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Immobilized capillary adenosine deaminase microreactor for inhibitor screening in natural extracts by capillary electrophoresis

Xiaowen Ji, Fanggui Ye, Pingtan Lin, Shulin Zhao*

The Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources (Ministry of Education), College of Chemistry and Chemical Engineering, Guangxi Normal University, Guilin 541004, China

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ABSTRACT

A novel strategy for the preparation of in-column adenosine deaminase (ADA) microreactor and rapid screening of enzyme inhibitors in natural extracts was demonstrated. In this approach, ADA was encapsulated in anionic polyelectrolyte alginate that was immobilized on the surface of fused-silica capillary via ionic binding technique with cationic polyelectrolyte polyethylenimine (PEI). On-line enzyme inhibition study was performed by capillary electrophoresis (CE). The substrate and product were baselined separated within 75 s. The enzyme activity was determined by the quantification of peak area of the product. Enzyme inhibition can be read out directly from the reduced peak area of the product in comparison with a reference electropherogram obtained in the absence of any inhibitor. The inhibition percentage was used to evaluate relative activity of ADA microreactor. A known ADA inhibitor, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was employed as a model compound for the validation of the inhibitor screening method, and the screening of ADA inhibitor in 19 traditional Chinese herbal medicines was performed.

1. Introduction

Adenosine deaminase (ADA) is an aminohydrolase which catalyses the deamination of adenosine to inosine [1]. Previous studies have shown that the increase of ADA activity was observed in several diseases, such as typhoid fever [2], infectious mononucleosis and tuberculosis [3]. In particular, the ADA level in T cells of chronic lymphocytic leukemia patients was 2–3 folds higher than normal subjects [4]. Therefore, the inhibition study of ADA activity and the discovery of new ADA inhibitors have received much attention [5–7].

Capillary electrophoresis (CE) methods have been proved to be a powerful approach for enzyme study and inhibitor screening [8]. Recently, Krylov and co-workers developed a CE method named inject-mix-react-separate and quantitate for screening enzyme inhibitors [9]. Iqbal et al. reported a highly sensitive CE method applying dynamic coating and on-line stacking for monitoring of nucleotide pyrophosphatases/phosphodiesterases and screening of inhibitors [10]. Kang's group has developed an electrophoretically mediated microanalysis technique for screening hexokinase and acetylcholinesterase inhibitors [11,12]. A CE-LIF assay was also reported for detection of adenylyl cyclase activity [13]. In all methods mentioned above, free enzyme technique was used.

The immobilized enzyme-based screening methods offer several advantages over the free enzyme-based screening methods including (1) the separation of the enzyme and reaction mixture was easy; (2) the screening cost can be reduced because the studied enzymes can be continuous reused or recycled; (3) the enzyme stability can be also promoted. Zhang's group demonstrated a high performance liquid chromatography (HPLC) [14] and an electrospray mass spectrometry (ESI-MS) [15] technique for screening enzyme inhibitors with immobilized enzyme on magnetic microspheres. Tang and Kang prepared an angiotensin-converting enzyme reactor based on the ionic binding immobilization approach in the head of the capillary [16]. They also reported an acetylcholinesterase microreactor based on layer-by-layer assembly technology for screening enzyme inhibitor [17].

Several CE-based methods have been developed for studying ADA activity. Saevels et al. described an electrophoretically mediated microanalysis technique to determine Michaelis constant and evaluate the behavior of the ADA inhibitor [18,19]. Tomas's group used transient capillary isotachophoresis technique for the measurement of ADA activity in human erythrocytes [20]. Hodgson et al. described the coupling of capillary-scale monolithic enzyme reactor columns directly to a tandem mass spectrometer for screening enzyme inhibitors [21].

In this work, a new strategy using on-column immobilized enzyme microreactor for screening ADA inhibitors in natural extracts by CE was developed. ADA was encapsulated in alginate and then immobilized on the surface of capillary head via the

* Corresponding author. Tel.: +86 773 5849646; fax: +86 773 5832294.
E-mail address: zhaoshulin001@163.com (S. Zhao).

