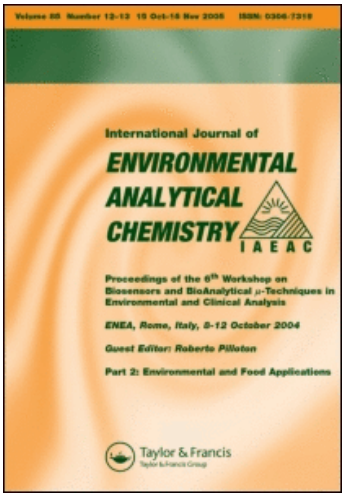

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Electrochemical flow injection immunoassay for cortisol using magnetic microbeads

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Online publication date: 20 January 2011

To cite this Article Muramatsu, Tadayoshi , Ohnuki, Hitoshi , Ushio, Hideki , Hibi, Kyoko , Igarashi, Maki , Hayashi, Tetsuhito , Ren, Huifeng and Endo, Hideaki(2011) 'Electrochemical flow injection immunoassay for cortisol using magnetic microbeads', International Journal of Environmental Analytical Chemistry, 91: 2, 161 — 173
To link to this Article: DOI: 10.1080/03067319.2010.500725
URL: http://dx.doi.org/10.1080/03067319.2010.500725

Intern. J. Environ. Anal. Chem.
Vol. 91, No. 2, 15 February 2011, 161–173

Taylor & Francis
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(*Received 31 July 2009; final version received 9 June 2010*)

We developed a novel flow injection assay for cortisol based on competitive immunologic reactions, magnetic separation, and electrochemical measurement. The proposed flow assay system was composed of two reaction units. An anti-cortisol antibody was immobilised on magnetic beads and injected into the reaction coil of a competitive reaction unit with a blood sample and a specific quantity of acetylcholinesterase-labelled cortisol (cort-AChE). After reacting in the reaction coil, the sample was separated magnetically using a neodymium magnet. The cort-AChE was detached from the magnetic beads and transferred into the enzyme reaction unit with acetylthiocholine (ATCh). ATCh was hydrolysed by the cort-AChE to produce thiocholine. The thiocholine was quantified downstream by electrochemical detection using a Pt-Ir electrode. The performance of the proposed flow assay system was optimised under the following conditions: pH 7.5, temperature 25°C, flow rate 170 µl min^{-1}, ATCh concentration in the substrate buffer 5 mmol L^{-1}. The output current was well correlated with the concentration of the cortisol standard solution (range: 7.8–500 pg mL^{-1}). The results obtained using the proposed flow method were compared with those obtained using conventional ELISA (correlation coefficient 0.9585 [$y = -0.9797 + 1.173(x), n = 11$]). These findings suggest that the EFIIA system can be used to analyse cortisol in fish plasma samples.

Keywords: cortisol; FIA; magnetic microbeads; immunoassay; fish; monitoring

1. Introduction

There are many studies on the effects of environmental factors on aquatic life. A general finding is that fish are acutely stressed by pollution of the aquatic environment due to chemical contaminants such as endocrine-disrupting compounds and several heavy metals. These pollutants disturb reproductive function and cause morphological deformities [1–4]. To prevent or reduce pollution-induced damage to the aquatic ecological system, it is important to develop an analytical technique to measure the effects of pollution of the environment on aquatic life. A large number of man-made chemicals with diverse chemical structures and environmental concentrations are pollutants. Therefore, it is difficult to

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ISSN 0306–7319 print/ISSN 1029–0397 online

DOI: 10.1080/03067319.2010.500725
http://www.informaworld.com

determine the influence of each pollutant on ecological systems. To address this problem, the development of analytical techniques to test for biomarkers of stress in fish blood is expected to facilitate evaluation of the effects of pollution on the aquatic environment.

Exposing fish to stressors, including chemical pollutants, elicits physiological changes that are collectively known as the stress response. Among these changes, cortisol levels are most commonly evaluated. Cortisol, a stress hormone, is a member of the glucocorticoid hormone family and is a key metabolic regulator. When fish are stressed, cortisol is released from the interrenal gland, located in the head-kidney, in response to adrenocorticotropic hormone (ACTH). Cortisol activates the central nervous system and induces an increase in glucose levels to cope with the stressors. Several studies have demonstrated that cortisol level fluctuations are related to stress following exposure to endocrine-disrupting compounds and heavy metals [5–8]. In addition, increases in cortisol levels are also induced by other stress factors, such as water acidification [9,10], hypoxic conditions [11] and temperature extremes [12]. Thus, a rapid rise in cortisol levels in fish indicates increased exposure to stressors in the aquatic environment. Moreover, because some studies have indicated that excessive secretion of cortisol might cause immunosuppression in fish [13–15], measuring cortisol levels in fish is important for comprehensive management of the aquatic environment.

