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Capillary electrophoretic enzyme immunoassay with electrochemical detection for cortisol

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Abstract

A novel capillary electrophoretic enzyme immunoassay with electrochemical detection has been developed and used for the determination of cortisol. In this method, after the competitive enzyme immunoreaction, the free enzyme (horseradish peroxidase, HRP)-labeled cortisol (HRP-cortisol) and the bound enzyme-labeled cortisol (HPR-cortisol–anti-cortisol) were separated in the separation capillary and then catalyzed the enzyme substrate [3,3,5,5-tetramethyl-benzidine dihydrochloride, TMB(Red)] in the reaction capillary. The product of the enzymatic catalysis reaction [TMB(Ox)] was amperometrically detected on a carbon fiber microdisk bundle electrode. A concentration limit of detection (LOD) of 1.7×10^{-9} mol/l, which corresponds to a mass LOD of 7.8 amol, was achieved with the relative standard deviation of 3.3%. The method has been verified using the cortisol controls.

Keywords: Immunoaffinity electrophoresis; Affinity electrophoresis; Cortisol; Steroids

1. Introduction

Since its advent in the late 1950s [1], immunoassay has become a primary analytical tool in clinical diagnostics. The methodology is mainly based on the inherent chemical specificity of an immunological reaction and the exquisite sensitivity in detecting labeled antigens or labeled antibodies, which allow fast, accurate, and precise quantification of a variety of analytes at very low concentrations in complex sample matrices. In the case of enzyme immunoassay, the enzyme molecule is used as the label that converts enzyme substrate into its product at a relatively high reaction rate. As the result of enzyme amplification, a significant amount of product can be produced for final detection. Therefore, assays that use enzymes as labels usually have excellent limits of detection [2].

CE has been proven to be a powerful tool for the separation of macromolecules, such as proteins and immunocomplexes [3,4]. With both superior separation power and high detection sensitivity, CE can separate free antibody and antigen from bound antibody and antigen rapidly and is especially suitable for immunoassays [5]. Actually, CE-based immunoassay, called capillary electrophoretic immunoassays (CEIA) or immunocapillary electrophoresis (ICE), has emerged as a new technique carrying out immunologic reactions in CE. CEIA offers

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several advantages over other conventional immunoassays. CEIA has a high selectivity and offers low reagent consumption. This assay methodology can be simplified by CE separation, in which many wash steps can be eliminated. In addition, the incubation time can be reduced for homogenous reaction and many components can be determined in a single capillary at the same time. Finally, CEIA can easily be combined with the existing CE detection techniques such as UV, laser induced fluorescence (LIF), electrochemical methods, mass spectrometry, etc. Recently, CEIA has been applied to determination of human growth hormone (HGH) [6], digoxin [7,8], insulin [4,9], drugs [10,11], thyoxine [12], theophylline [13], immunoglobulin G (IgG) [14], immunoglobulin A (IgA) [15], bovine serum albumin (BSA) [16–18], glucose-6-phosphate dehydrogenase (G-6-PDH) [19]. Although UV detection can be used for characterization of separation of immunocomplexes, the major disadvantage of the UV detector is the lack of sensitivity. The minimum detectable concentration by UV absorbance is around 10^{-6} mol/l [20], which is several orders of magnitude above the required sensitivity levels for many immunoassays. Moreover, UV detection is not available for many antigens that are small molecules without strong UV absorbance. LIF is a more general approach to improve sensitivity [20].

Cortisol is the major glucocorticoid steroid secreted by the adrenal gland. It shows anti-inflammatory activity and influences blood pressure and metabolism of proteins and carbohydrates. The determination of its concentration provides diagnostic information for adrenal malfunctions, e.g. Addison's disease (chronic adrenal insufficiency) and Cushing's syndrome (adrenal overproduction). Therefore, the determination of cortisol is meaningful. In this paper, we present a novel capillary electrophoretic enzyme immunoassay with electrochemical detection (CE–EIA–ED) for the determination of cortisol. The immunoassay was in a competitive format in which the labeled cortisol competed with the cortisol present for the limited number of anti-cortisol binding sites. After equilibrium was established, a small volume of incubate was injected into the separation capillary, whereupon free and bound labeled cortisol were separated by CE in the separation capillary and catalyzed the substrate 3,3,5,5-tetramethylbenzidine dihydrochloride [TMB(Red)] in the reaction capillary, which followed the separation capillary. The enzymatic reaction product [TMB(Ox)] was amperometrically detected on the carbon fiber microdisk bundle electrode at the outlet of the reaction capillary. Because of the amplification of the labeled enzyme, the concentration of TMB(Ox) was much higher than the labeled cortisol and immunocomplex. Therefore, it was much easier to detect TMB(Ox).

