Journal of Applied Pharmaceutical Science Vol. 8(04), pp 007-014, April, 2018
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2018.8402
ISSN 2231-3354

Application of capillary electrophoresis coupling with electrochemiluminescence detection to estimate activity of butyrylcholinesterase

Dongdong Wang, Fanlin Li, Ming Su*, Hanwen Sun

College of Chemistry and Environmental Science, Hebei University, Key Laboratory of Analytical Science and Technology of Hebei Province, Baoding, 071002, China.

ARTICLE INFO

Article history:
Received on: 08/03/2016
Accepted on: 07/04/2017
Available online: 29/04/2018

Key words:
Capillary electrophoresis, electrochemiluminescence, butyrylcholinesterase, enzyme activity.

ABSTRACT

A new method to estimate the butyrylcholinesterase (BChE, EC 3.1.1.8) activity by using capillary electrophoresis (CE) coupled with electrochemiluminescence (ECL) is described. The liberated N,N-dimethylethanolamine (DMEA) that is produced by BChE catalyzed hydrolysis reaction of tetracine hydrochloride was used as ECL coreagent to enhance $Ru(bpy)_3^{2+}$ ECL signals efficiently. The detection limit for DMEA was 1.98×10^{-8} M (S/N = 3), which is equal to 6.6×10^{-10} units of BChE used to catalyze substrate for 30 mins. The Michaelis constant K_m (1.16×10^{-3} mol/L) and the maximum reaction velocity V_{max} (2.71×10^{-7} mol/L/min) of BChE for tetracine hydrochloride are determined. Also, the reaction conditions including the concentration of metal ions, incubation temperature and pH were optimized. This method was successfully applied to detect BChE activity as a bio-marker in plasma and the results were in good agreement with that obtained by the clinical method.

INTRODUCTION

Butyrylcholinesterase (EC 3.1.1.8; BChE), also known as pseudocholinesterase, is a serine hydrolase with α/β hydrolase fold that hydrolyzes chemicals containing ester bonds (Fogle *et al*., 2016). The BChE has unique enzymatic properties and is widely distributed in almost all mammalian tissues, such as kidneys, intestine, lung and central nervous system, also localized on the red blood cell surface (Gorelick, 1997; Gómez *et al*., 2000). However, the 440 KDa tetrameric glycoprotein is mainly synthesized in the liver and is released into plasma immediately upon synthesis (Saxena *et al*., 1997). Usually, the enzyme is found predominantly in plasma (Carmona *et al*., 2000; Peters *et al*., 2012). The BChE has a few known functions in the body, and it is thought to be primarily responsible for detoxification reactions in the serum, liver, lungs, and intestinal mucosa, mainly acting as an endogenous bioscavenger for defensing against toxic compounds reaching the bloodstream (Ashani, 2000). A deficiency of BChE can result in delayed metabolism of various drugs, such as cocaine (Lynch *et al*., 1997), and treatment with doses of BChE can help in overcoming the physiological reaction to them. Moreover, as an activator enzyme, BChE converts administered prodrugs into functional therapeutics. However, the exact physiological function of BChE remains elusive (Johnson and Moore, 2012). According to the literature, the number of BChE molecules is about ten times more than the number of AChE which is a sister enzyme essential for functioning of the nervous system in the human body, BChE represents 0.1% of human plasma proteins with its concentration of 2-5 mg/L (Manoharan *et al*., 2007; Brimijoin and Hammond, 1988). So, the BChE is also called "plasma cholinesterase" and is generally used in reference to a clinical test that reflects levels of this enzyme in the blood (Peters *et al*., 2012). Clinical and laboratory blood test for the monitoring of butyrylcholinesterase activity in plasma can be

*Corresponding Author
Ming Su, College of Chemistry and Environmental Science, Hebei University, Key Laboratory of Analytical Science and Technology of Hebei Province, Baoding, 071002, China. E-mail: suminghbu @ 126.com

used as a liver function test (Buetis, 1999). The alterations of its activity have been associated with several pathological disorders of inflammatory disease (Tvarijonaviciute *et al*., 2013), liver injury (Blanloeil and Delaroche, 1996), including hepatitis, cirrhosis and hepatic carcinoma, and its inhibition is taken as a biomarker for indicating pathological processes.

