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Assays for serum cholinesterase activity by capillary electrophoresis and an amperometric flow injection choline biosensor

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ABSTRACT

A capillary electrophoresis method and a durable choline biosensor were developed for measuring serum cholinesterase (EC 3.1.1.8) activity, a useful clinical index for liver function. The former is based on separation of benzoate and benzoylcholine (the artificial substrate of cholinesterase) in an uncoated fused-silica capillary. The migration time of benzoylcholine and benzoate was 1.3 min and 5.5 min, respectively. By the peak areas of A_{233} signals, the linear dynamic ranges for both analytes were 0.01–50.0 mM, and the relative standard deviations of 1.0 mM benzoylcholine and benzoate were less than 4% and 6%, respectively.

The FIA-choline sensor was constructed with the working electrode of the flow cell covered with a natural chitinous membrane purified from Taiwanese soldier crab, *Mictyris brevidactylus*. The biomembrane served as the supporting material for enzyme immobilization (choline oxidase, EC 1.1.3.17), and also prevented protein adsorption on the electrode surface. The calibration curve was linear between 0.05 and 5.0 mM ($r = 0.999$). The relative standard deviations for 1.0 mM choline ($n = 7$) were less than 3%, and the activity of the bioactive membrane lasted for about 2 months. The analytical results of both methods correlated well ($r = 0.940$).

1. Introduction

Serum cholinesterase (EC 3.1.1.8, SChE) is synthesized mainly in liver, and its level is prone to decline for patients with hepatic diseases such as liver metastasis, chronic hepatitis and liver cirrhosis. On the contrary, serum cholinesterase activity tends to increase for patients with fatty liver [1,2]. The enzyme is relatively non-specific for several choline esters to release choline and the constituent acids. For the enzyme assay, the UV-absorbing substrate, benzoylcholine, is frequently used, but the absorption spectra of benzoylcholine and its hydrolysate (benzoate) are overlapped and cannot be resolved without a separation process such as HPLC. Therefore, thiocholine esters were introduced as the chromogenic substrates for direct quantification using Ellman reaction [3–7]. Although the chromogenic approach was extensively investigated, the complex reaction pathway of the Ellman reaction is easily affected by coexisting reducing substances in the serum. This seriously restricted its practical biomedical applications. Recently, benzoate-selective membrane electrodes were attempted to avoid the mentioned problems, but the signals suffered the interference from some physiological anions such as chloride [8,9].

Consequently, we propose here with a simple and rapid separation-based method and a durable biosensor using the most frequently used substrate, benzoylcholine, for routine clinical assay for serum cholinesterase activity. The former is based on capillary electrophoresis (CE) to simultaneously determine both benzoylcholine and benzoate in a single run of electrophoretic separation. The latter used an amperometric

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flow injection biosensor with the analytical enzyme immobilized onto a natural chitinous membrane which showed several advantages in clinical analysis. The analytical results obtained by both methods were compared.

2. Experimental

2.1. Reagents and materials

Buffers were prepared from sodium tetraborate ($Na_2B_4O_7$), hydrochloric acid, sodium hydroxide, sodium phosphate: monobasic (NaH_2PO_4) and dibasic (Na_2HPO_4), acetic acid and trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), all these chemicals were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Glutaraldehyde was also from Nacalai Tesque, Inc. as 25% (w/v) aqueous solution and was stored at 4 °C. Benzoylcholine chloride (purity: >98%), benzoate (purity: >99%) and choline chloride (purity: >98%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Choline oxidase (ChOx, EC 1.1.3.17 from *Alcaligenes*, 14 U mg^{-1} solid) was also from Wako Pure Chemical Industries, Ltd. and was stored at −20 °C. Potassium ferrocyanide ($K_4[Fe(CN)_6]$) was from Katayama Chemical Industries Co., Ltd. (Osaka, Japan). Human serum albumin (HSA) was from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Deionized water with conductivity less than 1.0 μS cm^{-1} was used for sample dilution and reagent preparation. Other chemicals were of analytical grade and used as received.