2. Experimental

2.1. Apparatus

The CE–enzyme immunoassay (EIA)–electrochemical detection (ED) system is illustrated in Fig. 1. It consists of six main parts, high-voltage power (Model 9323HVPS, Beijing Institute of New Technology, Beijing, China) (1), a running buffer reservoir (2), a liquid pressure buffer reservoir (3), a liquid pressure substrate reservoir (4), a Plexiglas catalysis reactor (5) and an electrochemical detector (6). In the system, the cylindrical running buffer reservoir (2) (12 mm diameter and 20 mm in depth) was made from Plexiglas with a rubber cover (9). There is a metal tubing (7) in the reservoir wall. The metal tubing linked up with the running buffer reservoir (2) and the liquid pressure buffer reservoir (3) through a plastic hose. On the hose there is a switch (8) to control the flow from the liquid pressure buffer reservoir. In this system a metal needle (13) (400 μm I.D., 680 μm O.D.) of a syringe with a hole (12) in the middle passed through the catalysis reactor (5). Both the poly(vinyl alcohol)-coated separation capillary (15 cm×50 μm I.D.×375 μm O.D.) (10) and the poly(vinyl alcohol)-coated reaction capillary (5 cm×50 μm I.D.×375 μm O.D.) (11) purchased from Hewlett-Packard Instruments were inserted in the needle with a gap (about 10 μm) between them. The separation capillary was connected to the running buffer reservoir through the rubber cover. The enzyme substrate [TMB(Red)] solution in the liquid pressure substrate reservoir (4) was introduced into the reaction capillaries (11) through the gap by means of the liquid pressure. There is another switch (8′) to control the flow from the liquid pressure substrate reservoir (4). A

