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Development of high performance liquid chromatography with immobilized enzyme onto magnetic nanospheres for screening enzyme inhibitor

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ABSTRACT

A novel-immobilized enzyme strategy created by magnetic nanospheres for monitoring enzyme activity and screening inhibitors followed by high performance liquid chromatography (HPLC) has been demonstrated. Through the reaction of the aldehyde groups with amine groups, α-glycosidase was simply and stably immobilized onto magnetic nanospheres by the cross-linking agent glutaraldehyde. In order to profiling the activity of the immobilized α-glucosidase, the natural substrate was hydrolyzed by it and the yield of product was determined by HPLC. Compared with traditional bioassay approach, the prepared immobilized α-glucosidase displays a high activity and stability which allows it to be easily reused for 10 times. Enzyme inhibition assays by known inhibitor glucobay and three candidate traditional Chinese medicines (TCMs) were then investigated using a similar methodology. This assay was able to readily detect the change of the immobilized enzyme activity based on measuring a decrease of product formation using HPLC. The approach is general and offers many attractive advantages including easy product isolation, inexpensive cost, and high efficiency in terms of reagent consumption.

1. Introduction

The biological activities of drugs are essentially associated with the binding to enzymes or receptors in vivo [1,2]. Thus, these enzymes play an important role in the development of new drugs [2]. The models focusing on the interaction between drug and enzyme or receptor have become one of the fundamentals for modern high-throughput screening of drugs [3,4]. So far, various methods have been developed to study activation and inactivation of enzymes [5]. Colorimetric bioassay is a common method used to investigate the inhibitory effects of herbal medicines [6], but it involves low reproducibility and large consumption of reagent. The fluorescence plate reader is another dominant tool for screening enzyme inhibitor [7–12]. However, these assays incorporating the fluorometric methods sometimes suffer from interference caused by the autofluorescence or the fluorescence quenching of the probe [13].

At present, immobilization of a protease on a solid support has been considered a potentially powerful procedure [14–17]. This is due to reduced autolysis of products, which allows for an effective protease concentration [18–23]. Several groups have explored the application of immobilized proteases, which would have a significant impact on large-scale proteomic research. Frechet and co-workers [24] reported that the enzymatic microreactor was prepared on a microfluidic chip by immobilizing trypsin on porous polymer monoliths. Tigrett et al. [25] bound trypsin to microparticles with a paramagnetic core, and utilized them in classical in-gel or in-solution digestion protocols, which replaces free trypsin. All of these findings are examples of how immobilizing a protease can contribute to proteomic research. Enzyme immobilization also plays an important role in pharmaceutical research. Mrksich and co-workers [26] established a peptide chip to measure protein kinases activities and to perform high-throughput inhibition screening. Such kinase-activity assays are important in the identification of lead compounds in drug discovery. Grasso et al. [27] immobilized the human matrix metalloproteinases (MMPs)-12 catalytic domain on the Au surface and demonstrated the applicability of the immobilized enzyme for rapid screening of MMP inhibitors. Srivastava and co-workers [28] introduced a novel immobilization technique for urease on glass-pH-electrode, and applied it to urea detection in blood serum.

Recently, the application of immobilized enzymes using magnetic polymer microspheres as carriers offers distinct advantages over other porous polymer monoliths or glass beads. One advantage is that they can be easily removed from the reaction mixture with the assistance of a magnetic field. This facilitates the separation and recycling of immobilized enzyme, as well as the purification of

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product. The possibility to recycle the studied enzyme or continuous reuse of enzyme is another outstanding advantage [29,30]. In our group, trypsin has been immobilized on magnetic microspheres through covalent binding. These novel-functionalized nanospheres were synthesized in earlier work by our group [31–35], and they show these advantages as an enzyme substrate in the application of on-plate digestion through its easy isolation, inexpensive cost [36–40].

Chromatography is one of the main techniques to separate complex mixtures due to its powerful separation efficiency. By coupling chromatography with different techniques, it provides a powerful tool for the inhibitor screening. On-line coupling of high performance liquid chromatography (HPLC) to a continuous-flow enzyme assay based on ESI-MS was established by Boer et al. [41]. More recently, a variety of chromatography-based immobilized enzyme methods have been developed for enzyme inhibitor screening. One method immobilizes an enzyme on silica parking materials, which was subsequently parked into a LC column as an enzyme reactor [42]. Kang and his co-workers created an on-column immobilized enzyme microreactor by an ionic binding technique, and applied such a microreactor for screening angiotensin-converting enzyme (ACE) inhibitors in a library of TCMs by capillary electrophoresis [4]. These are examples of how coupling chromatography with different techniques can be utilized to screen inhibitors.

