + 2H^+ + 2e^- \quad (2)$$

2.3. Fabrication of antibody-modified flat electrode

The flat electrode was formed by vapor deposition of a 1-nm-thick layer of platinum on a glass plate ($12 \times 20 \times 0.6$ mm). As a pretreatment, the platinum electrodes were cleaned of organic residue using piranha solution for 30 min. Nitrogen gas was sprayed onto the flat electrode to dry it. Immediately after, 5-carboxy-1-pentanethiol was used to modify the working electrode in order to form the SAM membrane (Ulman, 1996) used for subsequent immobilization of the anti-cortisol antibody. The flat electrode was soaked in a solution consisting of 100 mg/ml *N*-hydroxysulfosuccinimide and 100 mg/ml 1-ethy1-3-carbodiimide at 25 °C for 1 h to activate the SAM membrane. Next, the flat electrode was soaked in a solution consisting of 490 μl PBS and 10 μl anti-cortisol antibody (0.1 mg/ml and 30 p mol) for 0.5 h in order to immobilize the anti-cortisol antibody on the working electrode. After that, the flat electrode was soaked in 20% ethanolamine for 1 h to deactivate the remaining active ester. Finally, the whole surface of the flat electrode was blocked with 5% BSA.

2.4. Detected current characteristics

The detected current characteristics of the fabricated cortisol immunosensor were measured using an electrochemical analyzer (Model 842C, ALS Co., Ltd, Japan). Firstly, detected currents in the presence and absence of the GOD-labeled cortisol conjugate were compared. A 40 μl of PBS was used as the sample solution. Sample solutions with and without 6.65 pmol (= 0.05 mg/ml) of GOD-labeled cortisol conjugate dissolved in it were injected into the inlet. After the detected current had become stable (base detected current), 10 μl of glucose solution at 400 mg/dl (22.2 mM) was injected into the inlet. The relative detected current (ΔI_{max}) was determined by subtracting the base detected current from the maximum detected current.

Next, a calibration curve for the cortisol immunosensor was determined using standard cortisol solutions between 0.1 and 10 ng/ml. A 20 μl of sample solution and 20 μl of the conjugate solution (including 6.65 pmol of GOD-labeled cortisol conjugate) were dropped into the inlet and reacted for 30 min. A 10 μl of glucose solution at 400 mg/dl was used. The experiments were performed five times for each cortisol concentration.

2.5. Refreshing cortisol immunosensor

A method to refresh the cortisol immunosensor was examined. Firstly, the relative detected current was measured using 40 μl of GOD-labeled cortisol conjugate as a sample solution (equivalent to measuring 0 ng/ml of cortisol). After the measurement, the antigen was dissociated from the anti-cortisol antibody by dipping the cortisol immunosensor in 40 μl glycine–HCl buffer solution. A 30 min after that, the relative detected current was measured using the 40 μl PBS (without GOD-labeled cortisol conjugate) once more. This process was repeated twice.

2.6. Detection of human salivary cortisol

Using a study protocol approved by the Institutional Review Board, we conducted a feasibility study of the cortisol immunosensor by analyzing human salivary cortisol levels. After obtaining informed consent, whole saliva was collected from 15 healthy male subjects (22.1 ± 1.1 yrs). Unstimulated, pooled saliva over a 3 min period was collected by passive drool in polypropylene tubes.

Salivary cortisol levels were measured using cortisol enzyme-linked immunosorbent assay kits (cortisol ELISA, 1-3002, monoclonal antibody to cortisol, Salimetrics LLC, State College, PA) and a plate reader (450 nm measurement wavelength; ARVO MX; Perkin Elmer Life Science, Boston, MA). Two groups of cortisol concentration, 1.00 ± 0.20 ng/ml (ranged between 0.85 and 1.22 ng/ml, $n=7$, low concentration group) and 5.00 ± 0.20 ng/ml (ranged between 4.94 and 5.44 ng/ml, $n=7$, high concentration group), were used to evaluate of the cortisol immunosensor.