So far, the spectrophotometric method of Ellman is widely used to estimate butyrylcholinesterase activity (Waiskopf *et al*., 2011; Lucić *et al*., 2005; Ellman *et al*., 1961), using acetylthiocholine or butyrylthiocholine iodide as substrate. In this assay, the thiocholine that is produced enzymatically is coupled with Ellman's reagent to produce a coloured azo complex, whose absorbance is measured at 412 nm by UV-Visible spectrophotometry. The absorption intensity is proportional to the activity of BChE. However, the chromogenic reagent has poor chemical stability, enormous toxicity, carcinogenicity, and the sensitivity of UV detection is also limited.

CE in combination with ECL had been widely used, and mainly focused on the amine analysis (Li and Lee, 2007; Sreedhar *et al*., 2005), drug analysis (Deng *et al*., 2011; Yuan *et al*., 2010), monitoring the metabolism of drugs (Liu *et al*., 2010; Wang *et al*., 2011). However, only a few papers (Li *et al*., 2006; Yuan *et al*., 2006; Yuan *et al*., 2007; Su *et al*., 2013) about determination of bioactive enzymes using CE-ECL have been published. Herein, the CE-ECL method was successfully applied to detect BChE activity. Considering that BChE has high catalytic activity on ester bonds and is clinically important because of hydrolysis of the ester-type local anaesthetics, tetracine hydrochloride, containing both ester bond and a tertiary amine functional groups was chosen as a substrate for the enzyme catalyzed reactions. During the incubation procedure tetracine hydrochloride was hydrolyzed by the action of BChE, as shown in Fig. 1.

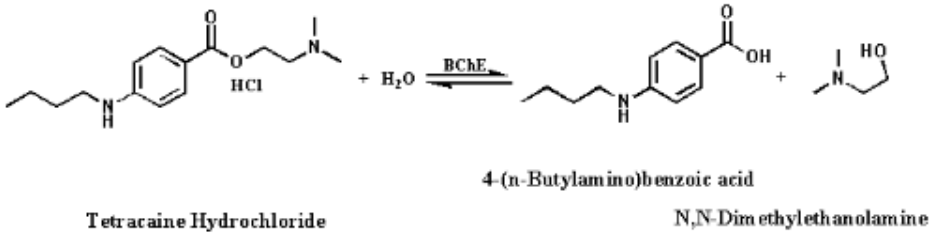

Fig. 1: Catalytic reaction of tetracine hydrochloride by butyrylcholinesterase(BChE).

Since the ECL signals of 4-(n-Butylamino)benzoic acid molecule are negligible, luminescent quantification of the enzymatic reaction will depend on the depletion of tetracine hydrochloride or the production of DMEA molecules. Finally, DMEA was chosen as quantitative probe because it enhanced $Ru(bpy)_3^{2+}$ ECL response significantly than tetracine hydrochloride. Several factors such as metal ions, pH, incubation temperature and time on the enzymatic catalysis reaction against tetracine hydrochloride were studied. K_m and V_{max} of BChE for tetracine hydrochloride were also studied. Monitoring of plasma butyrylcholinesterase should be recommended for patients with liver tissue injury as this may be a useful biomarker to predict and prevent health hazards of liver disease. The method was successfully used to assay BChE activity in plasma. Therefore this method can be used in the clinical diagnosis of relevant diseases.

MATERIALS AND METHODS

Chemicals and reagents

All reagents used were of analytical grade. Double-distilled water was used throughout the study. Tris(2,2′-bipyridyl) ruthenium(II) chloride hexahydrate ($Ru(bpy)_3Cl_2.6H_2O$, 99.95%), tetracine hydrochloride (99.5%), N,N-dimethylethanolamine (99.5%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Butyrylcholinesterase (10 untis/mg protein, from equine serm EC 3.1.1.8) was also from Sigma-Aldrich Co. and was stored at −20°C. Enzyme standard solutions were prepared and diluted with phosphate buffer solution (PBS). The blank plasma samples of healthy people and patients as gifts were collected from the Affiliated Hospital of Hebei University (Baoding, China). 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.