The work described herein introduces enzyme inhibitor screening in traditional Chinese medicines (TCMs) by high performance liquid chromatography using magnetic nanospheres to immobilize an enzyme. The immobilization was carried out through the reaction of the aldehyde groups with amine-functionalized nanospheres. The feasibility was demonstrated when α-glucosidase was selected as the experimental model. α-Glucosidase is a key target for treatment of Type 2 diabetes. It can hydrolyze the *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) into *p*-nitrophenol. The inhibition of α-glucosidase is an alternative strategy for Type 2 diabetes management [43]. The performance of the immobilized α-glucosidase was discussed, and the repeatability of the immobilized enzyme was also evaluated. Then, Glucobay, the known α-glucosidase inhibitor (Bayer) was selected as for inhibition assay. Finally to confirm their use in complicated systems, we also utilized these enzyme-immobilized nanospheres to test various TCM extracts used in traditional Chinese medicine receipts for chemotherapy of diabetes.

2. Materials and methods

2.1. Chemical and materials

Acetonitrile was chromatographic grade (Merk, Germany). The α-glucosidase Type I and *p*NPG were purchased from Sigma Chemicals (USA). Distilled water was purified by Milli-Q system (Milford, MA, USA). Glucobay (Bayer, Beijing) was from local medicine store. TCMs, *Honeysuckle*, *Radix astragali*, and *Huperzia serrata*, were purchased from local Chinese medicine stores in Shanghai province of China. Other reagents were analytical grade. If unspecified otherwise, the buffer is 67 mM phosphate buffer, pH 6.8.

2.2. Instrumentation

Liquid chromatography was performed using an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto-sampler, a binary pump, a vacuum degasser, and a ultraviolet detector. The ODS column (200 mm × 4.6 mm, i.d.) packed with 5 μm Hypersil-ODS silica, were purchased from Dalian Elite Company (Dalian, China). Ultraviolet/vis spectrophotometer (mini 1240) was from Shimadzu (Japan).

2.3. HPLC analysis

Separation was carried out on ODS column: sample was separated with linear gradient elution of A: water/0.1% formic acid (v/v) and B: acetonitrile/0.1% formic acid (v/v); starting from 5% B to 15% B in 20 min, then to 40% B in 8 min, after rapidly up to 100% B in 1 min holding at 100% B in 5 min. Flow rate was set at 1 mL min^{-1}. Detection wavelength was 254 nm.

2.4. Extraction of TCMs

About 10 g of each TCM was pulverized and heated to boiling for 2 h with 100 mL 50% ethanol, respectively. Then the solvent of each extract was removed by using the rotary vacuum evaporator at 80 °C. Each residue was stored at 4 °C in the absence of light for subsequent experiments. Then they will be dissolved in buffer before enzyme assays.

2.5. Preparation of the amine-functionalized magnetic nanospheres

The amine-functionalized magnetic nanospheres were synthesized in our laboratory according to the reported protocol [44]. Briefly, the magnetic nanospheres were synthesized through solvothermal reaction using $FeCl_3 \cdot 6H_2O$ as a single iron source and 1,6-hexadiamine as the ligand. The mixed reagents were heated at 200 °C for 6 h in a teflon-lined stainless-steel autoclave, and then, the products were obtained and washed by using magnetic force.

2.6. Immobilization of α-glucosidase onto the amine-functionalized magnetic nanospheres

Three milligrams of amine-functionalized magnetic nanospheres were transferred to a 1.5-mL Eppendorf tube, and the nanospheres retained by a magnet. The solution was removed, and the nanospheres were resuspended in 200 μL coupling buffer (CB: 50 mM NH_4OAc, pH 8.3, 1 mM $CaCl_2$, 1 mM $MnCl_2$) again retained by a magnet, and the solution was removed. The amine group of the nanospheres was activated at room temperature under gentle rotation for 1.5 h by 200 μL 5% glutaraldehyde solution in CB (pH ~7.0). The nanospheres were then retained by a magnet and the glutaraldehyde solution was removed, followed by four times washing each in 200 μL CB. α-Glucosidase (1 mg) was dissolved in 400 μL CB containing 1% $NaCNBH_3$, and the nanospheres were incubated with the protein solution for 3 h under rotation. After removal of the unbounded α-glucosidase solution, the nanospheres were incubated for 1 h with 200 μL 0.75% glycine, 1% $NaCNBH_3$ in CB. Finally the nanospheres were washed four times in 200 μL CB, before it was ready for use. After α-glucosidase immobilization procedure was conducted, the magnetic nanospheres were retained by a magnet. The whole procedure for α-glucosidase immobilization is shown in Fig. 1.