2.7. Statistical analysis

Within-group comparisons were performed using the Wilcoxon signed-ranks test. *P*-values $P < 0.01$ were taken to represent statistical significance. Unless otherwise stated, all data were expressed as the mean ± standard deviation (SD).

3. Results

3.1. Detected current characteristics

The baseline detected current stabilized 270 ± 95 s after dropping the sample solution. The absolute value of the base detected current was 557 nA. A 3 min was needed in order to remove the unreacted GOD-labeled cortisol conjugate in the lateral direction on the absorption pad by capillary action. When the sample solution with the GOD-labeled cortisol conjugate was used, the relative detected (maximum) current, ΔI, increased to 106.8 nA at 42 s (Fig. 3). On the other hand, when the sample solution without the GOD-labeled cortisol conjugate was used, the relative detected current was only 29.3 nA at 42 s. After 3 min, the relative detected currents had returned to 0 nA. The difference between the relative detected currents with and without the GOD-labeled cortisol conjugate was 77.5 nA.

In the competitive reaction between the sample cortisol and the GOD-labeled conjugate, the maximum relative detected currents at 400 mg/dl glucose concentration were 238.86 ± 15.20, 226.14 ± 12.24, 182.34 ± 18.10, 117.86 ± 16.50, and 51.34 ± 7.11 nA when the concentrations of the standard cortisol solution were 0, 0.1, 1, 5, and 10 ng/ml, respectively (Fig. 4a). Results of a linear regression analysis for the cortisol immunosensor showed the R^2 value was 0.98; the relationship between the relative detected current, ΔI, and the cortisol concentration, *Cort*, was given by $\Delta I = 230.78\ e^{-0.153Cort}$ (nA), and the coefficient of variation (CV) was 14.0%. The detected current was inversely proportional to the cortisol concentration in the sample solution. In this experiment, 1 min was needed to remove superfluous liquid using the fluid control mechanism.

Further calibration curves for the cortisol immunosensor were calculated from the same data indicating the initial slope of current, determined as the difference in current between 0 s and 30 s (Fig. 4b) and the area integrated current between 0 s and 90 s (Fig. 4c). The R^2 values of the initial slope and integration curves were 0.97 and 0.99, respectively. The CVs of the initial slope and the area integration curves were 25.3 and 19.6%, respectively. In this experiment, the calibration curve for the maximum detected current had the best CV value.

3.2. Refreshing cortisol immunosensor

The relative detected current, ΔI, for the GOD-labeled cortisol conjugate was measured (curve A in Fig. 5). After refreshing the immunosensor measurements of the PBS without the GOD-labeled cortisol conjugate were made (curve B in Fig. 5). These measurements were repeated to produce curves C and D. The maximum detected currents were as follows: A: 244.6 nA,

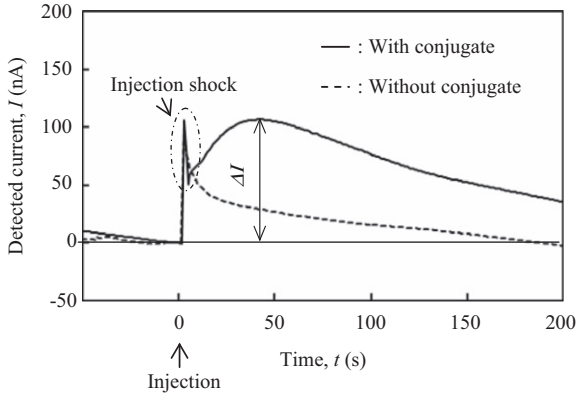

Fig. 3. Detected current characteristics of cortisol sensor with fluid control mechanism. (0 s: glucose injection).