Apparatus

All separation and detection were done on a Model MPI-B Multi parameter chemiluminescence analyzer systems (Xi'an Remax Electronic High-Tech Ltd, Xi'an, China). A programmable high-voltage power supply (0-20 kV) was applied to drive the electrokinetic sample injection and electrophoretic separation. All data were accomplished with a multifunctional chemiluminescence analyzer, an electrochemical and electrochemiluminescent spectrums were obtained by the MPI-B analysis software. The end-column ECL detection was installed with a three-electrode configuration, which was made up of 500 μm platinum disk electrode as working electrode, a platinum wire as counter electrode and Ag/AgCl (1.0 M KCl saturated by AgCl) electrode as reference electrode. An uncoated fused silica capillary with 40 cm length, 25 μm i.d. and 360 μm o.d. was used for separation (Yongnian Ruipu chromatogram equipment Ltd, Hebei, China). Amicon Ultra 0.5 mL centrifugal filters with MWCO 10 kDa were purchased from Millipore Corporation.

CE-ECL analysis

The new capillary was filled with 0.1 M sodium hydroxide over night before use. Prior to starting a series of analyses, the capillary was washed with 1.0 M sodium hydroxide for 5 min, followed by double distilled water for 5 min, and equilibrated with the running buffer for 5 min to maintain an active and reproducible inner surface. A solution of 5 mM $Ru(bpy)_3^{2+}$ in 80 mM phosphate buffer (pH 7.0) was directly injected into the detection reservoir, and the solution in the cell was replaced every 2 h in order to obtain good repeatability. The ECL signal was recorded by the MPI-B system. The input voltage of the photomultiplier tube was set at 800 V. Samples were injected in an electrokinetic mode at 10 kV for 10 s, separated in the capillary at 15 kV. Sample injections were made at the cathodic side of the capillary. Prior to CE analysis, the required sample solutions and PBS were filtered through 0.22 μm cellulose acetate membrane filters (Shanghai Xinya Purification Material Factory, Shanghai, China).

Enzyme catalyzed reaction

A 10 μL enzyme sample (2.1 U) was mixed with metal ions and tetracine hydrochloride, and then the mixture was incubated at a constant temperature, 37˚C for 30 mins. Although the enzyme reaction could be terminated by heating several minutes in boiling water, however, high temperatures can accelerate the hydrolysis of tetracine hydrochloride. So the transamination reaction was terminated by adjusting pH to 2.0 with 1.0 M HCl based on the protein acidic denaturalization in these experiments. Finally, the hydrolyzed products were transferred into Amicon Ultra filter device and centrifuged at 8000 rpm for 5 mins. The ultrafiltrate was diluted five-fold with phosphate buffer and then electrokinetically injected for CE-ECL measurements. Controls containing only substrate indicated that the assay buffer did not induce substrate hydrolysis. The unit of BChE activity used in this paper was unit per liter (U/L), which was defined as the amounts of enzyme that hydrolyzed 1.0 μM of tetracine hydrochloride per min at 37˚C. The hydrolysis of the substrate was monitored by detecting ECL intensity of DMEA.

Preparation of blood sample

Anticoagulant such as potassium oxalate, sodium citrate, or the disodium salt of ethylenediamine tetraacetate dihydrate always inhibited BChE activity. So, heparinized plasma was used in our experiment. About 2 mL of anticoagulant human whole blood samples were collected in a 5 mL centrifuge tube and centrifuged at 3000 rpm for 10 mins. The supernatants (the plasma) were then collected in a 1.5 mL microcentrifuge tube, stored at −20°C until use. They were diluted to 10-fold before CE analysis.