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ionic binding approach with polyethylenimine (PEI). The performance and inhibition kinetics of immobilized ADA were studied, and present method has been applied for screening ADA inhibitor in 19 traditional Chinese herbal medicines.

2. Materials and methods

2.1. Reagents and chemicals

Adenosine deaminase (ADA, EC 3.5.4.4), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) and sodium alginate were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Adenosine, inosine and polyethylenimine (PEI; M.W. 70,000, 30%, w/v aqueous solution) were purchased from Alfa Aesar (Ward Hill, USA). Sodium borate, calcium chloride and 2-propanol were obtained from China National Medicine Group Shanghai Corporation (Shanghai, China). All other chemicals and organic solvents used in this work were of analytical grade. Water was purified by employing a Milli-Q plus 185 equip from Millipore (Bedford, MA, USA), and used throughout the work. The running buffer was prepared with 25 mM borate and pH was adjusted with 1 M hydrochloric acid. The ADA solution containing 0.1 mg/mL sodium alginate was freshly prepared with running buffer. All solutions were filtered through 0.45 μm membrane filters before use.

2.2. Instrumentation

The following instruments were used to prepare natural extracts: RE-52AA rotavapour (Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China), DZF-1B vacuum drier (Shanghai Yuejin Medical Instrument Co., Ltd., Shanghai, China), SHB-bA water-circulation multifunction vacuum pump (Zhengzhou Great Wall Scientific Industry and Trade Co., Ltd., Zhengzhou, China), and ultrasound cleaning bath (Guangzhou Sonoc Ultrasonic Electronic Equipment Co., Ltd., China).

All separations and detections were performed on a HP^{3D} capillary electrophoresis system (Hewlett Packard, Waldbronm, Germany) with a dioade array detector (190–600 nm). Detection wavelength was at 254 nm. A HP chemstation was used for data acquisition and treatment. Uncoated fused-silica capillary 29 cm (effective length 20.5 cm) × 50 μm i.d. was used.

2.3. Preparation of natural extracts

Rhizoma chuanxiong was obtained from Guilin Pharmaceuticals Group of China (Guilin, China). Air-dried rhizoma chuanxiong was ground into a fine powder with a pulverizer. 10 g of ground material was extracted with 100 mL of 2-propanol in an ultrasonic cleaning bath for about 2 h at a frequency of 100 kHz and 60 °C. The solvent was evaporated using an RE-52AA rotavapour at 40 °C and a SHB-bA water-circulation multifunction vacuum pump. Finally, rhizoma chuanxiong extracts were dried in a DZF-1B vacuum drier at 30 °C and 0.07 MPa. The natural extracts were dissolved with 2-propanol aqueous solution giving a final concentration of 6 mg/mL. Other natural extracts were prepared in the same way.

2.4. Preparation of immobilized capillary ADA microreactor

Fig. 1 is a schematic representation of the immobilized enzyme microreactor profile.

The immobilized enzyme microreactor can be simply prepared using the three-step protocol. Before coating the capillary, an approximately 1 cm long plug of 1 M NaOH solution was injected into the capillary with a pressure of 50 mbar for 20 s. The NaOH solution was kept for 20 min and then the capillary was washed with deionized water for 5 min. Subsequently, following three steps