The UV absorption value of the supernatant solution was measured at λ = 280 nm and compared to the UV absorption value of the α-glucosidase solution before immobilization to calculate the amount of α-glucosidase immobilized on the magnetic nanospheres.

2.7. α-Glucosidase assay

The α-glucosidase-immobilized magnetic nanospheres were dispersed in 400 μL 67 mM phosphate buffer (pH 6.8) to

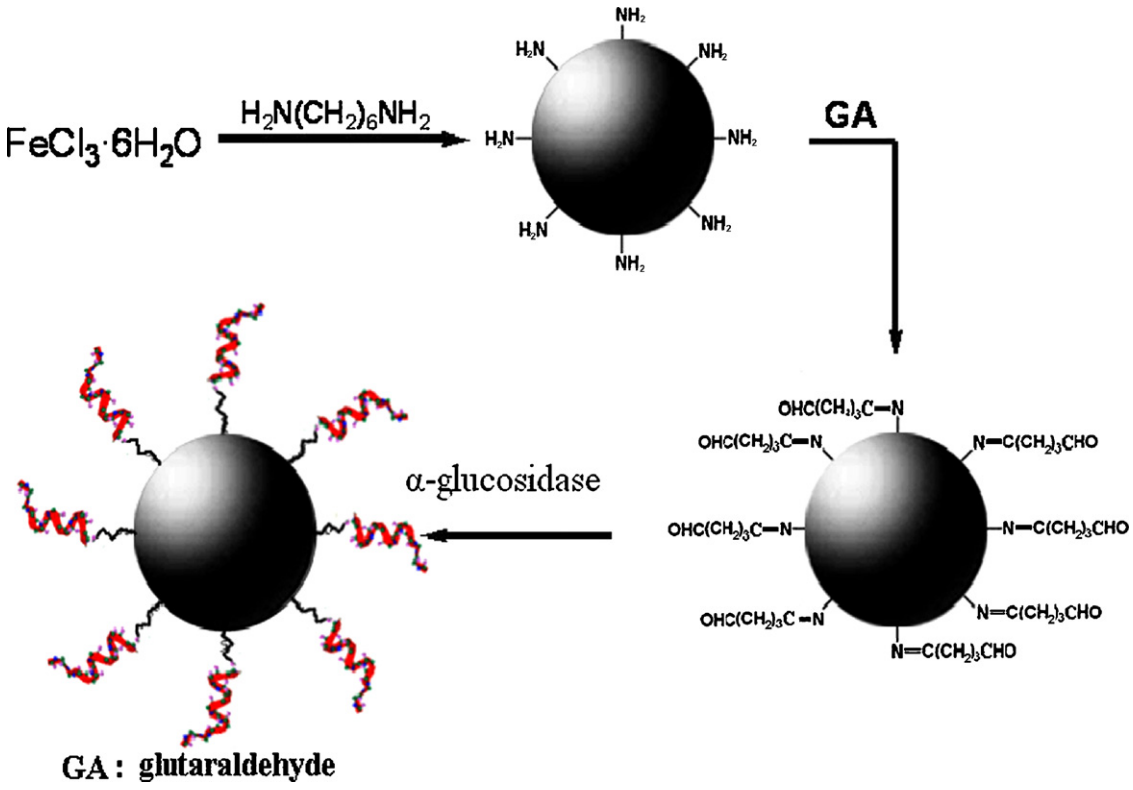

Fig. 1. Schematic illustration of α-glucosidase immobilization onto the amine-functionalized magnetic nanoparticles.

form a uniform suspension. Typically, the reaction mixture of α-glucosidase-immobilized nanospheres (3.75 μg enzyme) and substrate (1 mM *p*NPG), in the presence or absence of candidate medicines (5 mg), prepared in buffer to a final volume of 120 μL was incubated for 30 min at 37 °C. Then a magnet was used to gather and isolate the nanospheres. Finally, aliquot of 20 μL supernatant was analyzed by LC. For comparison, the assay was performed by free α-glucosidase in mixture according to the conventional procedure [6]: 100 μL mixture of 3.75 μg α-glucosidase and substrate (1 mM *p*NPG) was incubated for 30 min at 37 °C; then 20 μL acetonitrile was added to terminate the reaction.