RESULTS AND DISCUSSION

Detection of standard DMEA

DMEA can enhance the $Ru(bpy)_3^{2+}$ ECL efficiently. The ECL intensity of $Ru(bpy)_3^{2+}$/DMEA system was pH dependant and the optimum pH was found at pH 8.0 which agreed with the reported optimal pH range 6-9 for ECL reactions between $Ru(bpy)_3^{2+}$ and amines. On the other hand, 80 mM pH 7.0 PBS was used as the ECL detection buffer to offset possible pH change caused by the CE running buffer. Under the optimized conditions: ECL detection at 1.2 V, separation voltage at 15 kV, 30 mM PBS running buffer at pH 8.0, 5 mM $Ru(bpy)_3^{2+}$ dissolved in 80 mM PBS at pH 7.0 in the detection reservoir, the electrokinetical injection for 10 s at 10 kV, the linear range for DMEA extended from 3×10^{-8} to 6.6×10^{-7} M and from 1.6×10^{-6} to 6.4×10^{-5} M . The calibration curve equation was Y = (1.6E + 3)X-31.6793 (R = 0.9990) and Y = -21526.8lgX + 21772 (R = 0.9971), where X(M) and Y stood for the concentration of DMEA and the corresponding ECL intensity respectively. The detection limit of DMEA was 1.98×10^{-8} M (S/N = 3), corresponding to 6.6×10^{-10} U/L (9.43×10^{-3} μg/mL) of BChE catalyzing tetracine hydrochloride for 1 min. So, the corresponding LOD of BChE was 9.43×10^{-3} μg/mL in this method. 0.1 μM and 1.0 μM DMEA was injected consecutively six times to determine the repeatability of ECL intensity and migration time. Relative standard derivations of them were 3.5% and 1.2%, 3.0% and 0.3% respectively.

The activation of metal ions as cofactors on BChE

Usually, the BChE was fully activated by Ca^{2+} and Mg^{2+}. Metal ions played an important role in substrate binding. Thus, type of metal ion at the active site of BChE influenced enzymatic activity. The concentration effect of Ca^{2+} and Mg^{2+} on BChE activity against tetracine hydrochloride was investigated. As shown in Fig. 2, the enzyme was strongly activated by both Ca^{2+} and Mg^{2+}. At the low concentration, BChE activity increased with increasing concentration of both metal ions. BChE reached its highest activity at 2.0 mM Ca^{2+} and 1.2 mM Mg^{2+} respectively and decreased if the concentration of the metal ions further increased. Compared to Mg^{2+}, Ca^{2+} strongly stimulated enzymatic activity to 1.6 times under the optimized conditions. So, 2.0 mM Ca^{2+} was chosen in our experiments hereafter. Although Ca^{2+} stimulated BChE activity at concentrations between 0.4 and 2.0 mM, it inhibited the enzyme activity at higher concentrations. The reason is that excess amount of Ca^{2+} may change the conformation of its metal binding site. Based on inhibition of zinc ions on aminopeptidase (Auld, 2004), we propose that metal ions bind to the groups that have negative charges, such as the carboxyl gourp of the "Ser198-His438-Glu325" catalytic triad in the esteratic site, or Asp-70 in the peripheral anionic site of human butyrylcholinesterase, which reduce the charge on the metal, making it more difficult for the metal-bound water to ionize and for the metal to act as a Lewis acid catalyst.

Effect of pH on BChE activity

The BChE activity between pH 4.0 and 8.0 was determined in the presence of 2.0 mM Ca^{2+} and 1.0 mM tetracine hydrochloride. Fig.3 showed that the optimum pH was 7.5 The enzyme was relatively stable between pH 7.0 and 7.8 and lost activity rapidly when the pH was lower than 7.0 or higher than 7.8 These data reflected that the ionization of amino acid residues in the free enzyme influenced enzymatic activity and that BChE was a slightly basic esterase and worked in a narrow pH range. At the optimized pH, we speculate that tertiary ammonium group

of tetracine hydrochloride and of various active site ligands binds through a preferential interaction of quaternary nitrogens with the π electrons of aromatic groups, the active site was called an anionic substrate-binding site composed of Trp82. In addition, the pH specificity of the enzyme was similar to that of basic aminopeptidase from Common carp (Umetsu *et al*., 2003), in which pH influenced the disruption of the spatial structure of enzymes and the dissociation constant between enzymes and substrates.

Effect of incubation temperature on BChE

The BChE activities were measured at various temperatures. A series of aliquots of the BChE, 2.0 mM Ca^{2+} and 1.0 mM tetracine hydrochloride substrate were incubated for 30 min at various temperatures. The results of this experiment were depicted in Fig. 4. The enzyme activity increased with the increasing temperature. However, at temperatures greater than optimal temperatures, the enzyme is denatured due to which enzyme activity is denatured. In our experiment, the activity of BChE increases over the range 20°C-50°C and revealed an optimum temperature at 50°C. The activity declined obviously when the temperature increased further and came to its lowest peak at 70°C. It is noteworthy that the enzyme retained 70% of its optimal activity at 37°C, which is a suitable temperature for enzymatic activity.

