Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 1250–1253

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Screening α-glucosidase inhibitors from traditional Chinese drugs by capillary electrophoresis with electrophoretically mediated microanalysis

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ARTICLE INFO

Article history:
Received 16 April 2010
Received in revised form 26 July 2010
Accepted 27 July 2010
Available online 6 August 2010

Keywords:
α-Glucosidase inhibitor
Screening
Traditional Chinese drugs
Electrophoretically mediated microanalysis

ABSTRACT

In the present study, we report the study by a combination of electrophoretically mediated microanalysis method with a partial technique for screening α-glucosidase inhibitors from 21 traditional Chinese drugs. In the setup, substrates and enzymes were introduced into the capillary as distinct plugs, the electrophoretic conditions for enzyme reaction and separation of substrates and products were different in the composition and pH of the background electrolyte, which make more enzyme reactions possible. Part of the capillary was filled with the optimal buffer for the enzyme reaction, whereas the rest was filled with the background electrolyte optimal for the separation of substrates and products. With the optimal condition, the Michaelis–Menten constant and the inhibitive mechanism of acarbose were studied, which were in the same range as previous literature data. Furthermore, the inhibitory ratios of enzymatic activity (IRE) of 21 traditional Chinese drugs were determined. The classical method has superiorities over traditional assay methods, which not only minimizes the false-positive results but also simplifies the experimental processes. It could be used for screening inhibitors in natural extract.

1. Introduction

Diabetes mellitus is one of the most prevalent diseases in the world. In the recent years, the morbidity and mortality rates of diabetes rise rapidly. The main reasons for morbidity and mortality of diabetes are complications, such as diabetic retinopathy, nephropathy, neuropathy, microvascular complication and sexual dysfunction [1]. So diabetes has become the third largest health killer, which is only second to cancer and cardiovascular diseases. At present, the drugs for diabetes include sulfonylureas and biguanides. Both of them have some beneficial effects on hyperglycemia and also have some adverse effects. So it was an urgent need to have some new kinds of drugs to solve this problem until α-glucosidase inhibitors turned up. The α-glucosidase inhibitors which inhibit the enzyme at the brush border of small intestine are effective for delaying glucose absorption and preventing postprandial blood glucose level elevation, therefore they play a significant role in the therapy as chemotherapeutic agents for Noninsulin-dependent diabetes mellitus (NIDDM) [2,3]. Acarbose is the first α-glucosidase inhibitor [4,5] and miglitol and voglibose [6,7] are listed drugs of this kind on the market. However, few of these drugs are absorbed by small intestine and they have many adverse effects such as borborygmus, abdominal distention and sometimes diarrhea and stomach ache [8]. For these reasons, it is of great importance to exploit a new α-glucosidase inhibitor.

Ultraviolet spectrophotometry as the most classical analytical device was applied to assay the reduction of the enzyme activity in the presence of candidate compounds [9] to study the activity of the α-glucosidase inhibitors in vitro. The inhibitory ratios of enzymatic activity (IRE) were calculated [10–14] by detecting the values of absorbance of the products in the presence and absence of inhibitors. Fluorescence spectra (FS) and high efficiency liquid chromatography (HPLC) were used to search the inhibitive activity of traditional Chinese drugs [15]. The new procedure for the evaluation of enzyme reaction in CE, which was called electrophoretically mediated microanalysis (EMMA) method, has been reported for the analysis of the carbohydrate–enzyme reaction, especially the oligosaccharide-processing enzyme [16,17]. Kanie and Kanie described the assay for α-glucosidase and *p*-nitrophenyl-α-D-glucopyranoside (PNPG) by EMMA [18]. The method has combined enzymatic assays with capillary electrophoresis using regular capillary electrophoresis (CE) instrument or microfluidics devices. Such an approach has several advantages resulting from the unique combination of features of CE including the requirement for small quantities of both protein and sample, high resolution of CE, automation and multiplexing capabilities. EMMA is the analytical method of the on-column assays. It is of particular interest since all the steps of the assay take place in the capillary (including reaction, separation and detection) and it reduces the volume of the assay from μL to nL. One of the greatest advantages of EMMA is

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doi:10.1016/j.jpba.2010.07.041

that it provides separation of sample components after the chemical reaction with the substrates. In addition, the reaction products can be separated as well. Moreover, multiple simultaneous analyses of several analytes can be performed using this method [19–21].

