Screening of neuraminidase inhibitors from traditional Chinese medicine by transverse diffusion mediated capillary microanalysis

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A transverse diffusion mediated capillary microanalysis method has been developed for screening of neuraminidase inhibitors from traditional Chinese medicine. The enzyme, substrate and inhibitors were sequentially injected, mixed efficiently by transverse diffusion of laminar flow profiles, then incubated and separated in the same capillary. To enhance the mixing efficiency of reactants, running buffer was injected by alternately applying +5 kPa and -5 kPa at the capillary inlet and the procedure was repeated three times. The capillary electrophoresis (CE) separation conditions and reactants mixing conditions were optimized. Dual-wavelength detection was employed to eliminate the interference with natural compounds. The method has been applied to determine the kinetics constant of neuraminidase and screen 12 compounds from traditional Chinese medicine. Four compounds have been found to be positive for enzyme inhibition. The results are in good agreement with those reported in the literature. The method realized the mixing of substrate and enzyme with identical electrophoretic mobility. This novel CE method was simple, rapid, economic, and fully automated. Therefore, it was appropriate for neuraminidase inhibitors screening and could be extended to other high-throughput screening of active components from traditional Chinese medicine. © 2014 AIP Publishing LLC.

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I. INTRODUCTION

Neuraminidase (NA), one of the glycoproteins on the surface of influenza virus, cleaves the specific linkage of the sialic acid receptor to facilitate the release of influenza virus particles to infect new cells.¹ Due to the key role in the replication, infection, spread, and pathogenesis of influenza virus, NA has been regarded as an essential target for prevention and treatment of influenza infection.² Some synthetic neuraminidase inhibitors (NAIs) such as zanamivir and oseltamivir have been used in the clinic as first-line treatment drugs for decades, but have severe side effects and limitations.^{3–5} Therefore, more attention has been paid to natural compounds which act as anti-influenza agents in two ways: directly inhibiting the activity of NA to prevent the release of virus particle or indirectly resisting viral infection by regulating or enhancing immune function.^{6–8} The search for safe and effective NAIs from traditional Chinese medicine (TCM) is an increasingly important area in new anti-influenza drugs development.

The conventional screening for NAIs is usually performed by monitoring the conversion of the substrate to the product in solution and requires complicated step-by-step operations including reactants pre-mixing, off-line incubation, reaction quenching reagent addition. After extraction and centrifugation, the reaction mixture is generally detected without separation by fluorimetry,^{9,10} chemiluminescence,^{11,12} UV spectrometry,¹³ and mass spectrometry.^{14–16} These

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in-solution screenings suffer from large sample consumption (at least microliters of each reactant), tedious sample processing and interference of optical detection.

Reducing sample consumption and automated screening is essentially important for expensive reactant and for multiple assays in high-throughput screening. On this account, capillary electrophoresis (CE) is extensively applied for enzymatic assay as it is characterized by high efficient separation, low sample consumption, and versatile separation and detection modes.^{17–20} Two strategies based on CE have been proposed for online screening of enzyme inhibitors. The first is to immobilize the enzyme onto the capillary wall. In our previous work, an immobilized-enzyme microreactor (IMER) was fabricated for NAIs screening.²¹ The IMER improved the stability of NA and reduced the consumption of NA, but required 3 days to prepare. In addition, it has a disadvantage of requiring a large consumption of substrate as each run in the screening uses a mixture of substrate and inhibitor.

The second strategy is known as electrophoretically mediated microanalysis (EMMA), in which the reactants were successively injected into the capillary, then mixed by the applying of an electric field and incubated at zero potential to generate sufficient product.²² One of the greatest advantages of EMMA is that it integrates the enzymatic reaction, separation, and detection into one capillary to fully automate the system. Compared to the classical approach and IMER, EMMA method consumes only nanoliter volumes of reagents and does not require premixing of the substrate and inhibitors with enzyme, allowing a drastic reduction in reactant consumption per assay.