The chitinous membranes from Taiwanese soldier crabs, *Mictyris brevidactylus*, were purified as described previously [10] with deacetylation degree of ca. 25% [11].

2.2. Spectrophotometric apparatus

UV–vis spectra were measured with a double beam spectrophotometer (V-530, Jasco, Tokyo, Japan).

2.3. Hydrolysis of benzoylcholine by serum cholinesterase from human serum

Human blood was withdrawn from 12 healthy volunteers in National Taiwan University (Taipei, Taiwan). The whole blood samples were centrifuged at 3000 rpm (1500 g) for 10 min, and the supernatants (the serum) were stored at −20 °C until use. Prior to the experiment, 1.0 mM benzoylcholine solution was prepared by diluting a stock solution with phosphate electrolyte (30.0 mM, pH 7.0) to a final volume of 3.0 mL. After adding 10 μL of serum sample (diluting ratio: 1:300) into the benzoylcholine solution, 30 min of incubation was carried out at a constant temperature, 37 °C [12]. After the incubation, the mixtures in test tubes were chilled in an ice bath. A unit activity of serum cholinesterase (U) is defined as the amount of enzyme capable of hydrolyzing 1.0 μmol of benzoylcholine per min at 37 °C [13].

2.4. Capillary electrophoresis

CE was carried out using a commercialized system (G1600A, Agilent, MA, USA) equipped with an uncoated fused-silica capillary (34 cm × 75 μm i.d., Beckman Coulter, CA, USA). The software (Chemstation™, Agilent, MA, USA) was used for solution handling, separation, data processing and temperature control (25 °C). Detection was on-line by direct UV absorbance. The effective capillary length (from inlet to the detector) was 26.0 cm. New capillaries were first rinsed sequentially with 1.0 M NaOH (950 mbar × 50 min), deionized water (950 mbar × 20 min) and the buffer electrolyte (950 mbar × 50 min) before the following separation sequence. After flushing (950 mbar) with the buffer electrolyte for 6 min, sample was injected (30 mbar × 2 s), separated and monitored with a PDA detector. The capillary was then cleaned (950 mbar) sequentially with 1.0 M NaOH (3 min) and deionized water (3 min) after each determination.

2.5. Choline biosensor

An amperometric flow injection biosensor was constructed for choline determination as described previously [14]. This system consisted of a controllable peristaltic pump (SMP-23S, RIKAKIKAI Co., Tokyo, Japan), a low pressure injection valve (Model 5020, RHEODYNE, USA), a home-made amperometric flow cell, a potentiostat (CV-1, BAS, IN, USA) and a chart-pen recorder (101 A-1875, Cole-Parmer, IL, USA). The home-made amperometric flow cell was assembled with a platinum working electrode (14.0 mm^2), an Ag/AgCl reference electrode (RE-3V, BAS, IN, USA) and a stainless outlet-pipe (1.0 mm i.d. tube for HPLC) as the counter electrode. The platinum working electrode was covered with a chitinous membrane purified from Taiwanese soldier crab [10,15], and choline oxidase (ChOx, 14 U) was covalently immobilized on the surface of chitinous membrane [14].

2.6. Cyclic voltammetry and electrochemical impedance spectroscopy

Cyclic voltammetry (CV) was measured with GPES-module of Autolab PGSTAT-30, Eco Chemie, Utrecht, Netherlands; and electrochemical impedance spectroscopy (EIS) was measured with FRA-module of the same machine. Both measurements (CV and EIS) were carried out in 0.2 M phosphate buffer (pH 8.0) containing 0.01 M of $K_4[Fe(CN)_6]$ with/without 10 mg mL^{-1} HSA. The three-electrode electrochemical cell consisted of a platinum working electrode (CHI102, CH Instruments, Inc., TX, USA; 2.0 mm in diameter) mounted with the chitinous membrane from soldier crab, an Ag/AgCl reference electrode (RE-4, BAS, IN, USA) and a platinum wire auxiliary electrode (0.5 mm in diameter).