The aim of the present work is to develop a simple EMMA-based CE method for screening α-glucosidase inhibitors in traditional Chinese drugs. In the present method, the enzyme activity is directly assayed by measuring the peak area of products of *p*-nitrophenol with UV detection at 191 nm. Experimental parameters are optimized and the method is validated with acarbose (a commercially available α-glucosidase inhibitor). Furthermore, the inhibitory ratios of enzymatic activity (IRE) of 21 traditional Chinese drugs are determined by the method.

2. Experimental

2.1. Apparatus and conditions

All separations were carried out on an Agilent (USA) HP^{3D} capillary electrophoresis system equipped with DAD detector. Uncoated fused-silica capillary (Yongnian, Hebei Province, China) was 75 μm ID × 60 cm in the length (8.5 cm to the detector). Data were handled by HP Chemstation software. The voltage for separation was 15 kV. The capillary was thermostatic at 37 °C and the detector was monitored at 191 nm. Before each assay, the capillary was rinsed with 1 M NaOH for 10 min, then with deionized water for 10 min; it was then conditioned with running electrolyte for 10 min. Prior to each sample injection, the capillary was flushed with running buffer for 1 min.

2.2. Materials and reagents

The 21 traditional Chinese drugs were purchased from Tongren drugstore of Qingdao, Shandong province, China. The *p*-nitrophenyl-α-D-glucopyranoside (PNPG) and α-glucosidase were obtained from Sigma (St. Louis, Missouri, USA). All chemicals were of analytical-reagent grade from Beijing Chemical Factory (Beijing, PR China). The deionized water was used throughout. All solutions and samples were filtered through 0.45 μm syringe filter.

The reaction solutions containing certain amounts of H_3PO_4 were adjusted to pH 7.0 with 0.1 M NaOH. The buffer contained 20 mM borate were adjusted to pH 9.2 by mixing 0.1 M HCl or 0.1 M NaOH. The PNPG solution of 4.0 mM was prepared in the 40 mM of H_3PO_4 buffer (pH 7.0). The α-glucosidase was diluted in 40 mM of H_3PO_4 buffer (pH 7.0) to the concentration of 1.0 mg mL^{-1}. The acarbose solution of 5 mg mL^{-1} was prepared in deionized water. All of the solutions were filtered through a 0.45 μm membrane filter, and degassed by the same procedure. The different concentrations of the sample solutions were prepared by appropriate dilution from the stock solution with deionized water.

These traditional Chinese drugs (1 g) were crushed and dipped in 20 mL distilled water, then put on the electric hot plate (100 °C) for 1 h. After being filtered through a piece of filter paper, they were condensed to dryness with rotary evaporator. The extracts were dissolved with 1 mL phosphate buffer with the concentration of 40 mM.

2.3. Procedures for EMMA assay

The α-glucosidase solution (0.2 mg mL^{-1}) in 40 mM phosphate buffer (pH 7.0) was introduced by 50 mbar pressure for 10 s into the capillary equilibrate. Then the solutions of PNPG in the presence and absence of the inhibitors were introduced by 50 mbar pressure for 5 s. Then the 20 mM borate buffer (pH 9.2) was performed at 15 kV as the electrolyte to separate the products of *p*-nitrophenol from the unreacted substrates of PNPG. The enzyme activities can be qualified by the different peak areas of products of *p*-nitrophenol in the presence and absence of the inhibitors. The enzyme reaction is shown in Fig. 1.

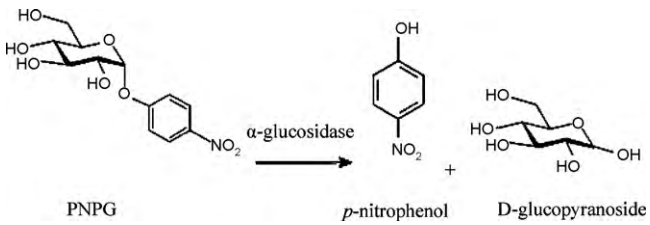

Fig. 1. The α-glucosidase-catalyzed reaction.