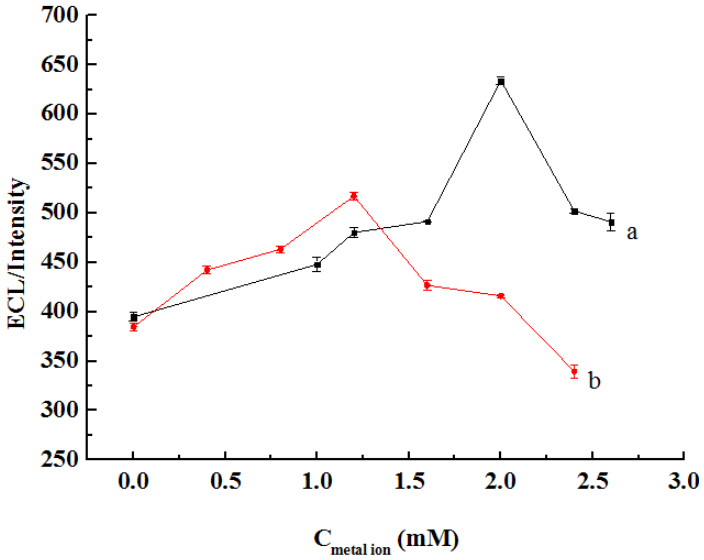

Fig. 2: Relationship between (ECL) intensity and concentration of Ca^{2+} (a) and Mg^{2+} (b). Conditions: 1.0 mM tetracine hydrochloride in 30 mM PBS at pH 7.5; incubation at 37°C for 30 min; 5.0 mM $Ru(bpy)_3^{2+}$ in 80 mM PBS at pH 7.0; detection potential, 1.2 V (vs Ag-AgCl).

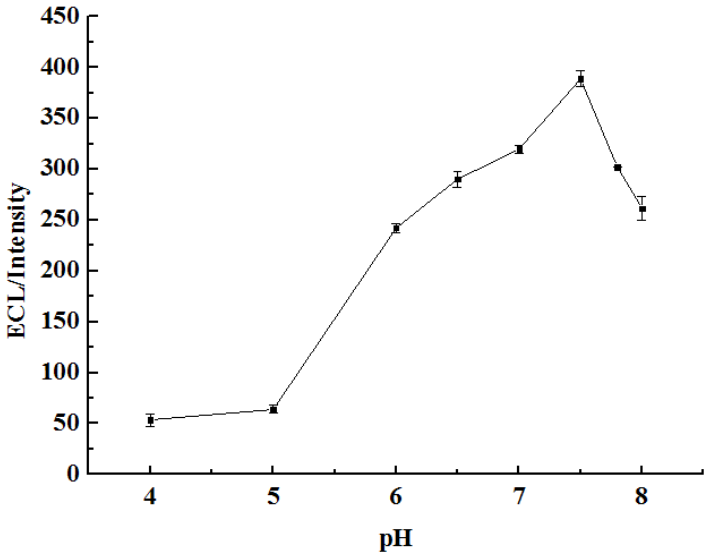

Fig. 3: Relationship between ECL intensity and pH. Conditions: 1.0 mM tetracine hydrochloride in 30 mM PBS; 2.0 mM Ca^{2+}; incubationat 37°C for 30 min; 5.0 mM $Ru(bpy)_3^{2+}$ in 80 mM PBS at pH 7.0; detection potential, 1.2 V (vs Ag-AgCl).