Cortisol can be analysed by several different methods, such as high-performance liquid chromatography [16,17], gas chromatography/mass spectrometry [18,19], and thin-layer chromatography. Although these assays are highly sensitive, they are very labour intensive. In addition, several immunochemical methods, such as radio immunoassay [20] and enzyme-linked immunosorbent assay (ELISA) [21,22], have been studied. Although radioimmunoassay is well established, special facilities and equipment are necessary due to the use of radioactive compounds. On the other hand, the ELISA method using a microtitre plate is widely used due to its safety and specificity. This method, however, is complicated, and time-consuming due to the multistage process, such as manual plate-washing and sample addition. Moreover, these methods are not suitable for the continuous measurements that are required for longitudinal research on the temporal changes in cortisol concentrations induced by pollutants.

New techniques for continuous assay have developed during the last decade [23,24]. The capillary electrophoresis [23] and magnetic separation [24] has been applied for determination of cortisol to eliminate wash steps. Each technique has several advantages such as high sensitivity and low regent consumption. However, a certain amount of incubation time for immunoreaction is required before each measurement is performed. On the other hand, recently, we also developed a novel method for measuring pathogenic bacteria using magnetic microbeads and flow cytometry [25,26]. The method, which utilises immunomagnetic separation to separate and concentrate an antigen from a sample solution, is highly sensitive. We developed a biosensor for cholesterol to function as a biomarker of disease resistance; this sensor system was based on flow injection analysis (FIA), which allowed for the rapid and convenient detection of cholesterol [27]. Therefore, using the know-how of our past study, the present study was aimed at developing a novel assay system for cortisol in fish based on FIA, competitive immunological reactions, immunomagnetic separation and electrochemical measurement. The analytical parameters (such as analysis time, sensitivity) of the developed electrochemical flow injection immunoassay system (EFIIA system) were evaluated. The proposed system was applied to the measurement of cortisol concentrations in fish plasma samples. The results were compared with those obtained using the conventional ELISA method.

2. Experimental

2.1 *Reagents*

Polyclonal anti-cortisol rabbit antibody, standard cortisol, and acetylcholinesterase-labelled cortisol (cort-AChE) were purchased from Cayman Chemical (Ann Arbor, MI). Magnetic microbeads coated with anti-rabbit antibody (Biomag@ Goat anti-Rabbit IgG, particle size 1.5 μm) were obtained from Polyscience (Warrington, PA). The magnetic beads were suspended in solution at a concentration of 1 mg ml^{-1}. Acetylthiocholine chloride (ATCh) and bovine serum albumin (fraction V, approximately 98%) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO). Potassium biphosphate (K_2HPO_4), monopotassium phosphate (KH_2PO_4), sodium azide (NaN_3) and sodium chloride (NaCl) were purchased from Kokusan Chemical Co. (Tokyo, Japan). 2-Phenoxyethanol and heparin sodium were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other reagents used for the experiments were commercial and laboratory grade.

2.2 *Preparation of anti-cortisol antibody immobilisation on magnetic microbeads*

Magnetic microbeads coated with anti-rabbit antibody (Biomag@ Goat anti-Rabbit IgG) were obtained as a suspension of particles approximately 1.5 μm in size that were covalently attached to goat anti-rabbit IgG antibody. After shaking vigorously, a 1.0 mL magnetic microbead suspension was added to 0.5 mL polyclonal anti-cortisol rabbit antibody. The resulting solution was incubated overnight at 4°C to immobilise the anti-cortisol antibody to the magnetic microbeads, then the solution was washed and magnetically separated. Finally, the magnetic microbeads with the immobilised anti-cortisol antibodies were suspended in 1.0 mL diluting buffer, comprising 0.01 % NaN_3, 2.34 % NaCl, 0.037 % EDTA, 0.1% bovine serum albumin with 0.1 M phosphate buffer, and stored at 4°C until the analysis was performed.

2.3 *Electrochemical flow injection immunoassay (EFIIA) system*

A schematic of the (EFIIA) system is shown in Figure 1. The system comprised micro-tube pumps (SH-1211L ATTO, Tokyo, Japan), a competitive reaction unit, an enzyme reaction unit, magnetic separation tube, neodymium magnet, a potentiostat (8031, Pinnacle Technology, Lawrence, KS), and a personal computer that served to control the potentiostat using the PAL software program for Windows. The electrochemical detector was constructed by placing a 20 mm long platinum iridium (Pt-Ir) electrode into an acrylic flow cell with a hole (φ 5 mm × 5 mm deep). A schematic of the electrochemical detector is shown in Figure 1(A). The working electrode was made using a 10 mm long Teflon-coated Pt-Ir (Pt 90% – Ir 10%) wire. The Teflon coating was stripped at one end to expose 1 mm of the Pt-Ir wire as the sensing element. The sensing element was dipped in 5.0% Nafion® solution and dried for 10 min. The prepared working electrode was inserted into a polyethylene tube (φ 5 mm), and the remaining space was filled with Ag/AgCl paste (BAS, Tokyo, Japan) as an electrode/counter electrode (Figure 1(A)), resulting in an electrode field area of 12.6 mm^2. The prepared detector was connected to the potentiostat and measurements were performed using a personal computer containing the PAL software program. A 650 mV potential (vs. Ag/AgCl) was applied by the potentiostat to the Pt-Ir working electrode for amperometric measurements.