3. Results and discussion

3.1. Optimization of EMMA conditions

3.1.1. Effect of phosphate buffer concentration

It was important to use the proper concentration of phosphate buffer for the successful enzymatic reaction. To verify the influence of buffer concentration on EMMA, experiments were performed using the H_3PO_4 buffers at pH 7.0 with different concentrations (20, 40, 60, 80, 160 and 200 mM). Fig. 2 shows the influence of the different buffer concentrations on the enzymatic reaction. The result indicated that the yields of the *p*-nitrophenol increased with the increasing phosphate buffer concentrations from 20 to 200 mM. This indicated that the enzymatic reaction is dependent on the phosphate concentrations. However, the use of higher phosphate concentrations was basically avoided due to the inaccuracy associated with a baseline disturbance. Taking account of analytical time and separation effect, 40 mM phosphate buffer was selected for the following experiment.

3.1.2. Effect of incubation time

In the EMMA method, the incubation time of the enzymes and substrates before the separation can influence the enzymatic reaction. So the influence of incubation times (0, 2, 4, 6 min) were also investigated. Fig. 3 shows the influence of incubation time. Longer incubation time meant more yields of the products. However, the experiment result indicated that some substrates have converted into the products without the extra incubation time and the peak areas of the products in the presence and absence of the inhibitor were different obviously. Taking account of analytical time, no extra incubation time was used in the present study.

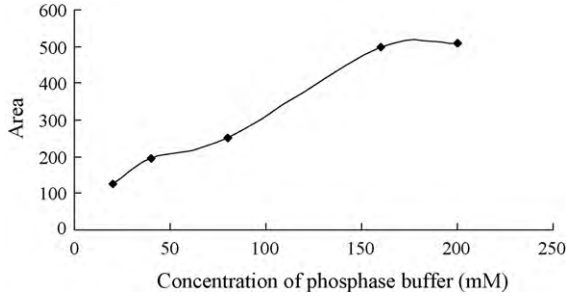

Fig. 2. The peak areas of *p*-nitrophenol changing with the different phosphate buffer concentrations. The buffer concentrations are 20, 40, 60, 80, 160 and 200 mM. Condition: uncoated fused-silica capillary, 50 μm ID × 60 cm in the length (8.5 cm to the detector); sample injection, enzyme 50 mbar for 10 s, substrate 50 mbar for 5 s; voltage for separation, 15 kV; running buffer, 20 mM borate buffer (pH 9.2), concentration of α-glucosidase, 0.2 mg mL^{-1}; concentration of PNPG, 0.5 mM; detection wavelength, 191 nm; column temperature, 37 °C.

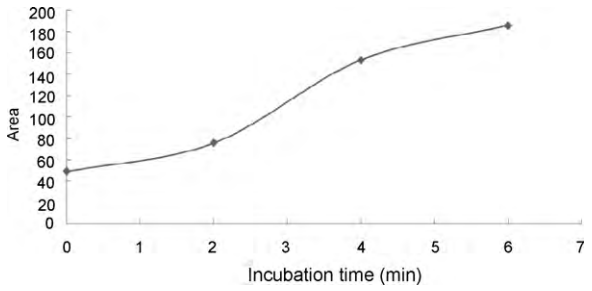

Fig. 3. The peak areas of *p*-nitrophenol changing with the incubation time of the enzyme and substrates. The incubation times are 0, 2, 4, 6 min. Condition: uncoated fused-silica capillary, 50 μm ID × 60 cm in the length (8.5 cm to the detector); sample injection, enzyme 50 mbar for 10 s, substrate 50 mbar for 5 s; voltage for separation, 15 kV; running buffer, 20 mM borate buffer (pH 9.2), concentration of α-glucosidase, 0.2 mg mL^{-1}; concentration of PNPG, 0.5 mM; detection wavelength, 191 nm; column temperature, 37 °C; the phosphate buffer concentration, 40 mM,(pH 9.2).

As can be seen, the optimal EMMA conditions were obtained .The reaction solution was 40 mM H_3PO_4 (pH 7.0) and no extra incubation time was used. The solution of α-glucosidase was introduced into the capillary, then the solution of PNPG was introduced. Then the 20 mM borate buffer (pH 9.2) was performed at 15 kV as the electrolyte to separate the products of *p*-nitrophenol from the unreacted substrates.

3.2. Kinetic parameters and the type of inhibition

We obtained Michaelis–Menten constant (K_m) of the reaction on the basis of the optimal conditions. The α-glucosidase solution was diluted at 0.2 mg mL^{-1}. Five different concentrations of PNPG were used, ranging from 0.4 to 1.2 mM. In the Michaelis–Menten plot, the corrected *p*-nitrophenol peak areas were presumed as initial reaction velocity. As shown in Fig. 4, the Michaelis–Menten constant was obtained by a Lineweaver–Burk plot. The K_m value for α-glucosidase was determined to be 0.63 μM. The value agrees with the literature values obtained by photometric assay method [18].