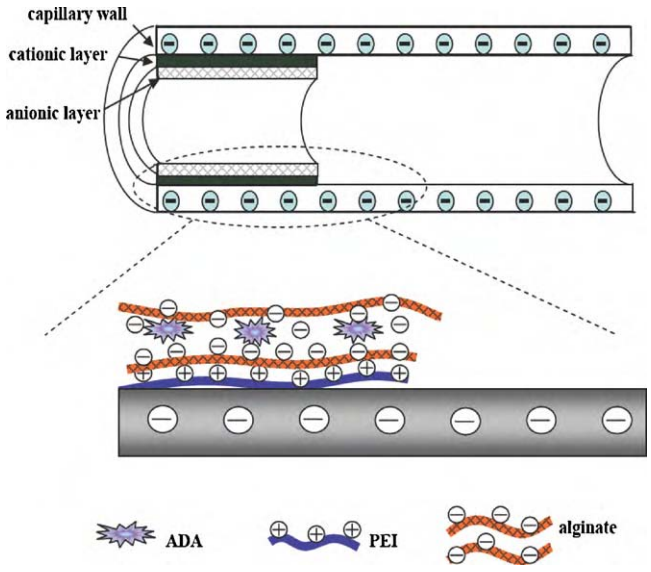

Fig. 1. Schematic representation of the immobilized capillary enzyme microreactor.

were accomplished for the preparation of immobilized enzyme microreactor. (i) The cationic polyelectrolyte PEI was injected into the capillary by pressure at 50 mbar for 10 s, giving a 0.4 cm long plug of the PEI solution. The PEI solution was left in place for 10 min to produce a positively charged coating on the surface of the capillary, and then the capillary was washed with deionized water by applying a pressure on the outlet end of the capillary; (ii) 45.5 unit/mL ADA containing 0.1 mg/mL sodium alginate with same plug length was injected into the capillary and incubated for 20 min to immobilize the enzyme on the capillary wall via electrostatic interaction. Unimmobilized enzyme was flushed out by deionized water; (iii) 1% $CaCl_2$ solution with the same plug length was injected into the capillary and remained for 10 min to make the immobilized enzyme reactors stable. If the activity of immobilized enzyme becomes poor, the reactor can be renewed conveniently by flushing the capillary with 0.1 M HCl, and 0.5 M NaOH, respectively, and then repeating the preparation protocol.

2.5. Immobilized enzyme activity assay and inhibition study

The activity of the immobilized enzyme was assayed simply by carrying out a CE separation. The capillary was filled with the running buffer. The adenosine solution (0.36 mM) was injected into the enzyme microreactor with a pressure of 40 mbar for 5 s. After incubation for 2 min, 20 kV voltages were applied to separate the product from unreacted substrate. The enzyme activity was then assayed by measuring the peak area of the product.

For inhibition study, the buffer containing the inhibitor (or natural extracts) was injected into the capillary and left in place for 3 min. Then the mixture of adenosine and inhibitor (or natural extracts) was injected and incubated. Finally, separation voltage was applied. The inhibition percentage was calculated according to the reduction of the enzyme activity.

3. Results and discussion

3.1. Preparation of the enzyme microreactor

ADA cannot be adsorbed on the inner surface of the fused-silica capillary due to negatively charged at pH 8.0 [22]. Therefore, it is necessary to modify the capillary wall by a strong cation exchanger. In this work, PEI was used for modifying the capillary wall. In order to keep the EOF unchanged, only the entrance of the capillary (about