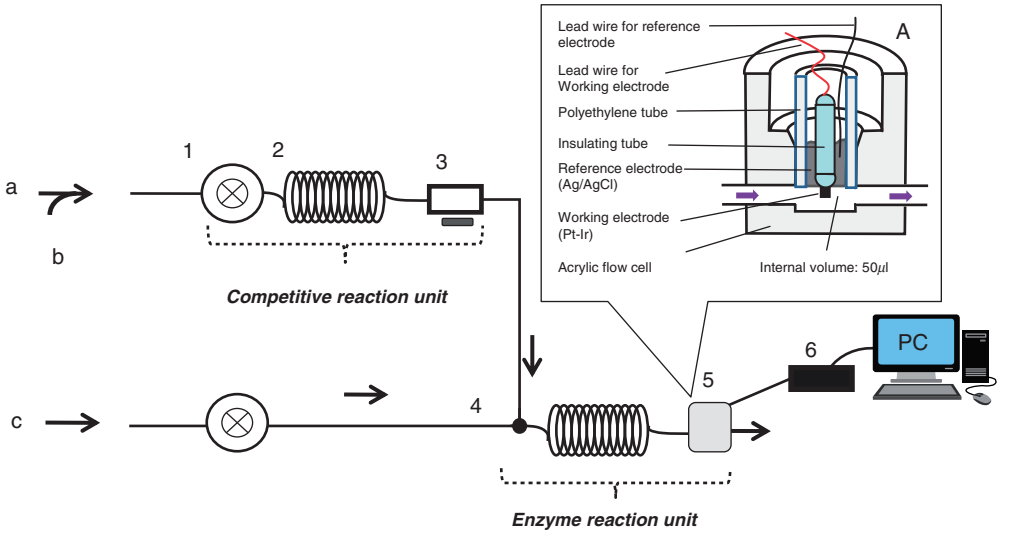


Figure 1. Schematic diagram of the electrochemical flow injection immunoassay (EFIIA) system. *a* carrier buffer, *b* sample solution, *c* substrate buffer, *1* micro-tube pump, *2* reaction coil, *3* magnetic separation tube and neodymium magnet, *4* connecting point, *5* electrochemical detector, *6* potentiostat, A: Schematic diagram of the electrochemical detector.

2.4 *Preparation of fish plasma samples*

We thought that Nile tilapia (*Oreochromis niloicus*) which are culturing all over the world was suitable as test fish. Moreover, it was easy for us to store them in the laboratory. So we selected Nile tilapia (*Oreochromis niloicus*) cultured at Tokyo University of Marine Science & Technology as test fish. The six fish were stored in a 50 L oxygenated tank with a controlled temperature of 25°C and kept under normal laboratory fluorescent lighting with a daily 12 h photoperiod. These fish (body length, ca. 18.9 ± 0.8 cm) were netted from the tank and anesthetised with 0.1% 2-phenoxyethanol by bath exposure for 3 min. Blood was collected from the caudal vein using a heparinised syringe fitted with a 23 G needle [15]. The blood samples (200–350 μl) were centrifuged ($550 \times g$, 12 min) to separate the plasma. Plasma samples (100 μl) were transferred into clean test tubes, and then diluted with 900 μl diluting buffer. The diluted plasma sample was again diluted 1/100 and frozen (−80°C) until the analysis was performed.

2.5 *Assay procedure of the proposed method*

The carrier buffer, comprising 1.0 M phosphate (pH 7.4) buffer and 0.02 vol% Tween-20, flowed uniformly through the competitive reaction unit (Figure 1(a)). The substrate buffer, comprising ATCh and the carrier buffer, was added through another passage (Figure 1(c)). Because ATCh might be hydrolysed spontaneously, each buffer should be purged by N_2 and should be used the day it was prepared to prevent from causing a positive error. Each buffer was joined at the connecting point (Figure 1(*4*)). After the sensor output stabilised, 50 μl of sample solution was injected into the reaction coil (Figure 1(*2*)) of the competitive reaction unit with 50 μl anti-cortisol antibody immobilised on magnetic beads and 50 μl

cort-AChE (termed 'mixed solution'). After the competitive reaction in the reaction coil (intratubular volume, ca. 785 µl), the mixed solution was separated magnetically using a neodymium magnet (φ 17.5 mm × 5.0 mm, C5 Arunet, Fukuoka, Japan). The concentration of unbound cort-AChE to the separated magnetic beads was proportional to the quantity of cortisol in the sample solution. The unbound cort-AChE was transferred to the enzyme reaction unit containing the substrate buffer. ATCh was hydrolysed by the cort-AChE to produce thiocholine. The thiocholine was detected downstream with the electrochemical detector using the Pt-Ir electrode.