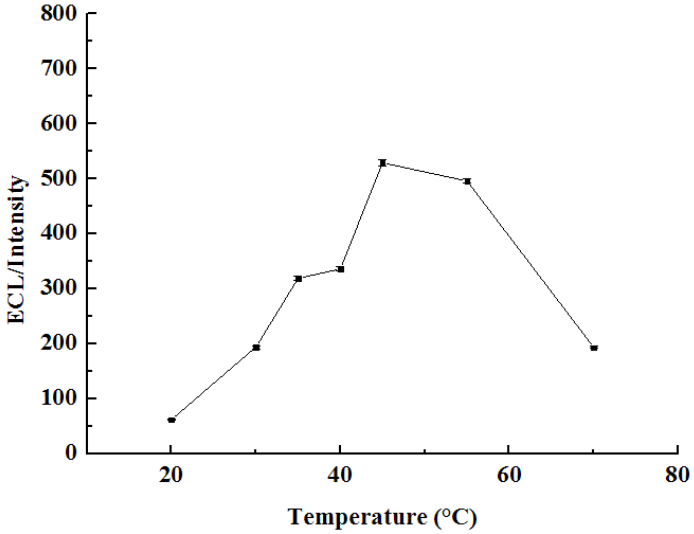

Fig. 4: Relationship between ECL intensity and temperature. Conditions: 1.0 mM tetracine hydrochloride in 30 mM PBS at pH 7.0; 2.0 mM Ca^{2+}; incubation time, 30 min; 5.0 mM $Ru(bpy)_3^{2+}$ in 80 mM PBS at pH 7.0; detection potential, 1.2 V (vs Ag-AgCl).

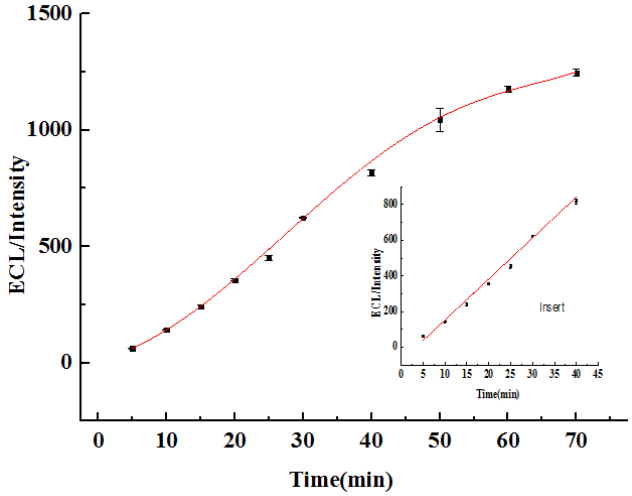

Fig. 5: Relationship between ECL intensity and incubation time within 70 and 40 min (inset). Conditions: 1.0 mM tetracine hydrochloride in 30 mM PBS at pH 7.5; 2.0 mM Ca^{2+}; incubation temperature, 37°C; 5.0 mM $Ru(bpy)_3^{2+}$ in 80 mM PBS at pH 7.0; detection potential, 1.2 V (vs Ag-AgCl).

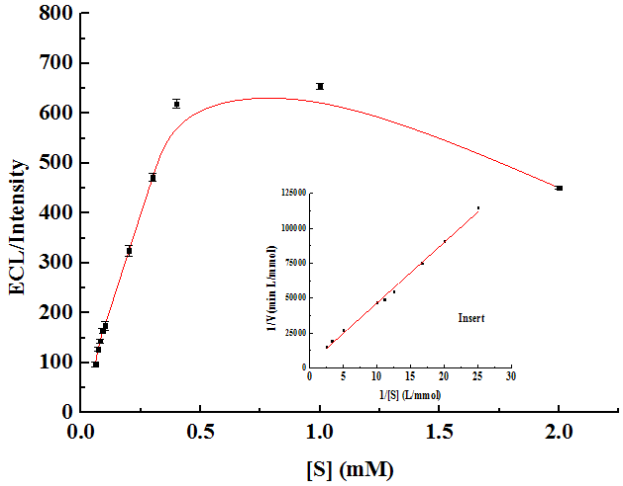

Fig. 6: The relationship between ECL intensity and substrate concentration. Inset: Lineweaver-Burk plot. Conditions: 1.0 mM tetracine hydrochloride in 30 mM PBS at pH 7.5; 2.0 mM Ca^{2+}; incubation at 37°C for 30 min; 5.0 mM $Ru(bpy)_3^{2+}$ in 80 mM PBS at pH 7.0; detection potential, 1.2 V (vs Ag-AgCl).