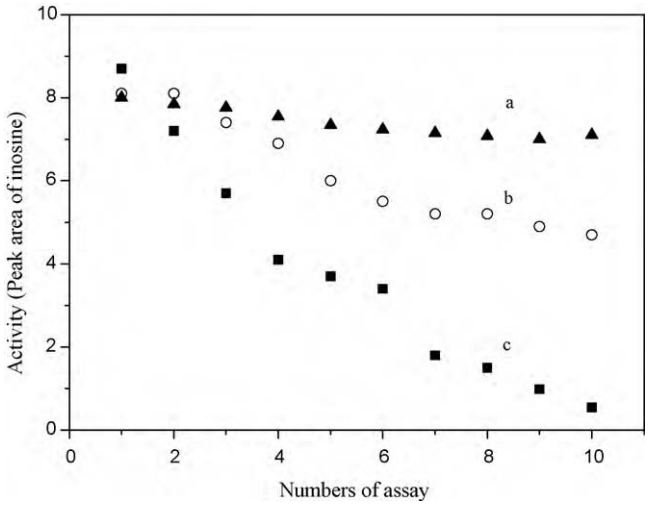

Fig. 2. Stability of the immobilized capillary ADA reactor. (a) After formation of "egg-box" structure of calcium alginate gel; (b) before formation of "egg-box" structure of calcium alginate gel; (c) in the absence of alginate. CE condition: running buffer, 25 mM borate (pH 8.0); column temperature, 25 °C; sample injection, 40 mbar for 5 s; incubation time, 2 min; concentration of adenosine, 0.36 mM; separation voltage, 20 kV.

0.5 cm long) was coated with PEI. When the enzyme was immobilized on PEI modified capillary wall, high enzyme activity was obtained. However, the immobilized ADA microreactor appeared to be not stable.

Alginate is a naturally occurring linear anionic polysaccharide which has become one of the most common biomaterials due to low/non-toxicity, low immunogenicity and good biocompatibility [23]. There are several unique properties for the entrapment of a variety of proteins with alginate. First, it offers a relatively inert aqueous environment within the matrix. Second, the encapsulation process is under a mild room temperature without organic solvents. Third, it possesses a high gel porosity which ensures high diffusion rates of macromolecules [24]. In this work, when the polyanion alginate, enzyme and polycation PEI were mixed, polyelectrolyte complexes were formed based on ionic interactions. The stability of the immobilized enzyme microreactor was reinforced with a little decrease in enzyme activity in the presence of alginate.

Alginate is composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues linked by a 1, 4-glycosidic bond with a varying G/M ratio. When Ca^{2+} was added in sodium alginate solution, the random coils structure of alginate chains was changed to the ordered ribbon-like structure due to the ionotropy effect between Ca^{2+} and Na^{+}. This entanglement of alginate chains finally contributes to the "egg-box" structure hydrogel which has a three-dimensional net structure [25]. Therefore, $CaCl_2$ was injected into the capillary in order to reduce the leaching of the immobilized enzyme molecules.

The stability comparison of immobilized enzyme reactor is shown in Fig. 2. It was found that relatively high stability of the enzyme reactor was obtained with "egg-box" structure. The immobilized enzyme reactor keeps its activity by 85% after 40 assays. The repeatability of the peak area of the product (inosine) in terms of run-to-run and batch-to-batch was evaluated, and RSDs% ($n = 6$) were 2.8% and 4.7%, respectively.

There are several advantages inherent in this immobilized enzyme reactor: (i) the immobilization procedure is simple, fast, and easy to automate; (ii) the enzyme activity can be maximally preserved during immobilization due to the biologically benign conditions (room temperature and neutral pH); (iii) because alginate had good biocompatibility, it can encapsulated many kinds of enzyme. Moreover, it is possible to immobilize several enzymes at the same time [26].

3.2. Optimization of CE conditions

Owing to the substrate and product exhibit an absorption maximum at 254 nm, so this wavelength was selected as the detection. The separation conditions such as pH of electrolyte solution, concentration of borate buffer, capillary length and the applied voltage were investigated to achieve an efficient separation. Because the ADA activity at pH 6.0 and 9.0 is approximately two-thirds of that at the optimal pH (6.5–8.0), so the effects of buffer pH in the range of 6.0–9.0 were tested. Result indicated that increasing buffer pH improve effectively the resolution between adenosine and inosine. Thus, pH 8.0 was chosen for further optimization. Resolution between adenosine and inosine was enhanced with increasing borate buffer concentration and capillary length. But high buffer concentration and long capillary length will increase analysis time. A higher applied voltage reduced the migration times due to an increase of EOF. However, high applied voltage may induce excessive Joule heating which ruins the resolution and repeatability. After a careful study, optimized CE conditions were as following: 50 μm i.d. × 29 cm long capillary, 20 kV applied voltages, and a running buffer containing 25 mM borate at pH 8.0.