2.6 *Assay procedure for the conventional ELISA method*

The conventional measurement of cortisol concentration was performed using an ELISA kit according to the manufacturer's instructions (Cayman Chemical). This test kit is based on the principles of a competitive binding assay. An ELISA 96-well microtitre plate was coated with mouse monoclonal anti-rabbit antibodies. Plasma samples and diluted standards were added to the wells of the microplate. The specific quantities of acetylcholine esterase-labelled cortisol (cort-AChE) and the anti-cortisol antibody were added to each well, respectively. The 'non-labelled' and 'labelled' cortisol competed for the limited number of binding sites of anti-cortisol antibodies overnight at 4°C. The overnight incubation in this study was the manufacturer's instructions. After the incubation period, unbound components were washed away with wash buffer comprising 0.1 M phosphate buffer (pH 7.4) and 0.05 vol% Tween-20. Ellman's Reagent containing the substrate for the esterase was then added to each well and developed for approximately 90 min. The activity of bound cort-AChE was measured by reading the absorbance at a wavelength of 405 nm using a plate reader (Multiskan JX, Thermo Scientific, Waltham, MA).

3. Results and discussion

3.1 *Typical response curve of the electrochemical detector*

To confirm the immobilisation of the anti-cortisol antibody on the magnetic microbeads, the response curve of a blank that included cort-AChE and non-immobilised magnetic microbeads was compared to the curve of a sample that included cort-AChE and magnetic microbeads containing immobilised anti-cortisol antibodies at pH 7.5, 30°C. The flow rate of each buffer solution was 170 µl min^{-1} and the ATCh concentration of the substrate buffer was 5 mmol L^{-1}. The reaction time in the reaction coil in the competitive reaction unit was 4.6 minutes when the flow rate of carrier buffer is 170 µl min^{-1}. Figure 2 shows a typical response curve of the EFIIA system. After injecting the blank solution, the output current of the system increased within 10 min, and one measurement could be completed within 20 min. This response indicated that the thiocholine hydrolysed by the AChE (Scheme 1(a)), was detected by the electrochemical oxidation reaction (Scheme 1(b)) at the sensing element of the Pt-Ir working electrode. The current increase from the baseline of the blank and the sample was termed I_0 and I_x, respectively. As shown in the figure, I_0 was larger than I_x. This difference in the response indicated that almost all of the cort-AChE in the blank solution was transferred to the enzyme reaction unit. That is, an immunologic reaction was not caused in the blank solution, because the blank contained magnetic microbeads that were not linked to the antibody. In this study, ΔI was calculated by subtracting I_x from I_0 and used as the analytical output signal of the EFIIA system.

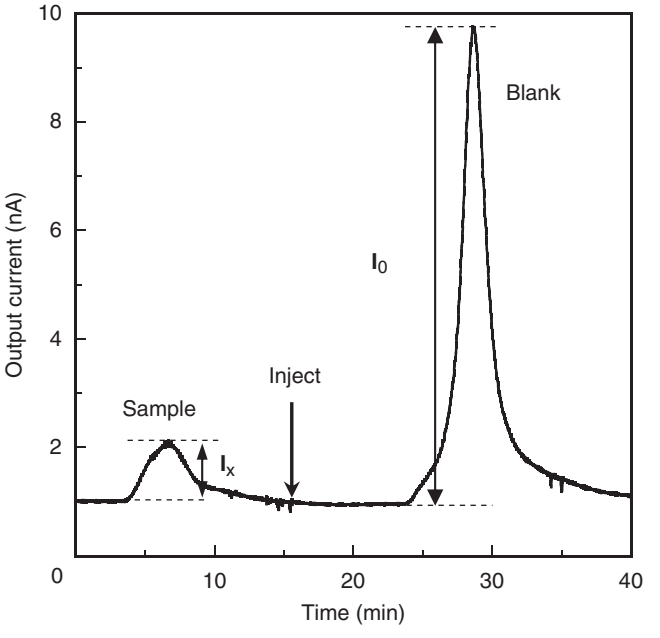
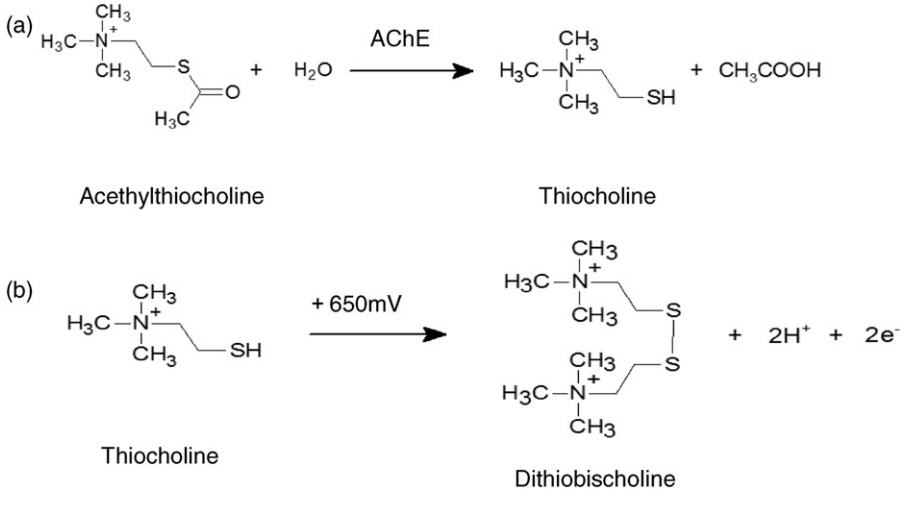