2.8. Calibration curve and quantification of p-nitrophenol

The nine-point calibration curve was constructed by plotting peak area ratio (y) of product (*p*-nitrophenol) to the external standard versus *p*-nitrophenol concentration (x). The regression parameters of slope, intercept and correlation coefficient were calculated by Origin 7.0 software. Concentrations for the *p*-nitrophenol were calculated from the resulting peak area ratios and the regression equation of the calibration curve.

3. Results and discussion

3.1. Performance of immobilized α-glucosidase

The transmission electron microscope (TEM) image and FTIR spectrum (Figure S1 and S2) indicated that the as-prepared magnetic nanospheres were about 50 nm in diameter with narrow size distribution and were well functionalized with amine groups. The small sizes of the nanospheres provides large surface to volume ratio while the amine groups on their surface offer abundant reaction sites for enzyme immobilization. The immobilization ability of magnetic nanospheres for α-glucosidase was studied by measuring the UV absorption value of the supernatant α-glucosidase solution at λ = 280 nm after the immobilization procedure and the amount of α-glucosidase immobilized on the magnetic nanospheres is calculated to be about 50 μg mg^{-1}.

α-Glucosidase can easily digest *p*NPG and meanwhile release *p*-nitrophenol, which displays a visible yellow color. But first, the optimal conditions such as the buffer, incubating temperature, and incubating time must be established for α-glucosidase assay, due to the enzyme's sensitivity to pH value and temperature. Here, three different buffers were compared. The pH values were 4 (50 mM potassium hydrogen phthalate), 6.8 (67 mM phosphate) and 9.18 (10 mM borate), respectively. In the borate buffer, the mixture was converted to yellow compound the instant the substrate was added. The color of product (*p*-nitrophenol) was most obvious under alkaline background. According to the regression equation of external standard, amount of product at pH 9.18 was not higher than that with phosphate buffer (shown in Fig. 2a). It can be deduced that as soon as the immobilized enzyme contacted with the substrate, a little product was released rapidly and turned the solution bright yellow. Such a prompt color change increased difficulties in monitoring and controlling the enzymatic reaction. And when the mixture of enzyme and substrate was incubated with potassium hydrogen phthalate buffer at pH 4.0 at a temperature of 37 °C for 30 min, the solution was still white. No signal of product in chromatogram (amount of product is shown in Fig. 2a) indicated that there was no product released with the buffer at pH 4.0, and the immobilized enzyme turned to be inactive. Therefore, 67 mM phosphate at pH 6.8 was fixed for the following experiments.

Fig. 2 illustrates the peak area of product under various conditions. As above, enzyme displayed optimal performance with the buffer at pH 6.8 (Fig. 2a). Moreover, the effect of the incubation temperature on the yield of product was investigated. It was obvious that at the temperature 37 °C, the enzyme showed the highest activity (Fig. 2b). Thus, a temperature 37 °C was selected for the following