To establish the EMMA method, the pivotal step is to mix reactants homogeneously in the capillary. Three methods have been proposed for mixing reactants inside capillary, namely, mixing by electrophoresis, longitudinal diffusion and transverse diffusion of laminar flow profiles (TDLFP).²³ Mixing by electrophoresis is built on the differences in electrophoretic mobilities of reactants, and therefore it is unsuitable for mixing reactants with identical electrophoretic mobilities. Moreover, it is difficult to mix more than three reactants by electrophoresis.²⁴ Mixing by longitudinal diffusion, also known as at-inlet technique, is based on longitudinal diffusion through transverse interfaces.²³ In the at-inlet method, the reactants were injected into the capillary by applying electric field or low pressure to render the front shape of plugs rectangular. It is only suitable for the mixing of small molecules because the longitudinal diffusion process is very slow.²⁵ In TDLFP, the reactants were successively injected into the capillary with pressure high enough to make the front shape of plugs parabolic. Then, plugs were mixed by transverse diffusion through the longitudinal interfaces. Since the transverse diffusion occurs very fast (completed at several or tens of seconds), TDLFP is considered as a generic method for reactants mixing and suitable for mixing more than four reactants as well.²⁵

In preliminary work, the NA and the widely used fluorogenic substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid sodium salt hydrate (4-MUNeu5Ac) were found to have identical electrophoretic mobilities. The purpose of the present work was to develop an automated CE method that consumes small quantities of each reagent by transverse diffusion mediated microanalysis to realize the enzymatic reaction in capillary and to screen potential NAIs from TCM. The activity of NA was determined by monitoring the conversion of the substrate 4-MUNeu5Ac to the product 4-methylumbelliferone (4-MU) and the peak area of 4-MU indicated the NA activity. The inhibitors were identified by the decrease of the peak area of 4-MU. The CE separation conditions of substrate and product were optimized. Dual-wavelength UV detection was employed to eliminate the detection interference of 4-MU. The effects of the injection sequence, injection time, and injection length of background electrolyte (BGE) on the online enzymatic reaction efficiency were investigated. Under the optimal conditions, the kinetics constant was tested and the inhibition activity of twelve compounds from TCM was screened.

II. EXPERIMENTAL

A. Chemicals and instruments

The substrate 4-MUNeu5Ac, the product 4-MU and the enzyme NA (EC 3.2.1.18) were purchased from Sigma-Aldrich (Steinheim, Germany). All natural compounds were obtained

from Shanghai Shunbo Bio-engineering Technology (Shanghai, China). For inhibitor assay, 4-MUNeu5Ac and NA were dissolved in deionized water and diluted by pH 6.0 phosphate sodium buffer to 125 μM and 0.5 U/ml, respectively. The natural compounds were dissolved in methanol and diluted by pH 6.0 phosphate sodium buffer to 60 μM . Phosphate buffer (10 mM NaH_2PO_4 , 10 mM Na_2HPO_4) as BGE for CE separation was fresh prepared in the lab with reagents obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

All CE experiments were performed on an Agilent 7100 CE system (Agilent, Waldbronn, Germany) equipped with a photodiode array detector. A fused-silica capillary (Ruifeng Chromatographic Devices, Hebei, China) with 50 μm i.d. and 365 μm o.d. was cut to 35.5 cm total length and 27 cm effective length. New capillary was flushed with 1 M NaOH, H_2O and BGE for 2 h, 0.5 h, and 0.25 h, respectively. Every five runs, the capillary was flushed 3 min with MeOH, 2 min with deionized water, and 3 min with BGE.

B. Principle of mixing by transverse diffusion of laminar flow profiles

The velocity (ν) of a pressure-driven flow inside the capillary can be calculated by Poiseuille's equation,²³

$$\nu(r, t) = \frac{(r_i^2 - r^2)\Delta p(t)}{4\eta L}, \quad (1)$$

where Δp represents the differential pressure (Pa); η , the fluid viscosity (Pa·s); L , the total length of the capillary (cm); r_i , the capillary inner radius (cm); r , the distance from the center of the capillary in the transverse direction (cm); t , injection time (s).

