Talanta 104 (2013) 17–21

Contents lists available at SciVerse ScienceDirect

Talanta

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

On-line solid phase extraction using organic–inorganic hybrid monolithic columns for the determination of trace β-lactam antibiotics in milk and water samples

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ARTICLE INFO

Article history:
Received 13 August 2012
Received in revised form
29 October 2012
Accepted 8 November 2012
Available online 17 November 2012

Keywords:
Epoxy-based monolithic column
On-line solid-phase extraction
Beta-lactam antibiotics
Milk
River water

ABSTRACT

A rapid and simple method with online solid phase extraction (SPE) has been developed for the simultaneous determination of beta-lactam antibiotics (BLAs) (amoxicillin, cephradine, and cefazolin sodium) in aquatic environment and milk. The epoxy-based organic–inorganic hybrid monolithic column was used as SPE sorbent to simultaneously monitor three analytes. The morphology of monolithic column and pressure drop across the columns were characterized. Excellent permeability and high selectivity were obtained. The linear range of the standard curve was from 2.0 to 500 ng/mL ($r^2 \geq 0.999$). Precision for inter- and intra-day assay showed acceptable results for quantitative assay with relative standard deviation (RSD) less than 11%. The accuracy and recovery were found to be within the range of 93–103% and 83–105%. The results indicated that the prepared monolithic column could provide excellent reproducibility and implied that the prepared monolith was feasible to be used as an on-line SPE sorbent material.

1. Introduction

In recent years, an increasing number of pharmaceuticals are being applied to animals and human beings. β-Lactam antibiotics have been the most widely used antimicrobial drugs for more than 80 years and still constitute the most important group of antibiotics [1]. Even the β-lactam which has poor stability is not possible to be excluded from the environment [2]. There are multiple sources for antibiotics entering the environment, including discharges from domestic river water treatment plants and pharmaceutical companies, dairy animal disease treatment [3]. Thus, the effluent that contains antibiotics may get diffused and accumulated in the surface waters, even ground water [4–6]. Those residual antibiotics have adverse effects on human health, such as sensitive individuals [7,8].

Amoxicillin, cephradine and cefazolin sodium are β-lactam antibiotics, which are widely used to treat bacterial infection of various organs [9], and the primarily distinguishing structural difference between penicillins and cephalosporins is the ring system fused to the β-lactam ring. Penicillins have a five membered thiazolidine ring while cephalosporins have a six membered dihydrothiazine ring (Fig. 1). Currently, a variety of chromatographic methods have been developed for the analysis of antibiotics. Sample pretreatment is typically achieved by gas chromatography (GC) equipped with a nitrogen phosphorus-specific, or a mass spectrometer (MS) detector, high-performance liquid chromatography (HPLC) combined with ultraviolet electrochemical fluorescence, liquid chromatography–mass spectrometry (LC–MS) or LC–MS–MS, enzyme-linked immunosorbent, liquid–liquid extraction, micellar liquid chromatography, and solid phase extraction (SPE) [10–16]. Solid phase extraction (SPE) is routinely used for preconcentration and clean-up in the analysis of biological and environmental samples. For the reason that it is simple, requires low volume of organic solvent, presents low contamination risk and can be used on-line [17–19]. Thus, SPE is often the sensitive and reliable analytical method to determine these compounds in milk and effluent river water [20–22].

In this paper, an organic–inorganic hybrid monolithic column prepared by hydrolysis/cocondensation of tetraethoxysilane (TEOS) and epoxy resin (EP) was used for the preconcentration and determination of β-lactam combined with C18 column in aquatic systems and milk. The morphology of the monolithic column has been characterized.

2. Experimental

2.1. Reagents and materials

Bisphenol A epoxy resin (ER) was purchased from Tianjin Hengtongda Co., ltd (Tianjin, China). Ethanol absolute was purchased from Tianjin Huadong Co., ltd (Tianjin, China). Tetraethylorthosilicate

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http://dx.doi.org/10.1016/j.talanta.2012.11.022