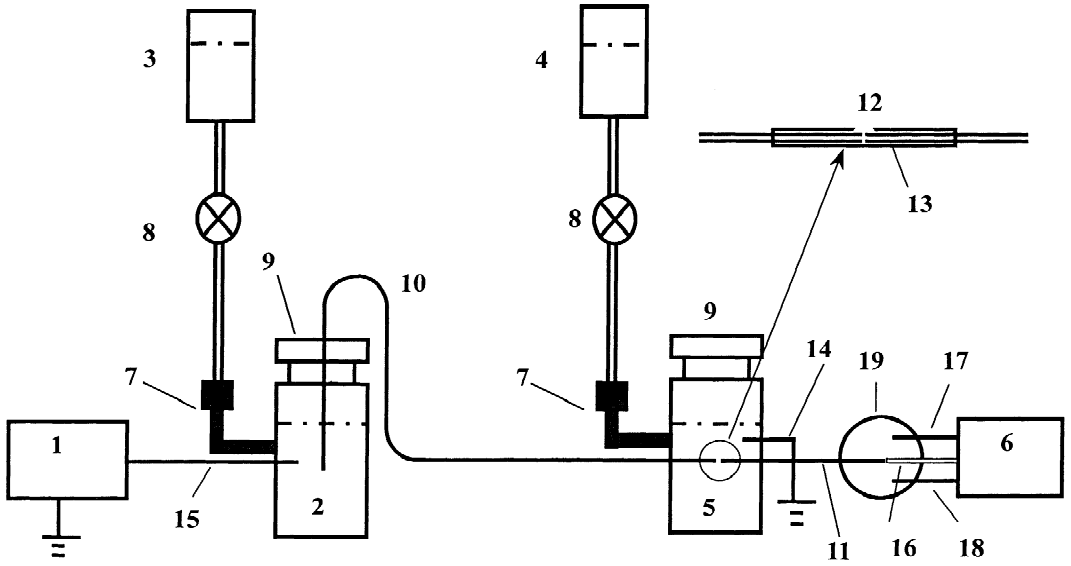


Fig. 1. Overview of the CE–EIA–ED system. 1, High-voltage power; 2, running buffer reservoir; 3, liquid pressure buffer reservoir; 4, liquid pressure substrate reservoir; 5, catalysis reactor; 6, electrochemical detector; 7, metal tubing; 8 and 8′, switch; 9 and 9′, rubber cover; 10, separation capillary; 11, reaction capillary; 12, hole; 13, syringe needle; 14, Pt cathode; 15, Pt anode; 16, working electrode; 17, reference electrode; 18, auxiliary electrode; 19, electrochemical cell.

platinum wire (14) served as the grounded electrode in contact with the substrate solution for the high potential drop across the separation capillary. Unless noted otherwise, the applied separation high-voltage was 10 kV. In the system, all the joints could be fixed with epoxy adhesive. ED at a constant potential was also performed with the electrochemical analyzer (Model CHI800, CH Instrument, Austin, TX). The detection cell, the detector and the catalysis reactor were housed in a Faraday cage in order to minimize the interference from external sources of noise. ED was carried out with a three-electrode system. It consisted of a carbon fiber microdisk bundle electrode as the working electrode, an SCE used as the reference electrode, and a coiled Pt wire (0.3 mm diameter, 5 cm in length) placed at the bottom of the cell as the auxiliary electrode. The arrangement of the electrochemical detection cell was illustrated in Ref. [21] in detail. The carbon fiber microdisk bundle electrodes used here were described previously [22]. Before use, all carbon fiber microdisk bundle electrodes with an area of ca. 8.5×10^{-4} mm^2 were cleaned in alcohol and washed with double-distilled water for 5 min by an ultrasonicator. During electrophoresis, the electrodes can be directly washed with alcohol and water in the detection cell. All disposable plastic wares and disposable micro pipette tips used in the assay were autoclaved prior to use in order to denature any contaminants.

2.2. Reagents and solutions

A DSL-10-2000 Active™ cortisol EIA kit was obtained from Diagnostic Systems Laboratories, Webster, TX. A series of cortisol standards, which contained 0, 5, 15, 40, 100, 200 and 600 μg/l cortisol in BSA with a non-mercury preservative, rabbit anti-cortisol serum in BSA, cortisol controls (containing low and high concentrations of cortisol in BSA) and cortisol enzyme conjugate concentrate [horseradish peroxidase (HRP)-cortisol] were packaged in the kit. The kit was stored at 2–8°C. Universal infection precautions were followed during the handling of the standards, controls and specimens. All kit reagents and specimens were brought to room temperature before use. Repeated freezing and thawing of the reagents was avoided. The reagents and samples were thoroughly mixed before

use by gentle inversion. Because the enzyme used as the label is highly sensitive to microbial contamination, fresh double-distilled water, and clean disposable pipette tips for each reagent, standard and control or specimen were employed in the assay. TMB(Red) was purchased from Sigma (St. Louis, MO). A stock standard solution of TMB(Red) (2.00×10^{-2} mol/l) was prepared in double-distilled water and kept in a dark bottle. Phosphate–citrate buffer (2.00×10^{-2} mol/l $Na_2HPO_4+1.00\times10^{-2}$ mol/l citrate, pH 5.0) was prepared by dissolving the appropriate amount of disodium hydrogen phosphate and citrate in water. All buffers were filtered through 0.45 μm cellulose acetate membrane filters (Shanghai Yadong Resin, Shanghai, China) before use. The substrate solution consisted of 4.00×10^{-4} mol/l TMB(Red) in the phosphate–citrate buffer. The running buffer consisted of 2.50×10^{-3} mol/l H_2O_2 in the phosphate–citrate buffer diluted (1.00×10^{-2} mol/l $Na_2HPO_4+5.00\times10^{-3}$ mol/l citrate, pH 5.0). TMB(Red) or H_2O_2 was added to solution just before the measurement. All buffers and solutions were stored at 4°C. Unless stated otherwise, all other reagents were of analytical grade or better and purchased from standard reagent suppliers. All solutions were prepared with double-distilled water. All solutions were prepared in disposable plastic ware using disposable pipette tips.