When the sample is injected into the capillary with high pressure, the profile of the flow is parabolic and the injection length can be calculated by the following equation:²³

$$l(r) = \int_0^{t_{inj}} \nu(r, t) dt = \int_0^{t_{inj}} \frac{(r_i^2 - r^2)\Delta p(t)}{4\eta L} dt. \quad (2)$$

Diffusion can be neglected if the pressure is high enough and the injection time is shorter than the transverse diffusion time t_r which can be calculated by the following equation:

$$t_r = \frac{r_i^2}{D}. \quad (3)$$

The equation shows that t_r depends on the capillary inner radius and the diffusion coefficient D ($\text{cm}^2\cdot\text{s}^{-1}$).

Based on the above liquid flow theory, Krylov *et al.*²⁶ proposed the TDLFP model for mixing reactants in the capillary and provided that the reactants should be injected at high pressure to ensure the profile of flow parabolic. After the reactants were separately injected into the capillary as distinct plugs, the parabolic plugs would penetrate into each other. The principle of mixing reactants in the capillary by TDLFP is schematically shown in Fig. 1. The group introduced the York number (Y_0) to predict the plug shape during injection. Y_0 can be calculated by the following equation:²⁷

$$Y_0 = \frac{D \times l}{\nu_{\max} \times r_i^2} = \frac{D \times (t_{inj} \times \nu_{\max})}{\nu_{\max} \times r_i^2} = \frac{D \times t_{inj}}{r_i^2} = \frac{t_{inj}}{t_r}, \quad (4)$$

where D is the diffusion coefficient ($\text{cm}^2\cdot\text{s}^{-1}$); l , injection length (cm); $\nu_{\max}$, the maximum velocity in certain injection time (t_{inj}). The equation shows that Y_0 is related to D , t_{inj} , and r_i . At certain injection time, Y_0 is inversely proportional to t_r . With the increase of Y_0 , t_r is shortened and transverse diffusion would occur during injection, leading to the low quality of mixing.

FIG. 1. The schematic of reactants mixing by transverse diffusion of laminar flow profiles. (a) The injection procedure and (b) the mixing profiles of plugs. E, enzyme; S, substrate; I, inhibitor; and B, background electrolyte.

C. Transverse diffusion mediated microanalysis

The reactants of NA, 4-MUNeu5Ac and natural compounds were sequentially injected into the capillary at 5 kPa for 6 s, followed by the injection of BGE at alternating +5 kPa and -5 kPa for 6 s and the BGE injection procedure was repeated three times. A 300 s incubation was then conducted. The unreacted 4-MUNeu5Ac, the generated 4-MU and natural compounds were separated at 20 kV with 10 mM pH 6.0 phosphate sodium buffer as the BGE. The capillary was thermostated at 30 °C with an air-cooling system. UV detection was operated at 320 nm with a reference wavelength of 260 nm for baicalein, baicalin, and vitexin assay and 320 nm with a bandwidth 4 nm for other compounds.

III. RESULTS AND DISCUSSION

A. Optimization of CE separation conditions

The standard solutions of 4-MUNeu5Ac and 4-MU were mixed to the final concentration of 125 μ M and 200 μ M, respectively, and separated by CE. The separation conditions were optimized by investigating the effect of pH (5.0–9.0) and buffer concentration (10–50 mM) on the migration time and peak height of the analytes. The peak height of 4-MU decreased with the increase of pH and reached the minimum value at pH 7.5 (Fig. 2(a)). This was mainly due to the fact that faster migrating compounds would remain shorter in the detector cell to give a lower peak height.²⁸ At pH 8.0, the peak height of 4-MU increased as a result of the transformation of 4-MUNeu5Ac to 4-MU. The migration time of 4-MUNeu5Ac and 4-MU was much sensitive to the pH of BGE. The migration time of analytes became shorter with the increase of pH. As the increase of pH, the concentration of Si-OH on the capillary wall will increase, leading to faster velocity of electroosmotic flow (EOF) and shorter migration time. However, 4-MUNeu5Ac was labile when pH was higher than 7.0, therefore, pH 6.0 was chosen as the pH value of BGE. Higher buffer concentration can increase the peak height of 4-MU, while lengthen the migration time of the analytes (Fig. 2(b)). In addition, high concentration of the buffer would generate high Joule heating and raise the temperature in the capillary, which was