Optimization of the incubation time on the yield of product inosine was investigated. When incubated over 2 min, a maximum yield could be achieved. So a 2 min incubation time was selected for further experiments.

3.3. Performance of the immobilized ADA

In order to make sure that no significant change on enzyme property was observed after enzyme immobilization, the kinetic behavior of the immobilized enzyme was evaluated. As the most important kinetic constant of the enzyme reaction, the Michaelis constant is determined conveniently by Lineweaver and Burk's plotting method [27].

$$\frac{1}{v} = \frac{K_m}{V_{\max}[S]} + \frac{1}{V_{\max}} \quad (1)$$

where v and $V_{\max}$ are the initial and maximal reaction velocity, [S] is the substrate concentration, and K_m is the Michaelis constant.

Six adenosine solutions ranging from 0.1 to 0.8 mM were injected into capillary. Each of six solutions was analyzed three times. And the peak area of inosine was used to express the initial reaction velocity. A double-reciprocal plot following Eq. (1) was constructed with values of 1/[adenosine] and $1/v$ in the x and y axis, respectively (Fig. 3). From the intercept on the abscissa, the K_m value for the enzymatic reaction was estimated to be 121 μM, which is consistent with previously report values obtained from enzyme reactor-MS assays (124 μM) [21]. The result indicated that no significant change on enzyme property was observed after enzyme immobilization.

3.4. Validation of inhibitor screening method

Under the optimal conditions, inhibition study of ADA activity was performed. A known inhibitor, EHNA was used as a model compound for the inhibition study. Before the equilibrium between the enzyme and inhibitor was achieved, the preincubation of enzyme with the inhibitor was required. Otherwise a significant amount of substrate may be converted to product [19]. Therefore, a buffer solution containing 12 nM EHNA was injected into the capillary and left in place for 3 min. Then the mixture of 0.36 mM adenosine and 12 nM EHNA was injected and incubated for 2 min. Finally, 20 kV

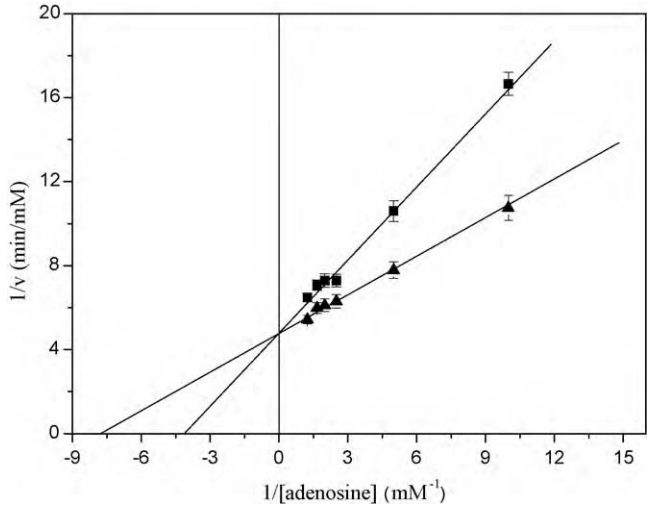

Fig. 3. Lineweaver–Burk plot of ADA in the presence or absence of inhibitor EHNA. The concentration of EHNA in the substrate solution was 0 nM (▲) and 12 nM (■). CE conditions were as in Fig. 2.