2.3. Procedure

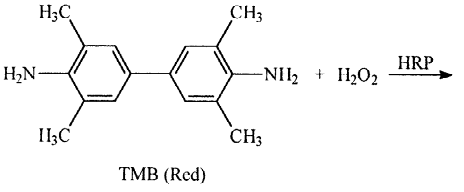
The immunoreaction protocol was a direct competitive format. The procedure followed the basic principle of enzyme immunoassay where there was competition between an unlabeled antigen and an enzyme-labeled antigen for a limited number of antibody binding sites. For the immunoreaction, 25 μl of the cortisol standard or control, 2 μl of HRP-cortisol and 100 μl of rabbit anti-cortisol antiserum were added to a 0.6-ml microcentrifuge tube. The solution was incubated for 30 min at room temperature and was diluted at a ratio of 1 part solution into 1 part running buffer before injection. In the assay, standards or samples were introduced hydrodynamically. Before injection, the level of the solutions in the sample vial (unshown in Fig. 1), the running buffer reservoir (2) and the catalysis reactor (5) were kept at the same height. The liquid pressure buffer reservoir (3) and the liquid pressure substrate reservoir (4) were put 40 cm over the running buffer reservoir and the catalysis reactor, respectively. The injection process was as follows: firstly, the liquid pressure substrate reservoir (4) was put down and keeps the solution level at the same height as that of the catalysis reactor (5). The switch (8′) of the liquid pressure substrate reservoir (4) was turned on. The switch (8) of the liquid pressure buffer reservoir (3) was turned off. Then hydrodynamic injection was performed by inserting the inlet of the separation capillary in the sample vial and raising the vial 6 cm in height for 20 s. After injection, the separation capillary was manipulated down, out of the sample vial, and then immersed in the running buffer solution in the running buffer reservoir (2). After that, the cover (9) of the running buffer reservoir was sealed. The switch (8) of liquid pressure buffer reservoir was turned on, and then the liquid pressure substrate reservoir was raised 40 cm. Finally, the separation high voltage was applied across the separation capillary, the detection potential was applied at the working electrode and the electropherogram was recorded. During the electrophoresis, the same liquid pressure from the liquid pressure buffer reservoir and the liquid pressure substrate reservoir was kept to prevent a distortion of the flat electrophoretic flow profile in the separation capillary. The reaction time could be controlled by the liquid pressure. Routinely, for a 50-μm I.D. reaction capillary, the applied liquid pressure height was 40 cm, giving a reaction time of 9.5 s.

In the electrochemical detection, the working microdisk bundle electrode was cemented onto a microscope slide, which was placed over a laboratory-made *xyz* micromanipulator and glued in place in such a way that the microdisk end protruded from the edge of the slide. The position of the microdisk bundle electrode was adjusted (under a microscope) against the outlet of the reaction capillary so that the electrode and the capillary were in contact. This arrangement allowed easy removal and realignment of both the capillary and the electrode. All potentials were measured against a saturated calomel electrode (SCE).

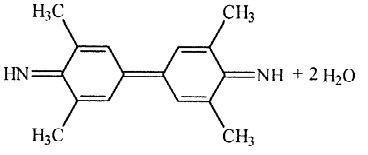
3. Results and discussion

3.1. Optimization of the experimental conditions

In the CE–EIA–ED assay, after the competitive enzyme immunoreaction, the free HRP-labeled cortisol (free HRP-cortisol) and the bound HRP-labeled cortisol (bound HRP-cortisol) are separated in the separation capillary and then catalyze the enzyme substrate TMB(Red) in the reaction capillary. The reaction is shown as follows [23,24]:



TMB (Red)



TMB (Ox)

The product of the catalysis reaction [TMB(Ox)] is amperometrically detected on the carbon fiber microdisk bundle electrode at the outlet of the reaction capillary according to their separation sequence. Therefore, to explore the optimum experimental conditions, the 1.5% HRP-cortisol concentrate diluted by the running buffer was used in the following experiments (unless stated otherwise). A typical electropherogram of HRP-cortisol is shown in Fig. 2. Because the peak slightly broadens on the pedestal, the area of the peak, rather than the peak height, was adopted to quantitate.