FIG. 2. Effect of pH and buffer concentration on the separation efficiency of substrate and product. CE conditions: fused-silica capillary, 50 μ m $\times$ 35.5 cm; BGE, 10 mM pH 5.0–8.0 phosphate sodium buffer; separation voltage, 20 kV; injection, 5 kPa, 6 s; UV detection, 320 nm; cartridge temperature, 30 °C; concentration of substrate and product, 125 and 200 μ M, respectively. Each point represents average value and error bars indicate SD; n = 3.

detrimental to peak shape and the separation efficiency. The migration times of the analytes were twice longer as buffer concentration increased from 10 mM to 20 mM. Hence, BGE consisting of pH 6.0 and buffer concentration of 10 mM was chosen as a compromise of short migration time and high peak height.

B. Optimization of plug mixing conditions

The most widely used in-capillary mixing technique is classical EMMA, which is based on the differences in electrophoretic mobilities of reactants. However, 4-MUNeu5Ac and NA had identical electrophoretic mobilities. Therefore, the TDLFP methodology was applied to mix the reactants in this study.

1. Injection sequence

It is important to determine the injection sequence for establishing the transverse diffusion mediated microanalysis method. The commonly used sandwich modes of enzyme-substrate-enzyme (E-S-E) and substrate-enzyme-substrate (S-E-S) were attempted. Fig. 3 shows the effect of different injection sequence on the peak area of 4-MU. No product was generated by the injection sequence of S-E and two splitted peaks of 4-Mu and 4-MUNeu5Ac were found by that of S-E-S, which may be attributed to the slow diffusion of NA and nonuniform mixing of plug zones. In turn, the injection sequence of E-S-E provided homogeneous mixing of reactants. At the same time, NA with small diffusion coefficient firstly injected into the capillary ensured transverse diffusion occurring at the end of the injection procedure. For inhibitors screening, to make sure the inhibitor in contact with NA at the same time as 4-MUNeu5Ac, the injection sequence of E-S-I was adopted.

2. Injection time

The TDLFP model regulated that the reagents should be injected into the capillary with high pressure and short time to obtain parabolic profiles.²⁶ It was reported that reactants injection at 3 kPa for 3 s,²⁹ 5 kPa for 3 s,³⁰ and 5 kPa for 6 s³¹ provided that profile. In this study, the injection at 5 kPa for 6 s was conducted. Then Y_0 value was calculated using Eq. (4) to make sure whether the profile of flow is parabolic. It was known that Y_0 was related to D , t_{inj} and r_i . The diffusion coefficient was estimated by the following equation:³²

FIG. 3. Effect of injection sequence on the online enzymatic reaction efficiency. E, enzyme; S, substrate. CE conditions: BGE, 10 mM pH 6.0 sodium phosphate buffer; concentration of substrate and enzyme, 125 μ M and 0.5 U/ml, respectively. Other conditions as in Fig. 2.

TABLE I. Parameters for efficient mixing by TDLFP.

	Injection time (t_{inj} , s)	Diffusion coefficient (D , $\text{cm}^2 \cdot \text{s}^{-1}$)	Y_0	Diffusion time (t_r , s)
4-MUNeu5Ac	6	1.03×10^{-5}	9.90	0.60
NA	6	5.50×10^{-7}	0.48	12.50
Inhibitor	6	1.14×10^{-5}	10.94	0.55

$$N = \frac{\mu_{app} \cdot V l}{2DL}, \quad (5)$$

where N is theoretical plate number; μ_{app} is the apparent electrophoretic mobility; V is the voltage applied to the capillary (V); L is the total length of the capillary (cm); l is the effective length of the capillary (cm). The diffusion coefficient of NA was reported to be $5.5 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$.³³ The results of the York number and transverse diffusion time were presented in Table I. The Y_0 value was in the range of 0.48–10.94, which demonstrated that the injection time of 6 s could provide parabolic profiles of plug zones. At the same time, relatively short transverse diffusion time was obtained in the range of 0.55–12.50 s, which showed that transverse diffusion occurred very quickly. The transverse diffusion time of NA is shorter than the injection time, which suggested that transverse diffusion did not occur during consecutive injection, which promoted a very effective mixing of reactants.