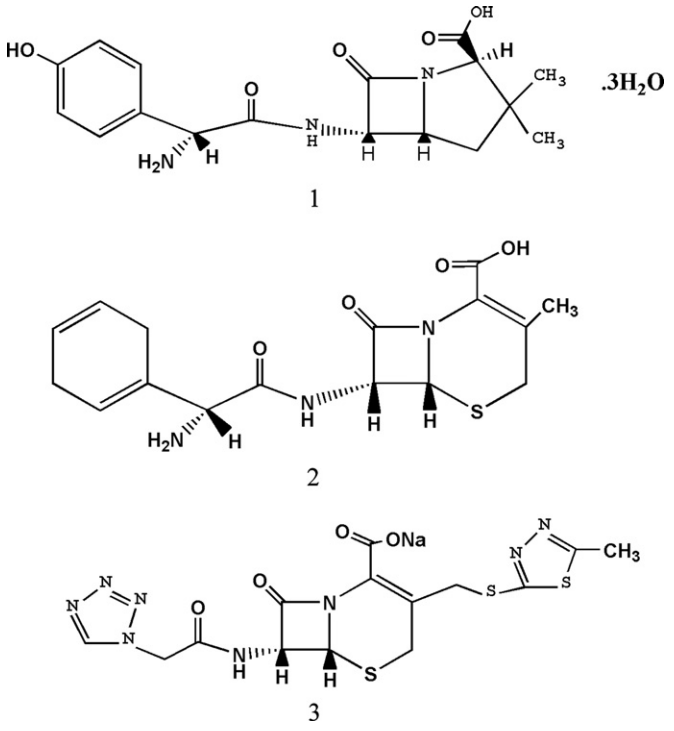

Fig. 1. Structural formula of amoxicillin (1), cephradine (2), and cefazolin sodium (3).

(TEOS) and γ-Aminopropyltriethoxysilane (KH550) were purchased from Hangzhou Guibao Co., ltd (Hangzhou, China). Acetic acid glacial was purchased from Tianjin Fuchen Co., ltd (Tianjin, China). Methanol was purchased from Tianjin Kemiou Co., ltd (Tianjin, China). Milk was purchased from a supermarket. River water obtained from a local river. Amoxicillin, cefazolin sodium and cephradine were purchased from Xingyao Science and Technology Co., ltd (Zhengzhou, China). Triple distilled water was used throughout all experiments. All reagents were of analytical reagent (AR) grade. All solutions used in HPLC were passed through a 0.45 μm nylon filter before use.

2.2. Preparation of calibration standards and samples

Amoxicillin, cephradine, and cefazolin sodium were dissolved in triple distilled water to obtain a concentration of 0.5 mg/mL each. Then, the mixed stock solution of amoxicillin, cephradine, and cefazolin sodium was also prepared at a concentration of 0.5 mg/mL in triple distilled water. The mixed stock solution was diluted with water to yield intermediate solutions of 5 μg/mL, 2 μg/mL, 1 μg/mL, 0.5 μg/mL, 0.25 μg/mL, 0.1 μg/mL, 0.05 μg/mL, and 0.02 μg/mL. These intermediate solutions were used to prepare standards at concentration of 500 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 10 ng/mL,5 ng/mL, and 2 ng/mL by diluting with blank milk and river water for milk samples and river water samples, respectively. Milk and river water were filtered through a 0.45 μm membrane. All the solutions mentioned above were stored at 4 °C until use. Quality control samples at three different concentration levels of 10 ng/mL, 50 ng/mL, and 500 ng/mL were prepared for evaluation of precision, accuracy and recovery in analysis of milk samples and river water samples.

2.3. Preparation and characterization of monolithic column

The organic–inorganic hybrid monolithic column was prepared briefly as follows: 0.6 g ER and 0.8 mL KH550 was mixed and shook for 1 min. The ER–KH550 mixture was stored in a refrigerator at 4 °C for 20 min. Then 0.8 mL EP–KH550 mixture, 1.0 mL TEOS and 0.6 mL 0.1 mol/L acetic acid glacial were all dissolved in 1.0 mL ethanol absolute. The mixture was sonicated and degassed for 10 min. Then, the mixture was poured into the 50 mm × 4.6 mm I.D. stainless steel column sealed at one end and then sealed at the other end. After the mixture was left to polymerize at 60 °C in a water bath for 48 h, the seals were removed from the tubes and the column was provided with fittings, attached to the HPLC system and washed the column with methanol for 1 h at a flow rate of 1 mL/min. The morphological properties of this monolith were studied by scanning electron microscopy (SEM) by Hitachi (Hitachi High Technologies, Tokyo, Japan) S-4300SEM instrument.

2.4. HPLC analysis

The HPLC measurements were performed using two LC-20AD pumps (loading pump and analytical pump) and a variable-wavelength SPD-20A detector (Shimadzu, Japan). Data processing was performed with an LC solution chromatography workstation (LC solution Software, Japan) The synthetic monolithic column was used as a pre-column and a C18 Dikma column (4.6 mm × 150 mm I.D.; 5 μm, Agilent, USA) was used as the analytical column. The mobile phase for enrichment was triple distilled water; the mobile phase for separation and analysis was methanol–water (63:37, v/v). The detection wavelength was set at 220 nm. The flow rate was set at 1 mL/min. The system was operated at ambient temperature.