Figure 2. Typical response curves of the EFIIA system. $\mathbf{I_0}$ sensor output of blank, $\mathbf{I_x}$ sample solution containing cort-AChE and magnetic microbeads with immoblised anti-cortisol antibody.



Scheme 1. (a) ATCh hydrolysis in the enzyme reaction unit. (b) Electrochemical oxidation of thiocholine in the electrochemical detector.

3.2 *Effects of analytical conditions on detector output*

The analytical output signal (ΔI) of the EFIIA system was based on an immunologic competitive reaction and enzyme reaction. Therefore, the output current was easily influenced by analytical conditions, such as pH, temperature, flow rate, and ATCh

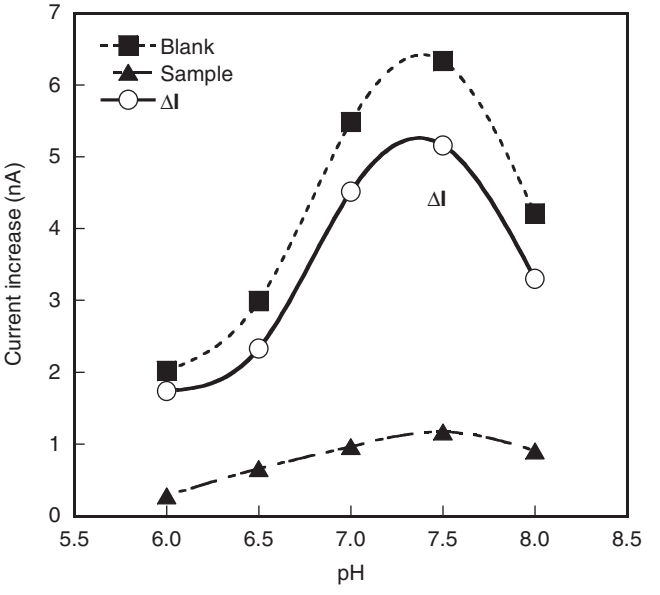


Figure 3. Effect of pH on detector output. Assay conditions were as follows: temperature 25°C, flow rate 170 µl min^{-1}, ATCh concentration in the substrate buffer 5 mmol L^{-1}.

concentration in the substrate buffer. The effects of these parameters on the output current of the EFIIA system were investigated. The effects of pH and temperature on ΔI of the EFIIA system are shown in Figures 3 and 4, respectively. The ΔI gradually increased with an increase in pH and temperature. Maximum ΔI was obtained at pH 7.5 and 25°C. The decrease in ΔI at pH 8.0 and 30°C indicated deactivation of the AChE or anti-cortisol antibody. The effect of flow rate on the output current of the EFIIA system was examined (Figure 5). The ΔI gradually decreased with an increase in flow rate. When the flow rate of the buffer was fast, the ratio of the formation of the enzyme substrate complex between the cort-AChE unbound to the separated magnetic beads and ATCh in the substrate buffer decreased, thus decreasing the generation of thiocholine. The competitive and enzyme reactions should be performed at the optimum flow rate. Therefore, the flow rate of each buffer solution was set to 170 µl min^{-1} to maintain a relatively fast measurement time. The effect of ATCh concentration in the substrate buffer on the output current of the EFIIA system is shown in Figure 6. The ΔI rapidly increased to 5 mmol L^{-1}, but there was only a small difference in ΔI at 5 mmol L^{-1} and 10 mmol L^{-1}. The optimum ATCh concentration in the substrate buffer was therefore 5 mmol L^{-1} to reduce the assay costs. Moreover, the standard deviation value ($n = 4$) of ΔI in each experiment was less than 0.04. These findings suggest that the performance of the proposed flow assay system was optimised under the following conditions: pH 7.5, temperature 25°C flow rate 170 µl min^{-1}, ATCh concentration in the substrate buffer 5 mmol L^{-1}.