Twenty cycles of linear cyclic voltage scan (between −0.3 V and +0.7 V at a scan rate of 50 mV s^{-1}) were conducted to stabilize the electrode surface, and the subsequent 10 scans were averaged to obtain a CV curve. EIS measurements were then followed in the same ferrocyanide–phosphate buffer solution from 100 kHz to 0.1 Hz at the formal potential of $[Fe(CN)_6]^{3-/4-}$ redox pair system (+0.3 V versus Ag/AgCl), the amplitude of the sinusoidal potential input was set to be 10 mV.

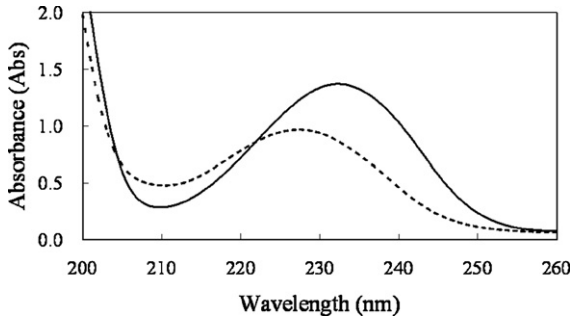

Fig. 1 – UV absorption spectra of 1.0 mM benzoylcholine (continuous line) and 1.0 mM benzoate (broken line) in 30 mM phosphate buffer, pH 7.0.

3. Results and discussion

3.1. *Optimization of CE parameters for the separation and detection of benzoylcholine and benzoate*

During the incubation procedure described in Section 2.3, benzoylcholine (BzCh) was hydrolyzed by the action of SChE existed in serum sample (Eq. (1)):

$$\text{benzoylcholine} + H_2O \xrightarrow{\text{SChE}} \text{benzoate} + \text{choline} \quad (1)$$

Since UV–vis absorption of choline molecule is negligible, photometrical quantification of the enzymatic reaction will depend on the depletion of benzoylcholine or the production of benzoate molecules. As the spectra shown in Fig. 1, the UV absorption of benzoylcholine and benzoate are overlapped with the λ_{max} at 233 nm and 228 nm, respectively. For the enzyme assay, A_{233} was selected for simultaneous determination of both the substrate (BzCh) and the product (BA); the extinction coefficient of benzoate at 233 nm was approximately 90% of its maximal value at 228 nm.

With separation voltage of 12 kV, the effects of pH on migration time and peak area were investigated using different buffer systems [16–18]: 20 mM acetate–tris buffer for pH 5.0–7.0, 20 mM phosphate buffer for pH 6.0–8.0 and 20 mM borate buffer for pH 9.0–11.0. The lower the pH, the longer the migration time and the poorer the peak symmetry, which led to a poor peak area reproducibility at acidic pH (RSD = 10% for benzoylcholine, RSD = 24% for benzoate at pH 5.0 in acetate–tris buffer, $n = 7$). However, in alkaline pH, the increased electroosmotic flow rate resulted in smaller peak area and the lower sensitivity. As the consequence, pH 7.0 phosphate buffer electrolyte was selected for the following experiments (RSD = 6% for benzoylcholine, RSD = 8% for benzoate, $n = 7$).

Typical buffer electrolyte concentrations for CE analyses are 10–50 mM; low electrolyte concentration may lead to adsorption of solutes on the capillary wall, particularly proteins [19]. As the concentration of phosphate buffer (pH 7.0) was changed from 10 mM to 50 mM, an increase in migration times and separation efficiency were obtained. The difference of migration times between benzoylcholine and benzoate increased from 3.32 min (10 mM) to 13.24 min (50 mM). Considering separation efficiency, analysis time and Joule heating