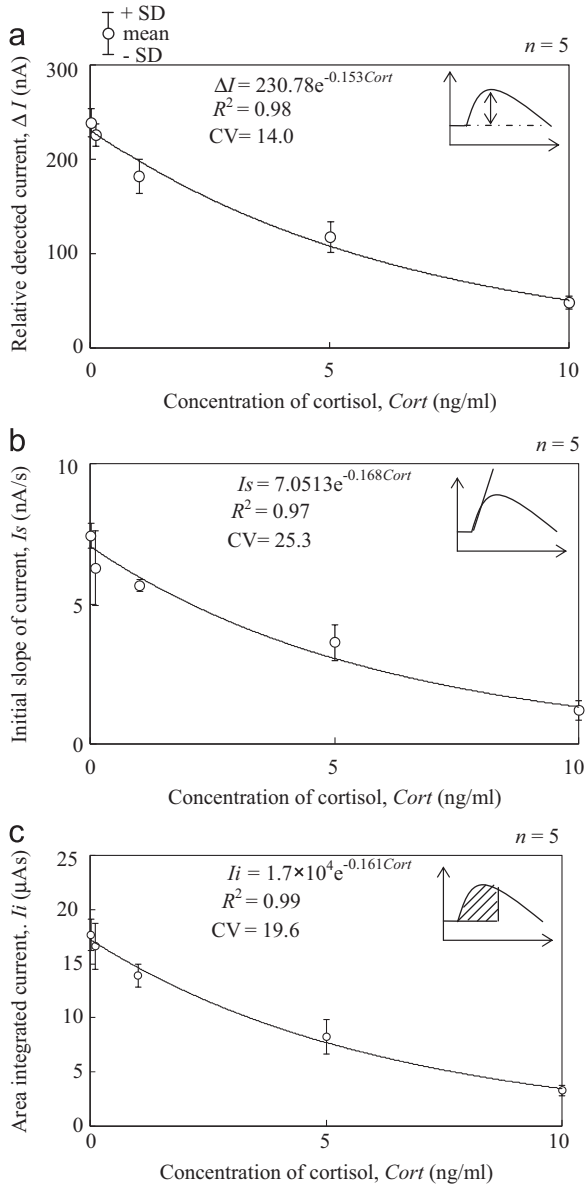

Fig. 4. Calibration curves for the cortisol immunosensor with competitive reaction. (a) Relative detected (maximum) current curve, (b) Initial slope curve and (c) Integration curve.

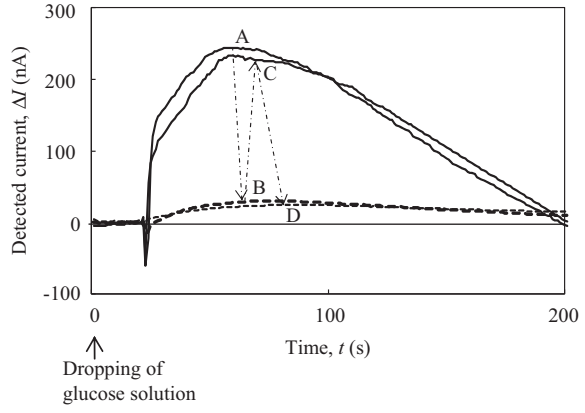

Fig. 5. Refreshing characteristics of the cortisol immunosensor evaluated by the relative detected current.

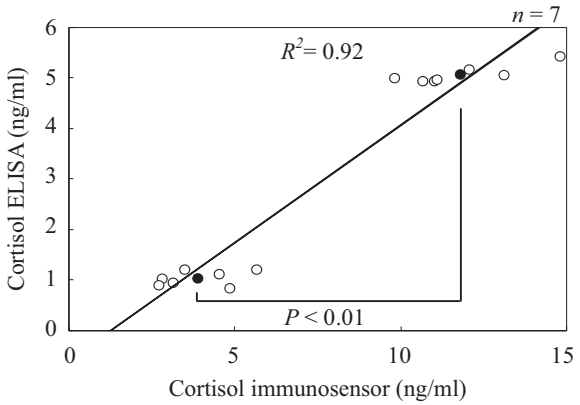

Fig. 6. Measured results of human salivary cortisol using the cortisol immunosensor (using calibration curve: Fig. 4a, ●: mean).