2.5. Investigation of the pretreatment ability of the monolith

The deproteinization ability of the monolithic column was tested by directly injecting blank milk into the empty column and the monolithic column and eluted with triple distilled water at 280 nm. Meanwhile, ability of drug enrichment on the monolith was also investigated by injecting 1 μL of 5 μg/mL amoxicillin, cephradine, and cefazolin sodium solution into the monolithic column at 220 nm.

2.6. SPE

The monolith, which was used as SPE column for sample enrichment, was equilibrated with triple distilled water at a flow rate of 1 mL/min for 5 min. Then, 50 μL of spiked milk and river water standards were directly injected into the SPE column in the "load" position of six-port injector valve. Then the SPE column was washed with triple distilled water by the loading pump at a flow rate of 1.0 mL/min to remove protein and other impurities in milk and river water for 5 min, meanwhile analytes were enriched on SPE column. Lastly, the retained analytes were eluted with methanol–water (63:37, v/v) from SPE column onto the analytical C18 column for 20 min. The SPE column was washed with methanol and triple distilled water for the next injection.

3. Results and discussions

3.1. Characteristic features of the monolith

To evaluate the characteristic features of the synthetic monolithic column, the column was flushed with methanol to remove for 1 h at a flow rate of 1 mL/min. Then, using a small fragment of monolith sputtered with gold to carry out SEM. Fig. 2 shows the porous skeleton structure of the resultant monolith. The porousness offered a large number of channels, which allowed the mobile phase to flow.

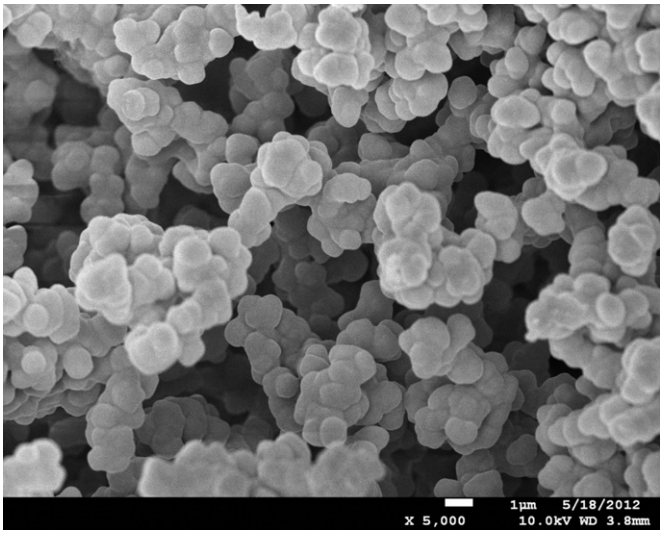

Fig. 2. SEM image of epoxy-based monolith.

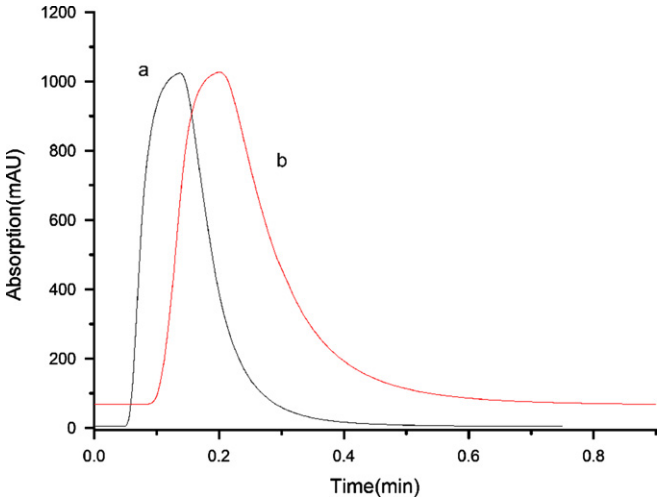

Fig. 3. Chromatograms of investigation of the sample pretreatment ability on the monolithic column. Column: 50 mm × 4.6 mm I.D.; flow rate: 1.0 mL/min; temperature: room temperature. 5 μL blank milk samples. (a) Empty column, (b) monolithic column; mobile phase: deionized water; detection wavelength: 280 nm.