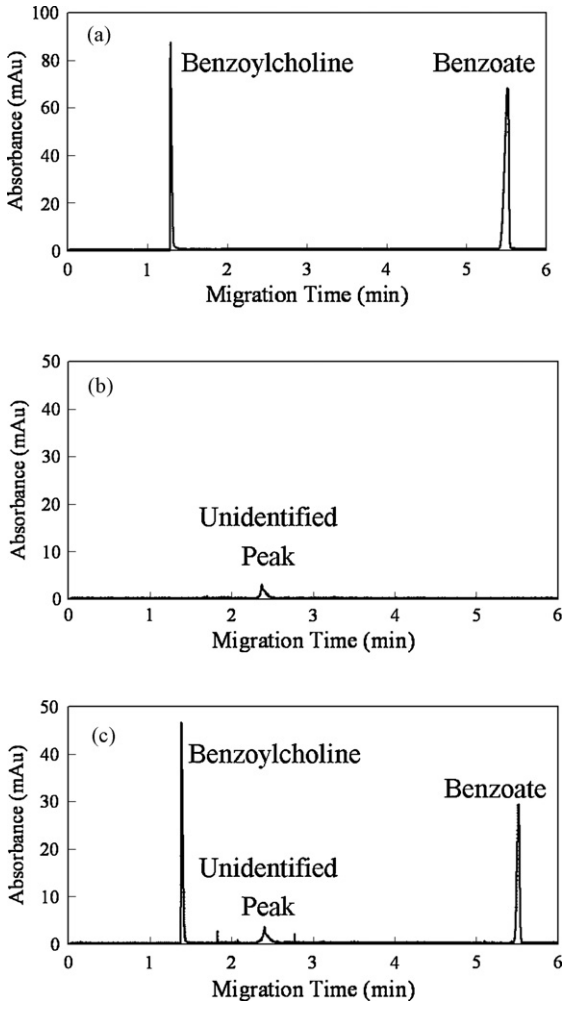

Fig. 2 – Electropherogram of (a) 1.0 mM benzoylcholine and 1.0 mM benzoate, (b) 300-fold diluted serum sample, (c) the reaction mixture of benzoylcholine and the diluted serum sample, the enzymatic reaction was performed as the description in the experimental section. Separation (16 kV, 25 °C) was performed with 30 mM phosphate buffer electrolyte, pH 7.0; the detection wavelength was 233 nm.

problem, 30 mM was chosen as the suitable concentration of phosphate buffer.

Joule heating inside the capillary may result in decreasing the viscosity of the carrier buffer and a distortion of electrophoretic profile, which will hamper the separation and quantification [20]. Deviation from Ohmic behavior (the linearity of I/V curve) was found with imposing voltage higher than 18 kV. Therefore, it is not adequate to work with a voltage higher than 18 kV under the aforementioned optimal conditions (pH 7.0, 30 mM phosphate buffer, 25 °C). For a higher theoretical plate number and shorter migration time, 16 kV was selected.

With the optimized separation and detection conditions for benzoylcholine and benzoate (30.0 mM phosphate buffer electrolyte, pH 7.0; 16 kV; 233 nm; 25 °C), a stable electrophoretic current (45 μA) and the electropherogram (Fig. 2a) was obtained by injecting a standard solution containing

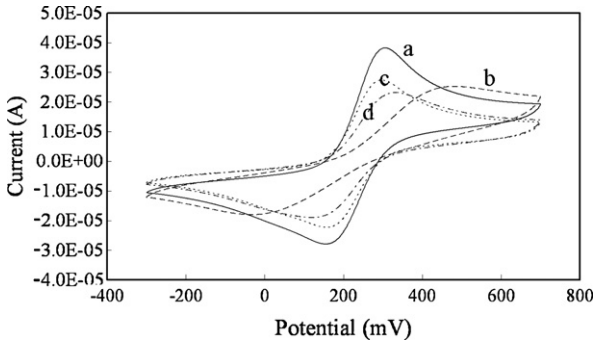

Fig. 3 – Cyclic voltammograms (scan rate: 50 mV s^{-1}) of 0.01 M $K_4[Fe(CN)_6]$ solution (phosphate buffer pH 8.0): (a) with bare platinum electrode; (b) with bare platinum electrode, and the solution contained 10 mg mL^{-1} human serum albumin (HSA); (c) with chitinous membrane-mounted platinum electrode; (d) with chitinous membrane-mounted platinum electrode, and the solution contained 10 mg mL^{-1} HSA. Each curve is the average of 10 scans.