The number of theoretical plates, N, almost does not change at different concentrations of running buffer, C_B. C_B affects the migration time, t_m, and the peak area, q. t_m increases with increasing C_B. When 1.00×10^{-2} mol/l phosphate–5.00×10^{-3} mol/l citrate (pH 5.0) was used as the separation buffer, q has a maximum and the reproducibility is the best. N almost stays constant at different separation voltages, V_s. V_s affects t_m and q. t_m decreases with increasing

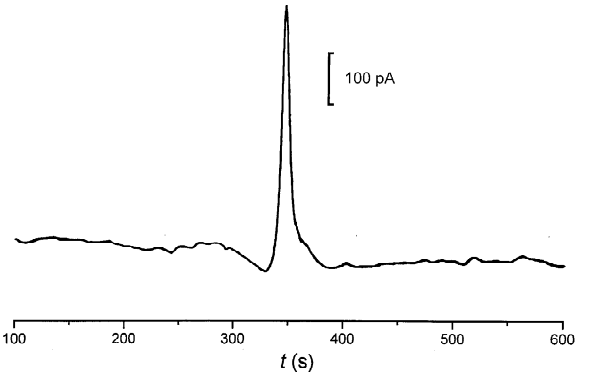


Fig. 2. Typical electropherogram of 1.5% HRP-cortisol concentrate. Separation buffer, 1.00×10^{-2} mol/l $Na_2HPO_4+5.00\times10^{-3}$ mol/l citrate buffer (pH 5.0); reaction buffer, 2.00×10^{-2} mol/l $Na_2HPO_4+1.00\times10^{-2}$ mol/l citrate buffer (pH 5.0); 2.50×10^{-3} mol/l H_2O_2; 4.00×10^{-4} mol/l TMB(Red); separation capillary, 20 cm×50 μm I.D.; reaction capillary, 5 cm×50 μm I.D.; liquid pressure height, 36 cm; separation voltage, 10 kV; hydrodynamical sample injection, 6 cm for 20 s; detection potential, 0.00 V (vs. SCE).

V_s. The maximum of q and the best reproducibility was obtained at 10 kV. Fig. 3 shows the relationship between q and the detection potential, E_d. When E_d is between 0.10 and −0.30 V, q is almost constant. When E_d is higher than 0.10 V, q decreases rapidly. However, the noise of the baseline increases rapidly with reducing E_d when the detection potential is less than −0.10 V. t_m, q and N depend on the liquid pressure height, h, for introducing the enzyme substrate into the reaction capillary. t_m decreases with

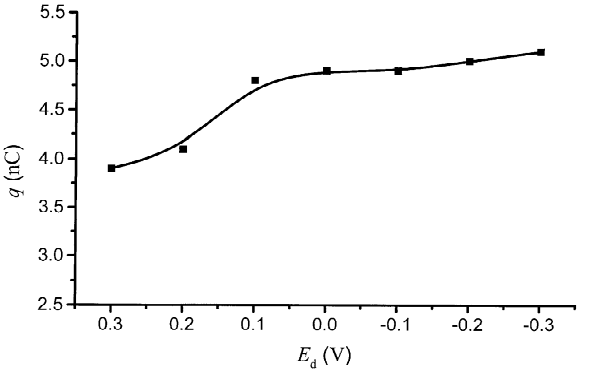


Fig. 3. Relationship between the detected peak area, q, and the applied potential, E_d. Conditions as in Fig. 1.

the increase of h, while q has a maximum at $h=36$ cm. Moreover, the reproducibility at this height is better than at other ones. N increases somewhat with increasing h. Fig. 4 shows the dependence of q on the concentration of TMB(Red), C_{TMB}, and H_2O_2, $C_{H_2O_2}$. When C_{TMB} is below 4.00×10^{-4} mol/l, q increased rapidly with increasing C_{TMB}. q remains almost a constant when C_{TMB} is higher than 4.00×10^{-4} mol/l, which indicates a substrate saturation for HRP. q increases rapidly with the increase of $C_{H_2O_2}$, when $C_{H_2O_2}$ is below 2.00×10^{-3} mol/l. Then it reaches a maximum at $2.50–3.00\times10^{-3}$ mol/l H_2O_2. When $C_{H_2O_2}$ is higher than 3.00×10^{-3} mol/l, q decreases with increasing $C_{H_2O_2}$. In our experiments, the optimum conditions are 1.00×10^{-2} mol/l phosphate–5.00×10^{-3} mol/l citrate (pH 5.0) for C_B, 10 kV for V_s, 0.00 V for E_d, 36 cm for h, 4.00×10^{-4} mol/l for C_{TMB} and 2.50×10^{-3} mol/l for $C_{H_2O_2}$.