B: 29.4 nA, C: 232.4 nA, and D: 24.0 nA. In the first and second measurements with the GOD-labeled cortisol conjugate (A and C), the relative detected current was close to the measurement with a cortisol concentration of 0 ng/ml. On the other hand, the relative detected current for the PBS without the GOD-labeled cortisol conjugate (B and D) was similar to that in Fig. 3.

3.3. Detection of human salivary cortisol

Two groups of cortisol concentration were analyzed using both the cortisol immunosensor and cortisol ELISA. The estimated values of the cortisol immunosensor were up to 3-fold higher than the cortisol ELISA (Fig. 6). The correlations measured by the cortisol immunosensor agreed well with the measurements made by the commercially available ELISA method ($R^2=0.98$). The difference between two groups of cortisol concentration could be distinguished using the cortisol immunosensor ($P<0.01$).

4. Discussion

The changes in the detected current characteristics in response to the presence or absence of the GOD-labeled cortisol conjugate (Fig. 3) indicated that the GOD-labeled conjugate was trapped by the anti-cortisol antibody through an antigen–antibody reaction. We determined that the absolute value of the maximum detected current exceeded 100 nA, a detection level that is sufficient for use as an electrochemical sensor. The turn-around-time for results reporting was approximately 35 min and included 30 min for the competitive antigen–antibody reaction, 1 min to remove surplus liquid using the fluid control mechanism, 3.5 min to stabilize the base detected current, and 0.5 min to reach the maximum detected current.

Comparison of the different calibration curves, including the relative detected (maximum) current, initial slope of current, and area integrated current, indicated that the curve using the relative detected current had the best $R^2=0.98$ and CV=14% values for the range of standard cortisol solutions between 0 and 10 ng/ml. The dynamic range of the cortisol immunosensor (1–10 ng/ml) is sufficient to allow measurement of the range of salivary cortisol concentrations (1–8 ng/ml) reported by Aardal (1995) in healthy adults (1 ng/ml=0.1 μg/dl=362 pmol/l).

Additionally, it is possible to reuse the cortisol immunosensor because the reacted antigen (GOD-labeled cortisol conjugate) could be refreshed by using glycine–HCl buffer solution as the dissociation liquid (Fig. 5). This renewable characteristic would allow the biosensing to be relatively inexpensive compared to single use unit.

In the evaluation of human salivary cortisol levels, the correlation measured by the cortisol immunosensor agreed well with the measurements made by the commercially available ELISA method ($R^2=0.92$). On the other hand, the estimated values of the cortisol immunosensor were to 3-fold higher than the cortisol ELISA. The difference suggests two causes. Firstly, the cortisol immunosensor has higher sensitivity than the cortisol ELISA causing viscosity of saliva. It was considered that in order to estimate the calibration curve, the viscosity of the cortisol standard solution should be prepared similar value to saliva. Secondly, the relative detected current reduction caused by remaining impurities should increase the estimated results of the cortisol immunosensor because the relative detected current is inversely proportional to the cortisol concentration in the sample solution. The cortisol correlation of two methods correlated well, so the remaining impurities may be a minor influence. Our findings suggest that the fluid control mechanism might contribute to the removal of other proteins in saliva as a refinement of immunosensor.

5. Conclusions

The analytical performance of our salivary cortisol immunosensor platform, coupling a competitive immunoassay with lateral and vertical fluid control mechanisms, allows for noninvasive and quantitative analyses of human salivary cortisol levels. The detected current, reflecting the competitive reaction between the sample cortisol and the GOD-labeled cortisol conjugate, was indirectly proportional to the amount of cortisol analyte present in the sample solution. The measurement range was sufficient to analyze the cortisol levels in the saliva samples. Compared to commercial ELISA methods, the short reporting time of 35 min underscores potential application for point of care measurement of cortisol. Our studies indicate that the biosensing platform could facilitate greater use of salivary cortisol as noninvasive biomarker of stress-related conditions and in therapeutic decision-making.

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