3. Injection length of BGE

To facilitate the mixing of reagents, BGE should be injected into the capillary at the end of the injection sequence. Krylov *et al.*²⁷ proved that the longer the injection length of BGE is, the higher the mixing efficiency is. However, this will engender a dilution effect and make the reactants distant from the capillary inlet and further reduce the separation efficiency. Generally, the length of the terminal plug of BGE was three times longer as the total length of reactants.²⁷ Hence, the mixing efficiency obtained by injecting BGE at 5 kPa for 50 s was investigated and compared with that obtained by shaking method of injection at alternating +5 kPa and −5 kPa for 6 s. As shown in Fig. 4, the peak area of 4-MU obtained by shaking method was increased by two times, which demonstrated that the shaking method increased the quality of mixing. It can be simply explained by the fact that the negative pressure reverses the parabolic profiles and the alternating backward and forward movement improves the mixing efficiency. In addition, shaking method has the advantage of decreasing dilution effect of reactants.²⁷

FIG. 4. Overlaid electropherograms obtained by BGE injection at ± 5 kPa for 6 s (a) and injection at 5 kPa for 50 s (b). CE conditions as in Fig. 3.

FIG. 5. Effect of incubation time on the peak area of 4-MU obtained by on-line enzymatic reaction. CE conditions were the same as Fig. 3. Each value is the mean of triplicate assays $\pm$ standard deviation.

4. Incubation time

After mixed in the capillary, the reactants need to be incubated for a while to generate adequate product for sensitive detection. The effect of incubation time in the range of 60–300 s on the peak area of 4-MU was investigated (Fig. 5). Every time point was assayed in triplicate. The peak area of 4-MU increased with the extension of incubation time and the plot of incubation time versus peak area of 4-MU was linear in 300 s. The conversion rate of 4-MUNeu5Ac to 4-MU reached 85%. Therefore, incubation time of 300 s was chosen for inhibitors screening.

C. Method validation

The standard solution of 100 μ M 4-MU was analyzed five times in one day or in five consecutive days to assess the intra-day and inter-day precision and the results were shown in Table II. The relative standard deviations (RSDs) of the migration time and peak area were less than 3%, which demonstrated that the CE separation method had good repeatability and good performance.

The enzyme and substrate were successively injected and reacted in the capillary under the optimal mixing conditions. The online enzymatic reaction by TDLFP was conducted five times in one day or in five consecutive days to evaluate the intra-day and inter-day precision. The RSDs of the migration time and peak area of 4-MU were less than 5% and 8%, respectively. The precision of peak area was a little inferior, which was most likely because the enzymatic reaction efficiency was susceptible to the ambient temperature variation.

D. Enzyme kinetics constant assay

The developed transverse diffusion mediated microanalysis was applied to assay the online NA kinetics constant (K_m). The peak area of 4-MU denoted the reaction velocity. The effect of

TABLE II. Intra-day and inter-day precision of standard 4-MU and on-line enzymatic reaction ($n = 5$).

	Intra-day precision (RSD%)		Inter-day precision (RSD%)	
	Migration time	Peak area	Migration time	Peak area
Standard	1.51	2.33	2.65	2.79
Online reaction	3.30	6.91	4.12	7.25

FIG. 6. Michaelis-Menten plot (a) and double reciprocal plot (b) for NA kinetics constant assay at varied concentrations of substrate ranging from 15 to 1000 μM by transverse diffusion mediated microanalysis. Incubation time, 60 s. Other conditions as in Fig. 3. Each value is the mean of triplicate assays $\pm$ standard deviation.