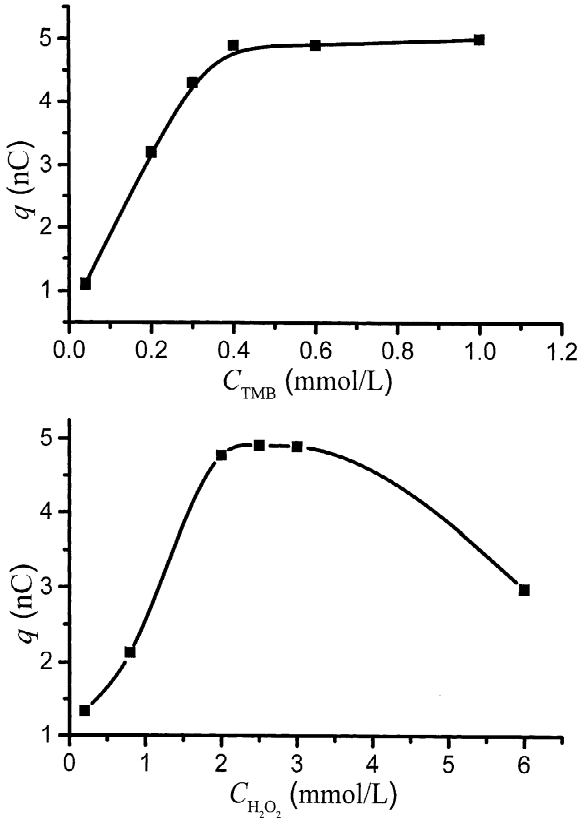


Fig. 4. Effect of concentrations of the substrate [TMB(Red)] and H_2O_2. Other conditions as in Fig. 1.

3.2. Identification of electrophoretic peaks

After injection, the solution after the immunoreaction contains the free and bound HRP-cortisol, which are resolved in the separation capillary. The typical electropherograms of the cortisol standards without and with 60.0 μg/l cortisol are shown in Fig. 5. Two peaks appear at 318 s and 350 s, respectively. Compared with Fig. 2, peak 1 can be identified as the peak of free HRP-cortisol, because they have the same migration time. With the increase in the amount of cortisol, the area of peak 1 increases, while the area of peak 2 decreases (see curve 2), which indicate the principle expected for a competitive immunoassay: an increase in the amount of cortisol standard leads to a decrease in signal for the bound HRP-cortisol and an increase in signal for the free HRP-cortisol. Therefore, the change of the

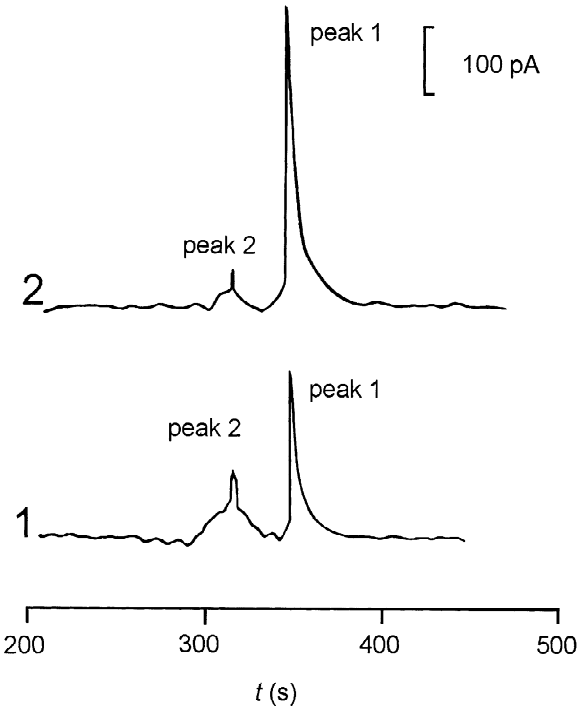


Fig. 5. Electropherograms of the solutions containing HRP-cortisol and anti-cortisol antibody (1) without and (2) with 60.0 μg/l cortisol standard. Conditions as in Fig. 1.