In the present research, acarbose (a commercially available α-glucosidase inhibitor) was employed as the model compound for the evaluation of our method. A series of experiments were carried out in varied concentrations of PNPG ranging from 0.1 to 0.8 mM at each different concentrations of acarbose. The data from these experiments were plotted by the method of Lineweaver–Burk. Lineweaver–Burk plot was obtained as shown in Fig. 5. The plot indicated that the acarbose was a competitive inhibitor, which is

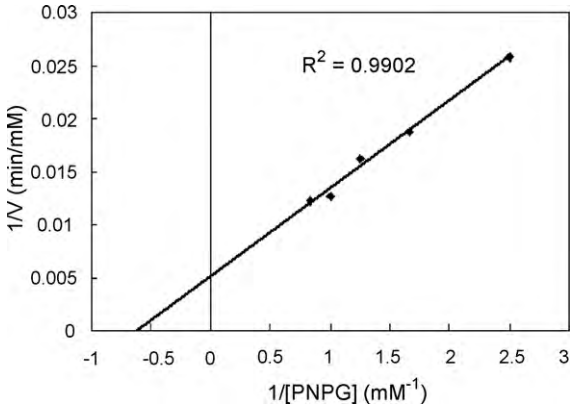

Fig. 4. Lineweaver–Burk plots of α-glucosidase assay. Concentration of the product [*p*-nitrophenol] was used instead of initial velocity. Concentration of α-glucosidase: 0.2 mg mL^{-1} and concentration of PNPG: 0.4, 0.6, 0.8, 1.0, 1.2 mM. Other conditions were similar to those shown in Fig. 3.

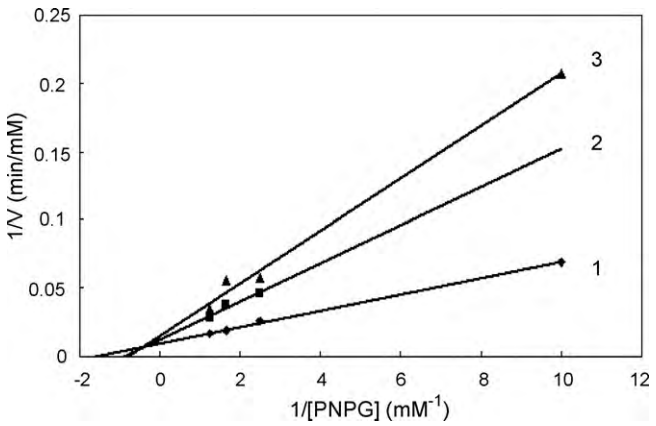

Fig. 5. Lineweaver–Burk plots of acarbose. The concentration of acarbose. 1: 0 mM; 2: 0.578 mM; 3: 1.156 mM. Other conditions are as shown in Fig. 3.

consistent with the literature [22]. K_i value of acarbose was determined as 1.088 mM by Dixon plot analysis. The IC_{50} (concentration of compounds at which the reaction was inhibited by 50%) with the in-capillary assay was 1.96 mM, derived from the K_i value calculated by Cheng and Prusoff's equation [23] giving the relationship between K_i and IC_{50}: $IC_{50} = K_i(1 + [S]/K_m)$. *S* is the substrate concentration, and K_m is the Michaelis–Menten constant. The IC_{50} was comparable with the literature values [22].

3.3. The inhibitory ratio of enzymatic activity (IRE) to α-glucosidase of the drugs

For the inhibition study and inhibitor screening, firstly the α-glucosidase solution was injected. Then the PNPG solution containing inhibitor or some natural extract with the final concentration 0.02 mg mL^{-1} was injected into the capillary. The concentrations of the α-glucosidase and PNPG were kept similar to the details mentioned-above. The electropherograms of acarbose and partial Chinese herbs for screening of α-glucosidase inhibitors are shown in Fig. 6. The areas of *p*-nitrophenol were identified in the presence and absence of acarbose and partial Chinese herbs. So the inhibitory ratios of enzymatic activity (IRE) of natural extract can be established, if the peak area of products of *p*-nitrophenol was reduced.