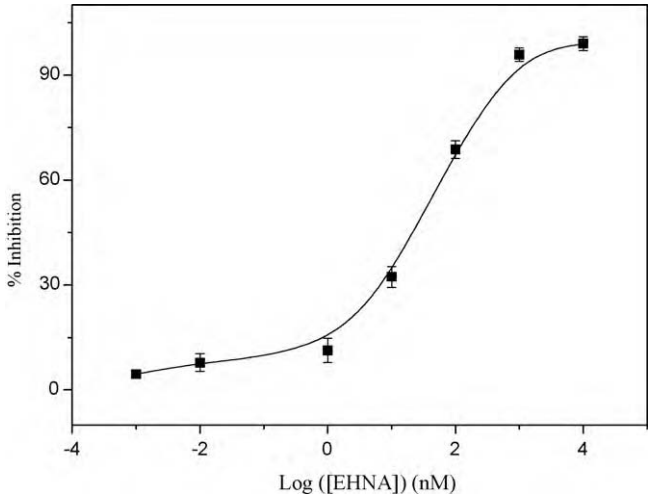

Fig. 4. The inhibition plot of ADA in the presence of inhibitor EHNA. CE conditions were as in Fig. 2.

Table 1
Extracts library used for inhibitor screening.

Extracts	Inhibition (%)	Extracts	Inhibition (%)
Aloe barbadensis	0	Radix notoginseng	0
Artemisia capillaris thumb	0	Radix sophorae tonkinensis	0
EHNA	76.8	Rhizoma chuanxiong	48.0
Flos chrysanthemi	0	Rhizoma coptidis	0
Fructus evodiae	0	Rhizoma corydalis	0
Fructus schisandrae	0	Rhizoma curcumae aeruginosae	0
Herba andrographitis	0	Rhizoma dioscoreae bulbiferae	0
Herba sedi	0	Semen ginkgo	0
Radix artemisiae annuae	0	Semen plantaginis	0
Radix et rhizomarhei	0	Tremolite asbestos	0

voltage was applied for the separation. The inhibition percentage was calculated using following equation:

$$\text{inhibition } (\%) = 100 - \left(\frac{x}{\text{blank}} \times 100\right) \quad (2)$$

where x and blank are the peak area of inosine determined with or without inhibitors, respectively.

The Lineweaver–Burk plots for the immobilized ADA in the presence of EHNA are shown in Fig. 3. The apparent $K_{m(\text{app})}$ was