peak area can also be used to identify the free HRP-cortisol and the bound HRP-cortisol. Thus, peak 1 corresponds to the free HRP-cortisol and peak 2 corresponds to the bound HRP-cortisol. The broadening of peak 2 results from the heterogeneity inherent in a polyclonal antibody, as was explored in the work of Schmalzing et al. [25].

3.3. Linear range and limit of detection

Since the peak of the bound HRP-cortisol broadens, the peak of free HRP-cortisol was used to establish the calibration curve. A standard curve for cortisol showed a linear response for area of the peak vs. concentration of cortisol over the range 0 to 60 μg/l (slope = 0.016 nC/μg l^{-1}, y-intercept = 0.78 nC, $r = 0.998$). For comparison, the calibration curve of the bound HRP-cortisol was obtained (slope = −0.0098 nC/μg l^{-1}, y-intercept = 0.90 nC, $r = 0.981$) in the same range of concentration. The points in the curves represent the average peak area detected from three runs. The calibration curves show the expected trend for labeled cortisol for competitive assays, with a higher signal for the free HRP-cortisol and a lower signal for the bound HRP-cortisol for higher concentration of cortisol. The operating concentration range of 0–60 μg/l was enough for the clinically significant range of 10–60 μg/l. The limit of detection (LOD) for the competitive assay can be calculated by determining the mean electric charge of the free HRP-cortisol for the zero-dose cortisol calibrator plus three times its standard deviation calculated from 10 trials of the calibrator [4]. A concentration LOD of 1.7×10^{-9} mol/l was obtained with the 3.3% RSD of 10 replicates of the blank by using peak 1. When peak 2 was used, an LOD of 1.2×10^{-8} mol/l was obtained with the 8.1% RSD of 10 replicates of the blank. As can be seen, the adoption for the peak area of the bound HRP-cortisol results in poor precision and low sensitivity for quantification. The injection volume calculated is 4.6 nl according to the Hagen–Poiseuille equation for the injection with 60 cm height for 20 s. Accordingly, a mass LOD of 7.8 amol (using peak 1) and 55.2 amol (using peak 2) were obtained. Since the volume of the cathodic buffer reservoir (also as the electrochemical cell) and the volume of the substrate eluting from the reaction capillary are ca. 500 μl and 4.6 nl, respectively, the product of the substrate reaction is diluted much more in the reservoir. Thus, the reaction of substrate does not increase background level.

3.4. Determination of cortisol

In order to verify the method, two cortisol controls provided by the kit were detected. The results are listed in Table 1. The concentrations of cortisol in the two controls obtained by the calibration curve method are 5.1 μg/l and 23.8 μg/l using peak 1, and 5.1 μg/l and 22.6 μg/l using peak 2, respectively, which agree with the values given by the kit (4.0±1.5 μg/l and 20±5.0 μg/l, respectively). The recovery was between 93% and 116% for peak 1 and between 80% and 129% for peak 2. Recoveries obtained using peak 1 are better than those using peak 2.

4. Conclusion

The developed CE–EIA–ED of cortisol has high sensitivity. Our work of monitoring cortisol indicates that CE–EIA–ED allows for rapid and quantitative determination of analytes of diagnostic relevance in complex sample matrices. The LOD of this method can be improved by improving the reproducibility of the peak areas or by improving the detection limit of the detector. Because many of the enzyme-linked immunosorbent assay (ELISA) kits with the labeled HRP on antigen or antibody are commercially available and TMB(Red) is used as the enzyme substrate in these ELISA kits, CE–EIA–ED can be always carried out based on the reduction of TMB(Ox) (oxidation product of TMB(Red)) on the carbon fiber microdisk bundle electrodes. In addition, this method is useful where the HRP enzyme label is available and a fluorescent label is not. We think that CE–EIA–ED will become a useful tool in immunological assays.