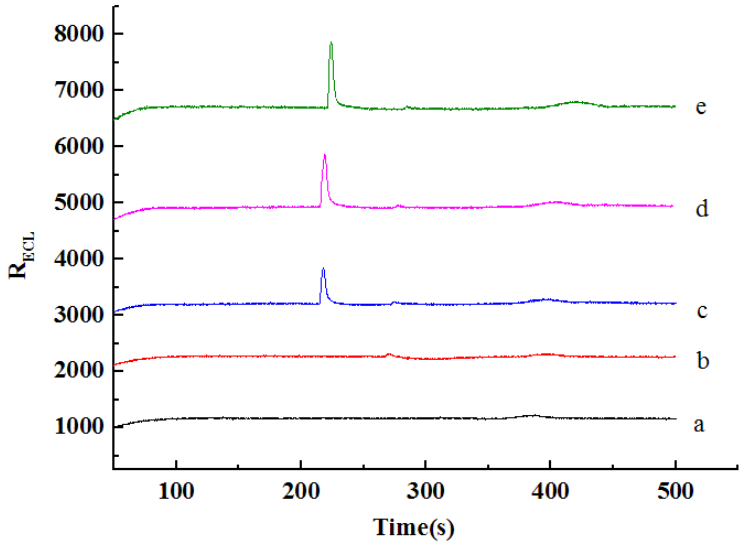

Fig. 7: Electropherograms of blank plasma without substrates (a); and plasma with addition of 1.0 mM tetracine hydrochloride before inbubation (b); plasmas from liver cancer patients(c), cholestasis hepatitis patients (d), healthy people(e) with addition of 1.0 mM tetracine hydrochloride after inbubation.

Effect of incubation time on BChE

Fig. 5 showed the change of DMEA ECL signal with the increasing incubation time. The ECL intensity increased faster rate for the first 30 min and then the velocity declines gradually. This is consistent with the enzyme reaction kinetics. In order to accurately reflect the enzyme activity using initial velocity, the hydrolysis reaction should be terminated before the decline of enzymatic reaction velocity. As showed in inset in Fig. 5, BChE activity was linearly related to incubation time in the first 30 minutes. So, 30 min as the incubation time was chosen.

Michaelis-Menten analysis

The Michaelis-Menten plots which reflect the relationship between the initial velocity (V) and substrate concentration ([S]) are presented in Fig. 6. The velocity of reaction increased quickly and was linear with increasing amounts of substrate at low substrate concentrations (<1 mM), obeyed first-order reaction model, because the enzyme was not saturated by substrates at low level. The reaction rate reached a maximum value when the substrate concentration increased to 1.0 mM, which was consistent with zero order reaction. So, the maximum activity of BChE was obtained when the concentration of tetracine hydrochloride was 1 mM. For enzyme-catalyzed reactions, the relation between initial reaction velocity (V) and substrate concentration [S] can be described by the Michaelis–Menten equation:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

where V_{max} is the maximum reaction velocity and K_m is the Michaelis constant, which may be taken as the substrate concentration at half the maximum reaction velocity if the amount of product is negligible. The Lineweaver-Burk reciprocal equation is obtained by inversion of this equation, which describes a linear relation between $1/V$ and $1/[S]$.

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

where $1/V$ means the concentration of DMEA divided by the reaction time. Six different concentrations of tetracine hydrochloride incubated with the BChE for 30 min, and then analyzed by CE-ECL. The Lineweaver-Burk plot lines for tetracine hydrochloride were shown in inset in Fig. 6. The K_m and V_{max} value were calculated to be 1.16×10^{-3} mol/L and 2.71×10^{-7} mol/L/min, respectively.

Application in evaluation plasma BChE activity

The proposed method has been used for the determination of plasma BChE activity in cholestatic hepatitis and liver cancer patients. To detect BChE activity, diluted plasma samples and tetracine hydrochloride were reacted at 37°C for 30 min and then were injected into the capillary. Sample pretreatment such as dialysis or extraction was not necessary. Fig.7 showed the electropherograms of plasma in the presence of tetracine hydrochloride with or without incubation.

Firstly, we carried out a blank test. There was only plasma without addition of substrate in the blank test. As shown in electropherograms a, there was no ECL peak produced, indicating that no intrinsic species and anticoagulant interfered in the plasma BChE activity assay. Secondly, electropherogram b represented a control assay in which only substrate solution was added to plasma sample but without incubation. Finally, electrophoregrams c, d, and e represented assay for plasma samples from liver cancer, cholestasis hepatitis patients and healthy people, respectively, after incubation with 1.0 mM tetracine hydrochloride under the optimal conditions. It was obvious that the DMEA content from healthy plasma was much higher than that from the diseased plasma, indicating that the activity of BChE had a great change for patients caught liver disease, especially in liver cancer patients.

Table 1: Results of BChE activities detected by CE-ECL and clinical method.