3.3 *Reproducibility of the EFIIA system*

The reproducibility of the EFIIA system was evaluated (Figure 7). To characterise the reproducibility of the EFIIA system, successive injections using 7.8 pg ml^{-1} cortisol

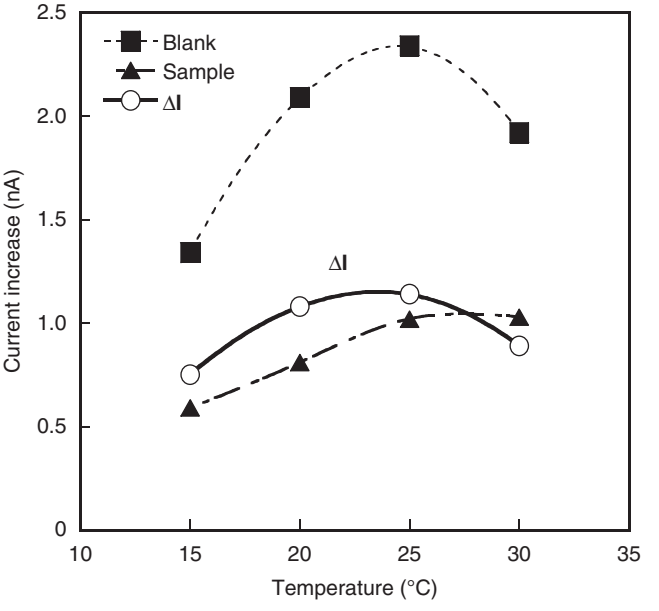


Figure 4. Effect of temperature on detector output. Assay conditions were as follows: pH 7.5, flow rate 170 μl min^{-1}, ATCh concentration in the substrate buffer 5 mmol L^{-1}.

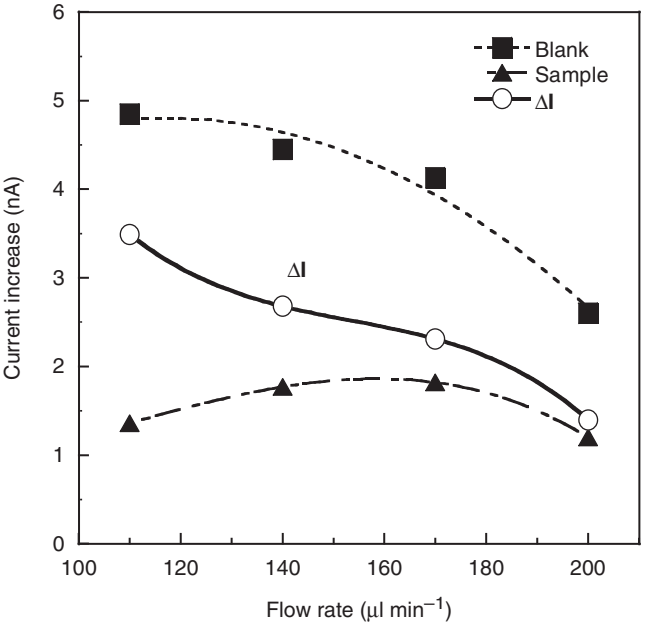


Figure 5. Effect of flow rate on detector output. Assay conditions were as follows: pH 7.5, temperature 25°C, ATCh concentration in the substrate buffer 5 mmol L^{-1}.

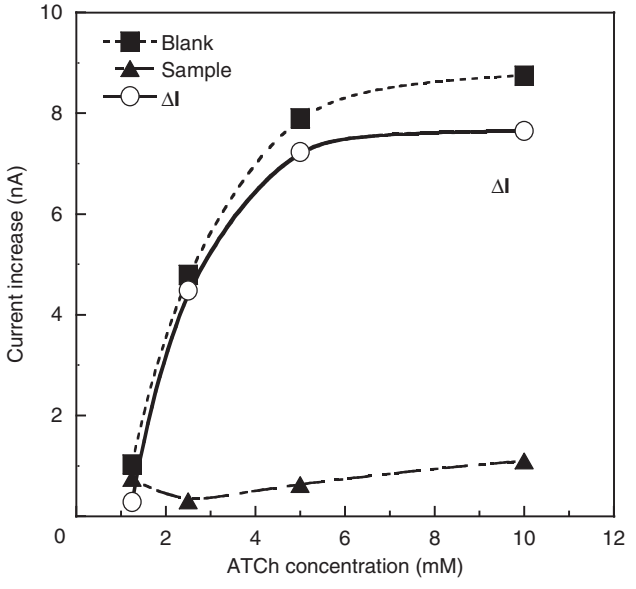


Figure 6. Effect of ATCh concentration in the substrate buffer on detector output. Assay conditions were as follows: pH 7.5, temperature 25°C, flow rate 170 µl min^{-1}.