Acknowledgements

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Table 1
The results detected and recovery of the cortisol controls. Based on (A) the peak of free HRP-cortisol (peak 1) and (B) the peak of bound HRP-cortisol (peak 2)

Control	Determined value (μg/l)	Average value (μg/l)	Stated value (μg/l)	Added value (μg/l)	Expected value (μg/l)	Observed value (μg/l)	Recovery (%)
(A)							
I	5.4			1.5	6.9	6.8	93
	4.8	5.1	4.0±1.5	4.0	8.8	8.7	98
	5.1			10.0	15.1	15.5	104
II	23.5			10.0	33.5	35.1	116
	22.9	23.8	20±5.0	20.0	42.9	42.2	96
	25.0			40.0	65	62.9	95
(B)							
I	5.2			1.5	6.7	6.4	80
	5.9	5.1	4.0±1.5	4.0	9.9	9.7	95
	4.1			10.0	14.1	16.1	120
II	23.0			10.0	33.0	35.9	129
	18.4	22.6	20±5.0	20.0	38.4	36.1	89
	26.5			40.0	66.5	77.3	127

Other conditions as in Fig. 1.

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References

[1] R.S. Yalow, S.A. Berson, Nature 184 (1959) 1648.
[2] N. Kaneki, Y. Xu, A. Kumavi, H.B. Halsall, W.R. Heineman, P.T. Kissinger, Anal. Chim. Acta 287 (1994) 253.
[3] Q. Weng, W. Jin, Electrophoresis 22 (2001) 2297.
[4] N.M. Schultz, R.T. Kennedy, Anal. Chem. 65 (1993) 3161.
[5] J. Bao, J.M. Fujima, N.D. Danielson, J. Chromatogr. B 699 (1997) 481.
[6] R.G. Nielsen, E.C. Rickard, P.F. Santa, D.A. Sharknas, G.S. Sittampalam, J. Chromatogr. 539 (1991) 177.
[7] X. Liu, Y. Xu, M.P.C. Ip, Anal. Chem. 67 (1995) 3211.
[8] F.-T.A. Chen, S.L. Pentoney Jr., J. Chromatogr. A 680 (1994) 425.
[9] N.M. Schultz, L. Huang, R.T. Kennedy, Anal. Chem. 67 (1995) 924.
[10] R.A. Evangelista, F.-T.A. Chen, J. Chromatogr. A 680 (1994) 587.
[11] J. Caslavska, D. Allemann, W. Thormann, J. Chromatogr. A 838 (1999) 197.
[12] D. Schmalzing, L.B. Koutny, T.A. Taylor, W. Nashabeh, M. Fuch, J. Chromatogr. B 697 (1997) 175.
[13] N. Chiem, D.J. Harrsion, Anal. Chem. 69 (1997) 373.
[14] O.-W. Reif, R. Lausch, T. Scheper, R. Freitag, Anal. Chem. 66 (1994) 4027.
[15] F.-T.A. Chen, J. Chromatogr. A 680 (1994) 419.
[16] J.P. Ou, Q.G. Wang, T.M. Cheung, S.T.H. Chan, W.S.B. Yeung, J. Chromatogr. B 727 (1999) 63.
[17] J.P. Ou, S.T.H. Chan, W.S.B. Yeung, J. Chromatogr. B 731 (1999) 389.
[18] Q.G. Wang, G.A. Luo, J.P. Ou, W.S.B. Yeung, J. Chromatogr. A 848 (1999) 139.
[19] Z. Rosenzweig, E.S. Yeung, Anal. Chem. 66 (1994) 1771.
[20] M.J. Gordon, X. Huang, S.L. Pentoney, R.N. Zare, Science 242 (1988) 224.
[21] W. Jin, Q. Weng, J. Wu, Anal. Chim. Acta 342 (1997) 67.
[22] W. Jin, D. Yu, Q. Dong, X. Ye, Electrophoresis 21 (2000) 925.
[23] P.D. Josephy, T. Eling, R.P. Mason, J. Biol. Chem. 257 (1982) 3669.
[24] G. Volpe, D. Compangnone, R. Draisci, G. Palleschi, Analyst 123 (1998) 1303.
[25] D. Schmalzing, W. Nashabeh, M. Fuchs, Clin. Chem. 41 (1995) 1403.