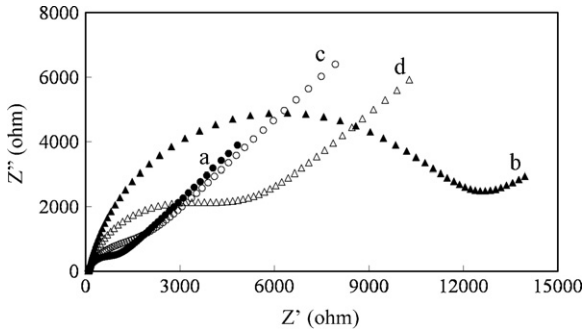

Fig. 4 – Complex plane impedance plots of 0.01 M $K_4[Fe(CN)_6]$ solution (phosphate buffer pH 8.0). Each plot was measured immediately after finishing the 10 cyclic scans conducted in Fig. 3. The curve notations are the same as Fig. 3. Applied voltage: +0.3 V; amplitude: 10 mV; frequency range: 100 kHz–0.1 Hz.

1.0 mM benzoylcholine and 1.0 mM benzoate (dissolved in 30 mM, pH 7.0 phosphate buffer). Sufficient separation with satisfactory peak symmetry was achieved in less than 6 min. The calibration curve for benzoylcholine and benzoate passed through the origin with good linearity ($R^2 = 0.998$ for benzoylcholine, $R^2 = 0.993$ for benzoate). The linear ranges were 0.01 mM to 50.0 mM for both benzoylcholine and benzoate; the RSD of the peak areas ($n = 10$) for 1.0 mM benzoylcholine and benzoate was less than 4% and 6%, respectively. The detection limits for both benzoylcholine and benzoate were 1.0 μM with the S/N ratio higher than 3. More than 1000 analysis could be performed without the need to replace the capillary.

3.2. *The advantages of natural chitinous membrane in electrochemical clinical analysis and the analytical performance of FIA-choline biosensor*

Electrode fouling by non-specific adsorption of serum proteins is a serious problem in electrochemical analysis; preliminary studies were therefore conducted using HSA as the model serum protein. Fig. 3 compares the CV curves of $K_4[Fe(CN)_6]$ taken with a bare electrode (curves a and b) and the chitinous membrane-mounted electrode (curves c and d). The faradaic current was seriously disturbed by the existence of serum albumin (curve b). After 10 scans of CV, the electrochemical impedances were measured and analyzed in the same solution system (Fig. 4, Table 1). As revealed by the dramatic increase in charge transfer resistance (R_{ct}), the non-specific HSA adsorption did impede the faradaic pathway of the electrode process.

With the electrode mounted with chitinous membrane (curve c and d in Figs. 3 and 4), the aforementioned interference was extensively reduced, so was the analytical results in Table 1 (the R_{ct} values of curves c and d). This electrode protection phenomenon may attribute to the electrostatic interaction between positively charged amino groups on the surface of chitinous membrane and negatively charged HSA particles (the iso-electric pH of HSA is 4.7 [21]). The problematic protein adsorption on the chitinous membrane can be effectively removed by washing with deionized water or an electrochemical cleaning process (linear scan between −0.3 V and +0.7 V). The protein adsorption problem will be further reduced if the following flow-injection strategy is adopted.

Sample solution containing choline was introduced into the carrier stream (0.2 M phosphate buffer, pH 8.0, flow rate: 1.5 mL min^{-1}) *via* the sample loop (ca. 20 μL) of the injection valve. Choline was oxidized with the catalysis of the immobilized choline oxidase (ChOx, Eq. (2)) on the chitinous membrane, and the evolved H_2O_2 was amperometrically monitored with the positively polarized (0.6 V versus Ag/AgCl) platinum working electrode [14]. The second substrate in the enzymatic reaction (oxygen, Eq. (2)) was in excessive amounts as dissolved in the carrier stream, which supported a pseudo-first order kinetic for choline as the previous report [14].