3.2. Investigation of the pretreatment ability of the monolithic column

Fig. 3 shows nearly identical peak area of blank milk samples on the empty column (a) and the monolithic column (b) when triple distilled water was used as the elution solution. In view of this result, it could be regarded that nearly all of biological matrix compounds have been removed when using triple distilled water as the elution solution. Thus, the milk samples need not be defatted by centrifugation and deproteinated before analysis. So the experimental process could be further simplified.

3.3. SPE–HPLC

The milk samples were made to flow through the SPE column with pure water as mobile phase and then analyzed on the RP-C18 column. The experiments proved that amoxicillin, cephradine and cefazolin sodium could be baseline separated on the RP-C18 column when eluted using methanol–water (63:37, v/v) as the mobile phase at a flow rate of 1 mL/min. If water was substituted by buffer salt solution, peak shape could be improved, but the effect is not significant and system piping is easily blocked. So, methanol–water was selected as an eluent. The total analytical run time was 20 min. Fig. 4 shows the chromatograms of the spiked milk samples (a) containing amoxicillin, cephradine and cefazolin sodium using on-line SPE–HPLC system and blank milk samples (b). The spiked river water samples (Fig. 5a) containing amoxicillin, cephradine and cefazolin sodium and blank river water samples (Fig. 5b) obtained uniform results according to the operation procedure of the milk samples. It could be seen that amoxicillin, cephradine and cefazolin sodium were separated very well in Figs.4a and 5a; meanwhile, no interfering peaks from endogenous matrix components were observed near the retention time of amoxicillin, cephradine and cefazolin sodium, which demonstrated the deproteinization and sample enrichment which could be achieved at the same time by this approach.

3.4. Method validation

Selectivity, linearity, accuracy, precision (intra- and inter-day), recovery and reproducibility were assessed for the on-line SPE–HPLC method.

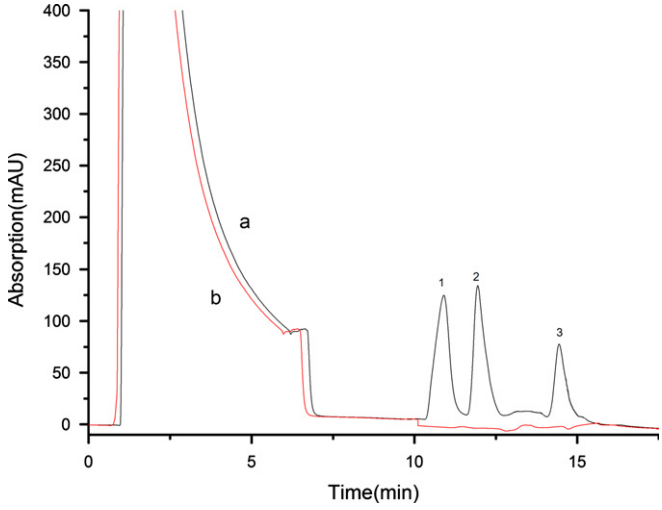

Fig. 4. Chromatograms for the gradient separation of mixture of amoxicillin (1), cephradine (2), and cefazolin sodium (3) in the milk sample at a concentration of 25 ng/mL (a) and blank milk sample (b). Elution: 63% methanol; Flow rate: 1.0 mL/min; Column: RP-C18 Dikma, 150 mm × 4.6 mm I.D.; UV detection: at 220 nm.

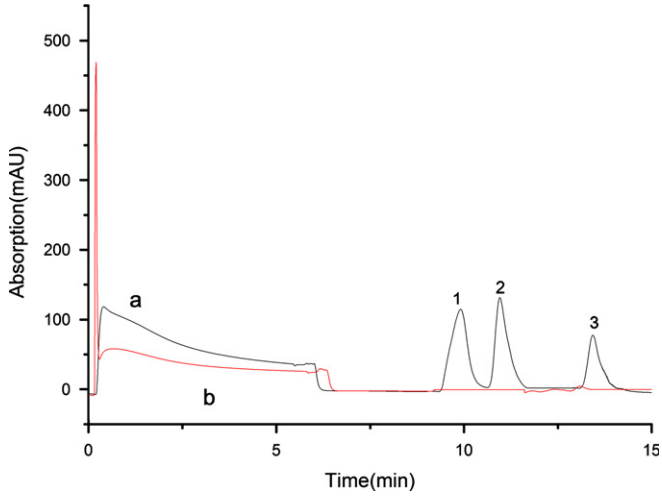

Fig. 5. Chromatograms for the gradient separation of mixture of amoxicillin (1), cephradine (2), and cefazolin sodium (3) in the river water sample at a concentration of 25 ng/mL (a) and blank river water sample (b). Elution: 63% methanol; Flow rate: 1.0 mL/min; Column: RP-C18 Dikma, 150 mm × 4.6 mm I.D.; UV detection: at 220 nm.