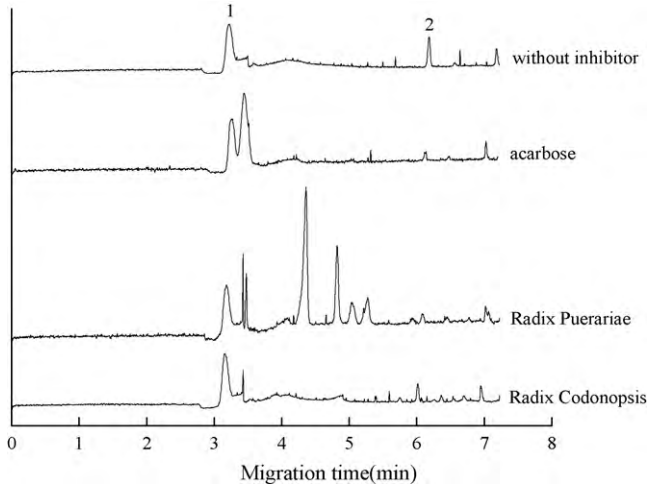

Fig. 6. Electropherograms for screening the α-glucosidase inhibitor. 1: PNPG; 2: *p*-nitrophenol. Conditions are as shown in Fig. 3.

Table 1
The inhibitory ratios of enzymatic activity for 21 different Chinese herbs.

Traditional Chinese drugs	IRE (%)	Traditional Chinese drugs	IRE (%)
Cortex Eucommiae	43.5	*Fructus Crataegi*	54.6
Fructus Forsythiae	45.5	*Radix Paeoniae Rubra*	
Radix Puerariae	69.7	*Caulis Spatholobi*	72.2
Folium Mori	63.3	*Radix Glycytthizae*	23.1
Raidix Sanguisorbae		*Rhizoma Phragmitis*	32.8
Rhizoma Polygoni Cuspidati	39.5	*Perillaseed*	23.1
Galla Chinensis	100	*Radix Rehmanniae*	39.1
Radix et Rhizoma Rhei	74.1	*Rhizoma Anemarrhenae*	33.7
Pollen Typhae	0	*Semen Alpiniae Katsumadai*	
Radix Codonopsis	49		
Flos Caryophylli	60.6		

The percentage of inhibition was determined according to the following equation:

$$\mathrm{IRE}\,(\%) = \frac{A_{(0)} - A_{(\mathrm{i})}}{A_{(0)}} \times 100$$

$A_{(0)}$ is the peak area value after reaction without sample. $A_{(\mathrm{i})}$ is the peak area value after reaction with sample.

To determine the inhibitory potency of α-glucosidase inhibitor with the developed in-capillary method, we compared the effect of 21 different traditional Chinese drugs at the same concentration of 20 mg mL^{-1}. The percentages of inhibition of 21 different Chinese herbs are summarized in Table 1. The products of *p*-nitrophenol in the *Semen Alpiniae Katsumadai*, *Radix Paeoniae Rubra* and *Raidix Sanguisorbae* were not separated from other components, so the IRE were not calculated. The IRE of *Galla Chinensis* attained 100%, the inhibitory activity was too strong, and may lead to hypoglycemia. If applied clinically, the dose of *Galla Chinensis* should be lower. The IRE of *Caulis Spatholobi*, *Flos Caryophylli*, *Radix et Rhizoma Rhei*, *Folium Mori*, *Radix Puerariae*, were between 55 and 75%, similar with acarbose and had potential as a source of α-glucosidase inhibitor. The IRE of the others were under 55%, which were too low. The IRE of the traditional Chinese herbs were consistent with the literature with ultraviolet spectrophotometry [13,24]. The difference in inhibitory potency of the inhibitor after in-capillary reaction implies that this method can be used for preliminary α-glucosidase inhibitor screening.

4. Conclusions

We have developed an EMMA-based approach for the screening of α-glucosidase inhibitors from traditional Chinese drugs by CE. The method not only simplified the screening procedure by directly assaying the reaction product, but also minimized the false-positive effect. The inhibitory activity of the traditional Chinese drugs as a whole can be easily found if the peak areas of the product are reduced. From the result, we can know that the EMMA is fit for the study of α-glucosidase enzyme activities and screening α-glucosidase inhibitors from natural extract. The method has proved to be rapid, simple, automatic, and robust.

Acknowledgement

This work was funded by the Chinese High-tech Project "863" (2006AA090401).

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