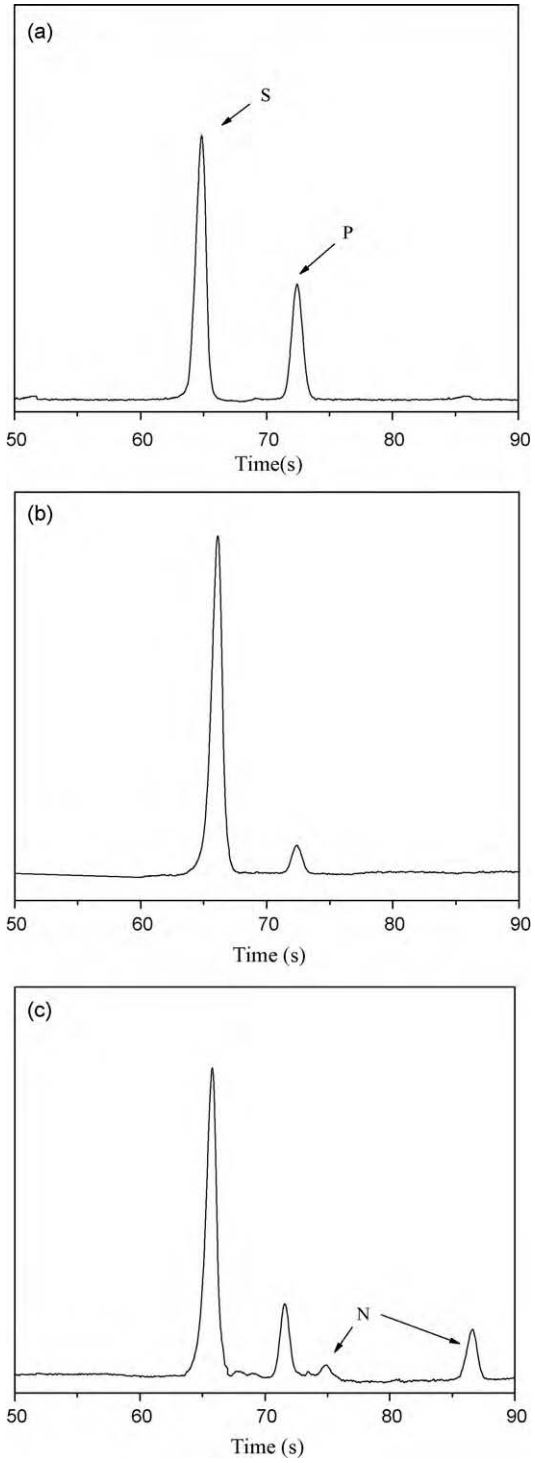

Fig. 5. Typical electropherograms for screening the ADA inhibitors. CE conditions were as in Fig. 2. Samples: (a) standard; (b) EHNA (12 nM); (c) rhizoma chuanxiong (0.6 mg/mL). Peak: S, substrate; P, product; N, unknown compounds.

estimated to be 241 μM. The *Ki* for EHNA was calculated according to Eq. (3). The resulting *Ki* of 12.1 nM was in agreement with the reported *Ki* values (1.5–16 nM) [22].

$$K_{m(\mathrm{app})} = K_m\left(1 + \frac{[\mathrm{I}]}{Ki}\right) \quad (3)$$

The inhibition plot of EHNA is shown in Fig. 4. The IC_{50} value was determined as 30.2 nM, which exactly matched with the literature value of 29.7 nM [21].

The quality parameter Z' factor, which was calculated according to Eq. (4), is recommended for evaluation of the accuracy of a drug screening system.

$$Z' = 1 - \frac{3\sigma_s + 3\sigma_c}{|\mu_s - \mu_c|} \quad (4)$$

σ_s and σ_c represent standard (no inhibition) and control assay (100% inhibition), respectively. While μ_s and μ_c are the mean of the signal of the standard deviations of standard and control assays, respectively. Z' factor value of higher than 0.5 implies an excellent quality for an assay method. In this work, the Z' factor value was calculated as 0.85.

3.5. Inhibitor screening in natural extracts

Because the increase of ADA activity was observed in several diseases such as typhoid fever, infectious mononucleosis, leukaemia and tuberculosis, and some natural extracts were usually used as traditional Chinese medicine for these diseases, therefore the present method was applied for ADA inhibitors screening in 19 natural extracts. In addition, EHNA were put into the chemical library for the purpose of method validation. The percentage of inhibition was determined according to Eq. (2), and the results were summarized in Table 1. EHNA (12 nM) was identified, and a natural extract of rhizoma chuanxiong was found to be positive for enzyme inhibition by the presented method.

Fig. 5 shows typical electropherograms for screening ADA inhibitors with the present method. Fig. 5a represents a control assay, in which only the substrate was injected. Fig. 5b and c represents the assays for the sample containing EHNA and a natural extract of rhizoma chuanxiong, respectively. The peak area of the product was reduced clearly in Fig. 5c. The experiment results indicated that some components in rhizoma chuanxiong were ADA inhibitors. Rhizoma chuanxiong is a medicinal herb for treating gynecological disorders, headache, and also in conjunction with cancer chemotherapy [28,29]. In particular, people use chuanxiong tea for chronic leukemia treatment in China. The enzyme inhibition may be responsible for the effect on leukemia treatment.

4. Conclusions

A method for preparing in-column adenosine deaminase microreactor based on an ionic binding approach has been developed for screening enzyme inhibitors. The present strategy can be potentially applied for immobilized various enzymes on capillary wall due to unique properties of alginate such as aqueous environment, mild immobilized enzyme process, and low/non-toxicity. The method displays advantages including very low cost and reusable enzyme, which not only saves on reagent costs but also provides an unprecedented internal control in that the level of enzyme is consistent for all assays. The performance and inhibition kinetics of the immobilized ADA were studied. Extracts of 19 herbs were tested.

Acknowledgments

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