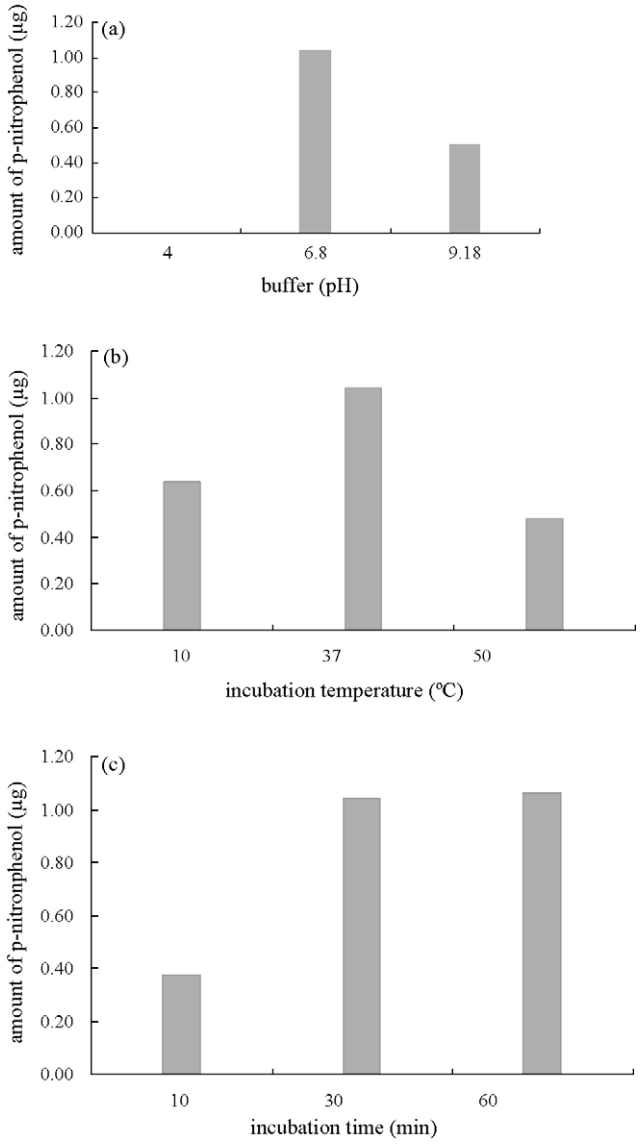

Fig. 2. Effect of incubation parameters against the amount of *p*-nitrophenol: (a) buffers; (b) incubation temperatures and (c) incubation time.

experiments. Additionally, optimization of the incubation time was also made. A 30-min incubation time should be used for the following since a maximum yield was achieved (shown in Fig. 2c). No significant increase of yield was observed as the incubation time was prolonged. Eventually in our experiments, we selected a 67 mM phosphate buffer, a reaction temperature at 37 °C, and a 30-min incubation time for all the experiments.

To demonstrate the performance of the immobilized enzyme, a free-enzyme reaction was investigated. With the same concentration of enzyme and substrate, the free α-glucosidase behaved a little less active than the immobilized enzyme. The assay for free enzyme was repeated for three times and the average amount of product was compared. The comparison of amount of product is shown in Fig. 3.

3.2. Recycle and reuse of enzyme

To allow continuous screening of inhibitors, the enzymes were recycled and reused. After enzymatic reaction, the supernatant was removed for analysis via LC. Three 200-μL washes of incubation

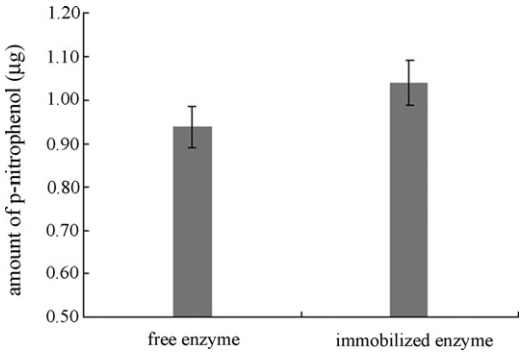

Fig. 3. Comparison of amount of product from free enzyme and immobilized enzyme. The error bar indicate standard deviations ($n = 3$).

buffer were added to the residual nanopheres followed by light shake, and removal of the supernatant. The immobilized enzymes were then denatured to release the bound compounds. Thus, the nanospheres could be used again for the next assay.

The repeatability of the immobilized enzyme was investigated by assaying the same immobilized enzyme activity for 10 times. The activity of immobilized enzyme in terms of the amount of the produced *p*-nitrophenol was evaluated through the LC with the comparison of regression equation. Ten chromatograms for α-glucosidase assays, which were operated with the same immobilized enzyme nanopheres were compared. No significant change of peak height was found. Typical chromatogram was shown in Fig. 4. The activity of the immobilized enzyme decreased a little at the eighth and ninth reaction. The RSD was calculated at 5.3%. The stability of immobilized enzyme was measured through inter-day assays. The capability of enzyme adhering to magnetic nanospheres was so strong that the enzyme still catalyzed the reaction very effectively in a long period of storage (5 days with RSD 3%).

It should be noted that although a little enzyme had been immobilized onto the nanospheres, its performance is sufficiently high enough to rapidly catalyze the reaction and keep a good repeatability. The immobilized enzyme can be used for at least 10 times.