$$\text{Choline} + 2O_2 \xrightarrow{\text{ChOx}} \text{betaine} + 2H_2O_2 \quad (2)$$

Typical FIAgram was demonstrated in Fig. 5 with a satisfactory reproducibility ($n = 10$, CV of peak height <3%). The

Table 1 – Impedance analysis based on Randies equivalent electrical circuit of electrode process

Curve	R_s[a] (kΩ)	C_{dl}[b] (μF)	R_{ct}[c] (kΩ)	Z_W[d] (mΩ)
a	0.13	2.21	0.71	0.22
b	0.21	2.45	10.23	0.24
c	0.14	2.36	1.23	0.13
d	0.18	2.27	3.83	0.14

[a] R_s: solution resistance.
[b] C_{dl}: electric double layer capacitance.
[c] R_{ct}: charge transfer resistance.
[d] Z_W: Warburg impedance.
Each datum is the average of three repeated experiments with a standard deviation <3%. Curves a–d refer to those in Fig. 4.

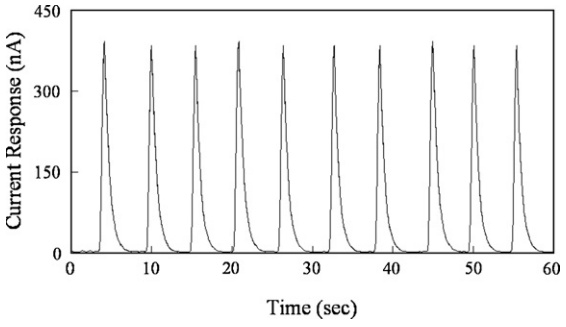

Fig. 5 – Typical FIAgram obtained by injecting 1.0 mM choline solution (20 μL) into the carrier stream (0.2 M phosphate buffer, pH 8.0; flow rate: 1.5 mL min^{-1}). The bias potential applied to platinum working electrode was +0.6 V versus Ag/AgCl reference electrode.

linear dynamic range for choline quantification was up to 5.0 mM with a correlation coefficient of 0.999, the detection limit was estimated to be 10.0 μM with a signal-to-noise ratio higher than 3. With 1.5 mL min^{-1} flow rate, the peak reversion time was 3 s and the sample throughput was ca. 10 determination min^{-1}. Typically, the biosensor could be used for at least 2 months.

3.3. *Measuring serum cholinesterase activity by CE and FIA-biosensor*

For applying CE in clinical analysis, cares should be taken to avoid the adsorption of proteins and the other biomolecules in the serum matrix on the negatively charged capillary wall. We found that a simple dilution process worked well as the sample pretreatment procedure, and a hundreds-fold dilution of serum was enough to avoid the adverse matrix effects with sufficient signals of benzoylcholine and benzoate.

Fig. 2b shows the electropherogram of 300-fold diluted serum. From the spectral information of the built-in PDA detector of the CE system, the unidentified peak might be attributed to the protein-like materials in the sample matrix. However, the separation can fully resolve the unwanted signal

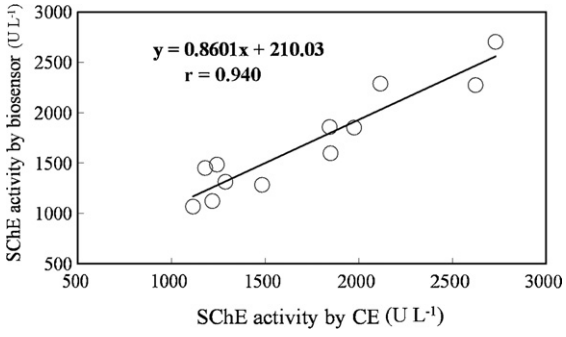

Fig. 6 – Comparison of the enzyme assay results obtained by the CE approach and those with amperometric FIA-choline biosensor. The average of five repeated experiments was taken for each datum. For every data point, the relative standard deviations of CE and biosensor were less than 8% and 6%, respectively.

(Fig. 2c, unidentified peak). The relative standard deviations for migration time and peak area were less than 1% and 10%, respectively ($n = 7$).