Pathogeny	Sample (No)	CE-ECL method (U/L)	Clinical method (U/L)	(CE-ECL method—Clinical method)/ Clinical method)
Liver cancer	1	3257	3453	-5.68%
	2	3350	3485	-3.87%
	3	3856	3687	4.58%
	4	3150	3315	-4.98%
	5	3589	3425	-4.80%
	6	4105	4312	-4.80%
	7	3875	3698	4.79%
	8	3683	3565	3.31%
	9	3323	3463	4.04%
	10	3415	3569	4.31%
Cholestasis hepatitis	1	5263	5389	-2.34%
	2	5845	6012	-2.78%
	3	5135	5325	-3.57%
	4	5216	5020	3.90%
	5	5985	5760	3.91%
	6	6158	6435	-4.30%
	7	5945	6124	-2.92%
	8	6210	6547	-5.15%
	9	5673	5400	5.06%
	10	5286	5486	-3.65%
Normal control	1	7218	7563	-4.56%
	2	7536	7789	-3.25%
	3	7680	7415	3.57%
	4	7510	7686	-2.29%
	5	7376	6970	5.82%

Table 2: Plasma leucyl aminopeptidase activity in patients with liver diseases.

Group	n	BChE activity (U/L)	Number of positive	Positive rate (%)
Liver cancer	26	3865.44 ± 135	19	73.08%
Cholestasis hepatitis	23	5982 ± 225	16	69.57%
Normal control	5	7464 ± 156	--	--

The activity of BChE in plasma of 5 normal subjects, 10 cholestasis hepatitis patients, and 10 liver cancer patients were determined and the results were listed in Table 1. It was demonstrated that plasma BChE activity from hepatopaths was much lower than that from healthy people. This is mainly due to the degrees of degeneration and necrosis for liver. The more serious liver tissue damaged, lower the activity of the enzyme. Especially, plasma BChE activity from liver cancer patients was much lower than that from cholestasis hepatitis patients. These results were in good agreement with that from clinical method based on spectral absorption. The errors were from −5.68% to 5.82%. The correlation between enzyme activity and liver injury is accordance with the previous report (Carello *et al*., 1999). All these results indicated that the proposed method for BChE activity determination was accurate and reliable.

These results were calculated by statistical analysis. It is assumed that the two-tailed p values were significantly <0.05, plasma BChE activity (mean ± SD) in the groups was shown in table 2. Mean ± SD were 3865.44 ± 135 U/L for twenty-six liver cancer plasma samples and 5982 ± 225 U/L for twenty-three cholestasis hepatitis plasma samples respectively. All the obtained data were much lower than the normal reference interval 7464 ± 156 U/L. There was also a significant positive correlation between elevated BChE activity in plasmas and liver lesion diseases. The positive rate was 73.08% in twenty-six plasma samples for liver cancer and 69.57% in twenty-three plasma samples for cholestasis hepatitis.

CONCLUSION

In this study, the CE coupled with ECL detection was used to investigate the BChE activity for diagnosis of liver disease. The kinetic study of enzymatic-catalyzed tetracine hydrochloride hydrolysis was investigated and both K_m and V_{max} value of enzymatic reaction were determined. The low detection limit, wide linear range, and good reproducibility for the indirect detection of DMEA made it become a suitable signal probe for enzyme analysis. More importantly, the experimental process is simple and environmental friendly since this method does not use the chromogenic reagents with poor chemical stability, enormous toxicity, and carcinogenicity which are typically used in the clinical analysis method. The proposed method has been successfully applied to detect BChE activity in the clinical plasma samples. Results demonstrated that a highly significant increase in BChE activity in liver diseases patients, which agreed well with the results detected by clinical method.

ACKNOWLEDGEMENTS

The project is supported by Natural Science Foundation of Hebei Province (B2015201193), Science and Technology Research Project of Hebei Higher Education (QN2016087), Science and Technology Research Program of Baoding City (16ZF190), Graduate Student Research and Innovation Program of Jiangsu Province (CX10B_068Z).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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How to cite this article:
Wang D, Li F, Su M, Sun H. Application of capillary electrophoresis coupling with electrochemiluminescence detection to estimate activity of butyrylcholinesterase. J App Pharm Sci, 2018; 8(04): 007-014.