various concentration of substrate in the range of 15–1000 μM on the reaction velocity was investigated. Each concentration was analyzed in triplicate. The data were fitted to Lineweaver-Burk equation to obtain the double reciprocal curve of $1/[\text{substrate}]$ versus $1/[\text{velocity}]$ by Origin Pro 8.0 software (OriginLab, Northampton, MA, USA). The linear regression equation was $y = 3.6672x + 0.0234$ with a determination coefficient of 0.9976 (Fig. 6). The K_m value for NA was calculated by the slope and the intercept on the ordinate to be $156.72 \pm 15.22 \mu\text{M}$ which was consistent with the values reported.^{34–36}

FIG. 7. Typical electropherograms of the screened compounds detection at 320 nm (a) and at 320 nm with a reference wavelength of 260 nm (b). CE conditions as in Fig. 3.

FIG. 8. Typical electropherograms for inhibitors screening by transverse diffusion mediated microanalysis. Baicalein, baicalin, and vitexin were detected at 320 nm with a reference wavelength of 260 nm and chrysin was detected at 320 nm. Other CE conditions as in Fig. 3.

E. Inhibitors screening

Before inhibitors screening, 12 natural compounds, substrate, and product were separately analyzed under the optimal separation conditions. The results showed that the migration times of 4-MU, baicalein, and vitexin were identical and those of 4-MUNeu5Ac and baicalin were identical (Fig. 7(a)), which suggested that these inhibitors would interfere with the detection of 4-MU. In previous study, dual-wavelength detection was employed to eliminate the interference.²¹ As seen in Fig. 7(b), when detected at 320 nm with a reference wavelength of 260 nm, the peaks of baicalein, baicalin, and vitexin disappeared while the peak height of 4-MU scarcely changed.

To demonstrate the feasibility of the method for drug screening, 12 compounds from TCM at constant concentration of 60 μ M were screened for NA inhibition activity. Each compound was measured in triplicate. The inhibition of NA activity can be directly read out if the peak area of 4-MU is reduced compared with that obtained by online enzymatic reaction in the absence of inhibitor. The inhibition percentage was calculated by the following equation:

$$\text{Inhibition\%} = \left(1 - \frac{A_I}{A_S}\right) \times 100\%, \quad (6)$$

where A_S and A_I represent peak area of 4-MU obtained by online enzymatic reaction in the presence of substrate and inhibitor, respectively. It was found out that four of the natural compounds displayed potent inhibitory activity of NA. The typical electropherograms for inhibitors screening are shown in Fig. 8. The inhibition percentages of natural compounds are expressed as average value $\pm$ SD and summarized in Table III. The ranking of inhibitory potency was baicalein > baicalin > chrysin > vitexin, which was consistent with the structure-activity relationship study in literature.³⁷ The results imply that transverse diffusion mediated microanalysis is well suited for preliminary screening of NAIs.

TABLE III. Inhibition percentages for NAIs screening by transverse diffusion mediated microanalysis (n = 3).

Compounds	I%	Compounds	I%
Baicalein	68.35 $\pm$ 4.14%	Resveratrol	0%
Baicalin	64.07 $\pm$ 2.76%	Aconitine	0%
Vitexin	45.88 $\pm$ 2.99%	Lycorine	0%
Chrysin	47.30 $\pm$ 2.84%	Galanthamine	0%
Oleanolic acid	0%	Matrine	0%
Ursolic acid	0%	Oxymatrine	0%

IV. CONCLUSIONS

A CE method based on transverse diffusion mediated microanalysis was developed for the screening of NAIs from TCM. The kinetic constant of NA and the ranking of inhibition potency of four identified inhibitors were consistent with the results reported in literature, indicating that the method was suitable for NAIs screening. Distinct from the conventional screening methods, this method not only enabled the system full automation by integrating the multiple screening procedures into one capillary but also minimized the false positive results by CE separation with dual-wavelength detection. In particular, compared with other EMMA method, the method realized fast mixing of reactants with identical electrophoretic mobilities and reduced the screening cost by separately injection of the substrate, enzyme, and inhibitors. The research provided an automated fast approach for active constituents screening from TCM.

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