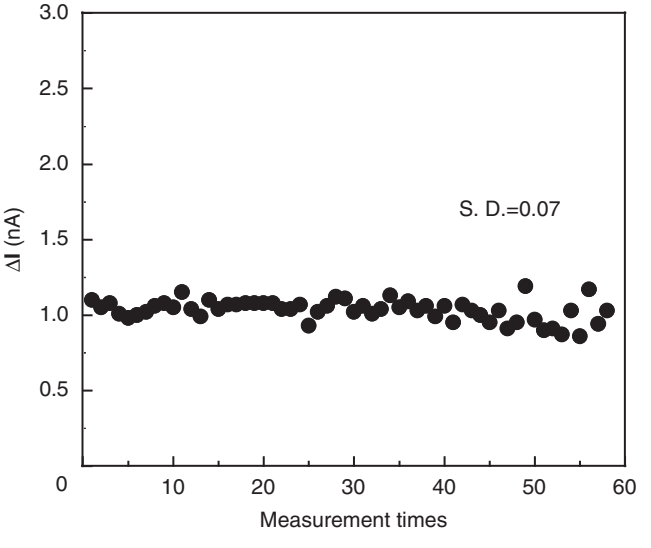


Figure 7. Reproducibility of the EFIIA system. Measurement was performed using a 7.8 pg mL^{-1} cortisol standard solution. Assay conditions were as follows: pH 7.5, temperature 25°C, flow rate 170 µl min^{-1}, ATCh concentration in the substrate buffer 5 mmol L^{-1}.

standard solutions were performed and the blank was measured before each sample was injected. Measurements were taken under the optimum conditions; pH 7.5, temperature 25°C flow rate 170 µl min^{-1}, ATCh concentration in the substrate buffer 5 mmol L^{-1}. The output current of the EFIIA system was reproducible for 58 injections, with

a standard deviation value of 0.07. After 59 injections, it became impossible to confirm the response of the EFIIA system because the baseline became unstable. This reason seems to be influence of an accumulation of magnetic microbeads in the separating tube or a deterioration of the electrochemical detector. This result indicates that sequential measurement of up to 58 injections is possible without the need to change the separating tube and the electrode. In this system, the separating tube and the electrochemical detector could be replaced with a new one each time the reproducibility decreased. After the replacement of each one, good reproducibility was obtained again. The exchange of the tube is very easy and the time required only 15 min.

3.4 *Calibration curve*

The relationship between ΔI and the concentration of the cortisol standard solution was investigated under the optimum conditions (pH: 7.4, temperature: 25°C flow rate: 170 μl min^{-1}, ATCh concentration in the substrate buffer: 5 mmol L^{-1}). As shown in Figure 8, the calibration curve for the cortisol was linear from 7.8 to 500 pg mL^{-1} ($y = 2.213 - 0.43 \times \log(x)$, $R = 0.9814$), which was almost the same as that of the conventional ELISA method. The range of fluctuation of the cortisol concentration in fish was approximately 10 to 300 ng mL^{-1}. Therefore, considering the calibration range of proposed method, it is suitable that the dilution rate of actual plasma sample is 1/100.

3.5 *Application of the EFIIA system of actual fish plasma samples and pseudo samples*

To evaluate the potential use of the EFIIA system for actual samples, the system was applied to determine the cortisol concentration in fish plasma (Nile tilapia) and was

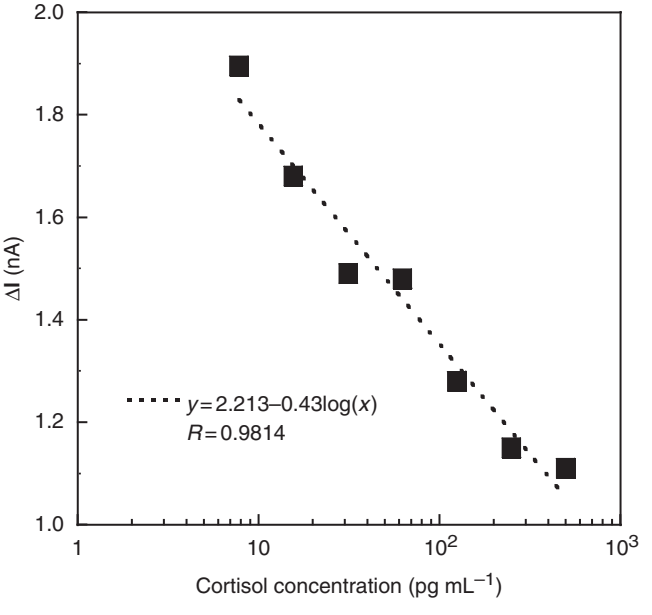


Figure 8. Relationship between analytical output signal of the EFIIA system and cortisol concentration in standard solutions.