Finally, the serum cholinesterase (SChE) activities of 12 human serum samples were determined separately by the CE approach and the amperometric FIA-choline biosensor, the reliability of the sensor approach was confirmed by the separation-based analysis (Fig. 6). The healthy range of SChE activity was reported to be within 1130–1960 U L^{-1}; the SChE activity of fatty liver patients may reach to a 50% increase while patients with acute hepatitis can lead to a 50% drop [1,2,7,9]. The dynamic ranges of both the CE and biosensor methods are sufficient for diagnostic purposes.

4. Conclusions

Our group developed two promising serum cholinesterase activity assays based on CE separation technique and a durable biosensing system. The enzyme activities of serum samples could be directly determined after simple dilution process. Both methods are highly selective, rapid and simple for use in routine clinical diagnosis. Our recent experiments revealed additional encouraging bio-analytical benefits of the natural chitinous membrane from soldier crab; the membrane can act as a sizing barrier to repel the interference from blood cells. The possibility of whole blood assay is currently under investigations.

REFERENCES

[1] M.J. McQueen, Clin. Chim. Acta 237 (1995) 91.
[2] L. Davis, J.J. Britten, M. Morgan, Anaesthesia 52 (1997) 244.
[3] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, Biochem. Pharmacol. 7 (1961) 88.
[4] J.J. Ceron, F. Tecles, J.C. Espin, Res. Vet. Sci. 67 (1999) 261.
[5] M. Yamada, Y. Marui, C. Hayashi, Y. Miki, S. Takemura, Clin. Chem. 47 (2001) 1962.
[6] T. Furlanello, G. Simonato, M. Caldin, D. DeLorenzi, G. Lubas, D. Bernardini, L. Solano-Gallego, Vet. Res. Commun. 30 (2006) 723.
[7] S. Osawa, K. Kariyone, F. Ichihara, K. Arai, N. Takagasa, H. Ito, Clin. Chim. Acta 351 (2005) 65.
[8] T. Katsu, T. Kayamoto, Anal. Chim. Acta 254 (1991) 95.
[9] T. Katsu, N. Hanada, Anal. Chim. Acta 321 (1996) 21.
[10] B.C. Hsieh, T.J. Cheng, T.Y. Wang, R.L.C. Chen, Mar. Biotechnol. 5 (2003) 119.
[11] H.Y. Hsiao, C.C. Tsai, S.M. Chen, B.C. Hsieh, R.L.C. Chen, Macromol. Biosci. 4 (2004) 919.
[12] A. Mosca, R. Bonora, F. Ceriotti, C. Franzini, G. Lando, M.C. Patross, M. Zaninotto, M. Panteghini, Clin. Chem. Lab. Med. 41 (2003) 317.
[13] M. Kondo, T. Hada, K. Fukui, A. Iwasaki, K. Higashino, K. Yasukawa, Clin. Chim. Acta 243 (1995) 1.
[14] B.C. Hsieh, K. Matsumoto, T.J. Cheng, G. Yuu, R.L.C. Chen, J. Pharm. Biomed. 45 (2007) 673.
[15] P.C. Chen, B.C. Hsieh, R.L.C. Chen, T.Y. Wang, H.Y. Hsiao, T.J. Cheng, Bioelectrochemistry 68 (2006) 72.
[16] J.J. Chen, Y.C. Lee, H.C. Lin, R.L.C. Chen, J. Food Drug Anal. 4 (2004) 332.

[17] M. Chiari, N. Dell'Orto, L. Casella, J. Chromatogr. A 745 (1996) 93.
[18] M.A. Kelly, K.D. Altria, B.J. Clark, J. Chromatogr. A 768 (1997) 73.
[19] B.Y. Gong, J.W. Ho, Electrophoresis 18 (1997) 732.
[20] R.J. Nelson, A. Pailus, A.S. Cohen, A. Guttman, B.L. Karger, J. Chromatogr. 480 (1989) 111.
[21] L.B. Avalle, O.R. Camara, F.Y. Oliva, J. Electroanal. Chem. 585 (2005) 281.