3.3. Inhibitor screening

Glucobay contains a structural motif related to the substrate *p*NPG which may create reversible competing inhibition on α-glucosidase. Before performing the inhibitor screening, 5 mg Glucobay was selected to testify the application of immobilized

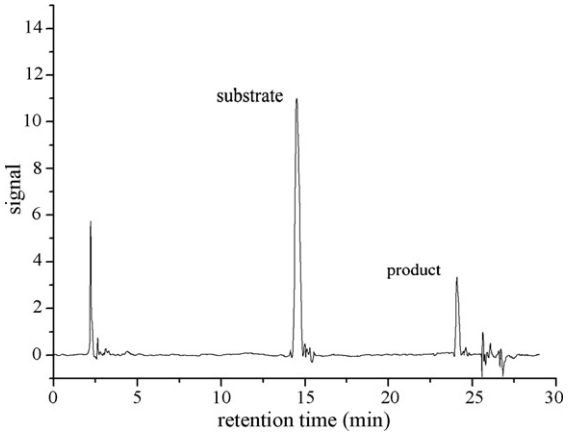

Fig. 4. Typical chromatogram of α-glucosidase assay reacted with the immobilized enzyme nanopheres. Conditions are shown in Section 2.

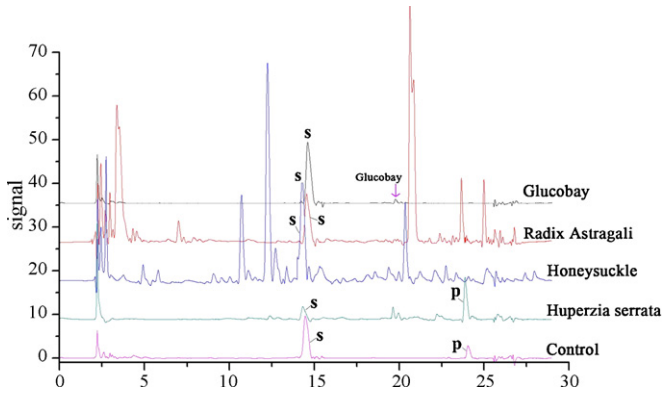

Fig. 5. Typical chromatograms for inhibition assay with Glucobay and screening inhibitor in TCMs (s, substrate; p, product). Conditions are same as Fig. 3.

enzyme. The typical chromatogram was shown in Fig. 5. Consistent with its pharmaceutical study and clinical application, here glucobay definitely inhibited α-glucosidase since the peak of product was disappeared. This proof offered the possibility of further inhibitor screening among complex system or library.

Then, three different TCMs, *R. astragali*, *Honeysuckle* and *Huperzia serrata* dissolved in the buffer were employed to evaluate the present immobilized enzyme. *Radix astragali* is recommended in Chinese pharmacopoeia for treatment of diabetes. Potential inhibitors are quickly identified by comparison of the spectra after incubation with or without TCMs; UV signal abundance of the product *p*-nitrophenol decreases or disappears after incubation if the candidate TCM inhibits the enzyme. Non-inhibitors show no change in peak area of product after incubation.

Finally, *Honeysuckle* and *R. astragali* were found to be inhibitors, while *Huperzia serrata* was not. This was consistent with the literature [45]. As shown in Fig. 5, the peak of *p*-nitrophenol was not presented after the substrate was incubated with the *Honeysuckle* or *R. astragali*. This indicated that these two herbals contain several active compounds inhibiting the α-glucosidase. More proof will be demanded for the further study.

3.4. Quantification of p-nitrophenol

We use the external standard method since it is simple, fast and accurate for sample preparation. The repeatability of retention time about *p*-nitrophenol during the precision studied was found to be excellent for all the solutions. The calibration curve for *p*-nitrophenol was constructed by analyzing a series of *p*-nitrophenol standard sample in the concentration range from 0.1 μg to 20 μg mL^{-1} and by plotting concentration versus peak area. The calibration curve showed good linearity in the 1–20 μg mL^{-1}. The regression equation was $y = 149.02x - 25.24$. Linear regression analysis of the data yielded a correlation coefficient (R^2) of 0.996.

4. Conclusions

A new strategy using α-glucosidase immobilized with magnetic nanospheres for screening enzyme inhibitors and monitoring enzyme activity has been developed. Compared with the conventional bioassay approach, this immobilized enzyme offers the advantages of easy isolation, inexpensive cost, and high efficiency. In particular, the immobilized enzyme can be recycled and lowers the consumption of reagent. Therefore, the screening cost can be dramatically reduced. In conclusion, the potential to screen enzyme inhibitors in a complex sample matrix, such as TCM extracts, would be the great benefit from the presented method.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2008.06.036.

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