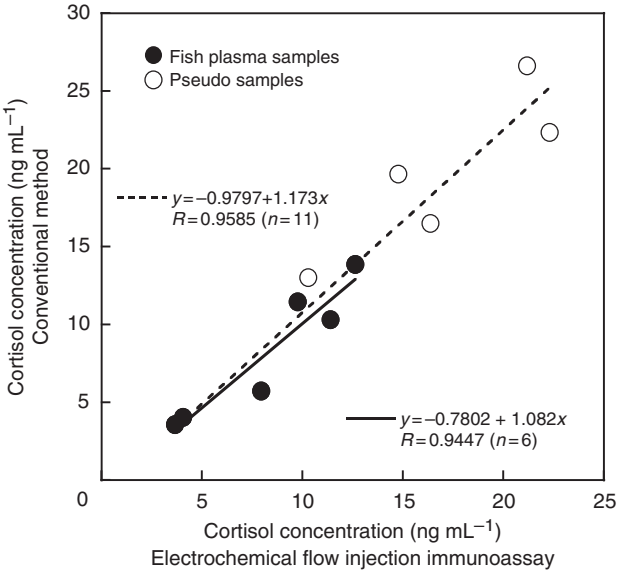


Figure 9. Correlation between the values determined using the EFIIA system and conventional methods.

compared with the conventional method (ELISA). A large number of samples were needed to expand the detection range to examine the correlation between the proposed method and ELISA. Therefore, each pseudo sample was made by adding 10 µl standard cortisol (1000 pg mL^{-1}) to 190 µl of a 1/100 diluted plasma sample from five fish except for one fish from which it was not possible to gather a lot of blood. The amount of cortisol in six actual fish plasma samples and five pseudo samples was determined using the EFIIA system, and calculated from the calibration curve shown in Figure 8. We examined each correlation of total sample data (including plasma samples data and that of pseudo samples, $n = 11$) and plasma sample data ($n = 6$) that obtained using the proposed method and the conventional ELISA method (Figure 9). In this experiment, each actual sample was diluted 1/100 using buffer before each measurement was performed, because the calibration curve for the cortisol obtained by the proposed method and ELISA method was linear in the range of 'pg mL^{-1}'. In Figure 9, therefore, each result of a measurement was shown as a corresponding value (ng mL^{-1}), which is the value of the sample before dilution. The values determined using the proposed flow assay were linearly correlated with those obtained using the conventional ELISA (Figure 9). The correlation coefficient of the total data was 0.9585 [$y = -0.9797 + 1.173(x)$, $n = 11$], and that of the plasma sample data was 0.9447 [$y = -0.7802 + 1.082(x)$, $n = 6$]. This result indicated that the effects of the proteins and other contaminants found in real plasma samples are minimised by diluting the plasma samples 1/100, and suggest that the EFIIA system can be used to analyse cortisol concentrations in fish plasma samples.

4. Conclusion

The present findings confirm that cortisol concentrations in actual fish plasma can be rapidly and conveniently measured using an electrochemical flow injection system.

The optimum analytical condition for the EFIIA system was pH 7.5, temperature 25°C flow rate 170 µl min^{-1}, ATCh concentration in the substrate buffer 5 mmol L^{-1}. The calibration curve for cortisol was linear in the range from 7.8 to 500 pg mL^{-1}. Each assay could be completed within 20 min. Good reproducibility of the EFIIA system was obtained in 58 assays at optimum conditions without the need to change the separating tube and the electrochemical detector. When the EFIIA system was applied to determine cortisol concentrations in fish plasma samples, there was a good correlation ($R = 0.9447$) between the values determined using the proposed system and those obtained using the conventional ELISA-based method. The conventional ELISA method required long incubation times (several hours or more) and a degree of washing process. In the case of our proposed method, the washing process is not necessary. In addition, the competitive reaction and the enzyme reaction can be performed within only 20 min. Preparation of the magnetic bead immobilised antibody, which requires overnight incubation, was not included in the assay time, because it is possible to keep the modified magnetic beads stable at 4°C for 2 weeks. Therefore, the proposed method is not a 2 day assay. To miniaturise the sensor and automate the injection sample for the EFIIA system, further studies are required to enable even more rapid determination.

Acknowledgements

This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology.

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