Table 1
Calibration curve, LOD and LOQ of amoxicillin, cephradine , and cefazolin sodium from milk and river water samples.

Analyte	*Samples solution*	*Calibration equations*	*Correlation coefficient*	*LOQ* (ng/mL)	*LOD* (ng/mL)
Amoxicillin	Milk	$Y=3.86\times10^3x+2.22\times10^4$	$R^2=0.9993$	10	2
	River water	$Y=3.32\times10^3x+2.98\times10^4$	$R^2=0.9992$	4	1.5
Cephalexin	Milk	$Y=9.03\times10^3x+2.83\times10^4$	$R^2=0.9991$	10	3
	River water	$Y=1.07\times10^3x+2.75\times10^4$	$R^2=0.9991$	5	2
Cefazolin sodium	Milk	$Y=3.43\times10^3x+7.54\times10^4$	$R^2=0.9993$	6	2
	River water	$Y=2.84\times10^3x+7.19\times10^4$	$R^2=0.9997$	4	1.5

3.4.1. Selectivity

The selectivity of the method was evaluated by comparing the chromatograms of the spiked samples containing amoxicillin, cephradine and cefazolin sodium with those blank milk and river water samples. As shown in Figs.4 and 5, they were free from significant interfering endogenous substances at the retention times for the selected drugs. These results showed that the developed method is selective and specific.

3.4.2. Linearity

The calibration curve was constructed from standard solutions at different concentrations (500 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 10 ng/mL, 5 ng/mL, and 2 ng/mL) of amoxicillin, cephradine and cefazolin sodium in milk and river water. Each sample of different concentrations was injected at least three times. As can be seen from Table 1, the results showed a linear relationship in the selected range. The correlation coefficients of all the equations were above 0.999. The limit of detection (LOD) and limit of quantification (LOQ) were calculated with signal-to-noise ratio equal to 3 and 10, respectively.

3.4.3. Precision and accuracy validation

The precision and accuracy of the method was determined using quality control (QC) samples at low, medium and high levels. The precision including the intra-day and inter-day experiments were applied to evaluate the relative standard deviation (RSD) of analytes concentration. The intra-day precision studies were performed at the optimal extraction condition for samples containing analytes of three concentration (10, 50 and 500 ng/mL) levels, respectively; each level was investigated at least in triplicates; while inter-day precision was determined by repetitive analysis of three concentration levels over five consecutive days. The accuracy of this method was obtained by the measured concentrations of amoxicillin, cephradine and cefazolin sodium in milk and river water samples according to the on-line SPE to those targeted concentrations. Table 2 shows good intra- and inter-day precision with RSD and accuracy values less than 11%. The results indicated that the reproducibility of method was excellent.

3.4.4. Recovery and reproducibility

The absolute recovery was measured by comparing the peak area measured after SPE–LC analysis of spiked urine and plasma samples to the peak area obtained by direct injection of amoxicillin, cephradine and cefazolin sodium dissolved in triple distilled water without SPE pretreatment. To check the reliability of this method, the method recovery was measured by comparing the concentration of analytes obtained from the calibration curve to the initial concentration of analytes in the spiked milk and river water standard. The results of recovery are shown in Table 3. The results showed that the recoveries were satisfactory and the method was acceptable for the analysis of milk and river water samples. Three monolithic columns were prepared with the same polymerization process as described in Section 2.2 and used the same process and conditions. The RSD of the retention time for the peak area were in the range of 2.6–3.9%, and the RSD for peak area were all less than 6%. These data revealed that the prepared monolithic column could provide excellent reproducibility and be use as an on-line SPE sorbent material.

Table 2
Intra- and inter-day precisions and accuracies of amoxicillin, cephradine , and cefazolin sodium from milk and river water samples at three different concentrations.

Analyte	*Samples solution*	*Concentration* (ng/mL)	*Precision RSD* (%)		*Accuracy* (%)
			Intra-day	*Inter-day*	
Amoxicillin	Milk	10	8.52	10.82	101.5
		50	7.48	8.18	101.7
		500	4.48	4.37	101.8
	River water	10	3.37	5.26	102.7
		50	4.50	5.26	93.1
		500	2.89	6.90	96.6
Cephalexin	Milk	10	6.79	5.32	102.9
		50	4.99	3.95	101.9
		500	3.80	3.40	102.9
	River water	10	3.38	4.20	96.2
		50	3.31	5.71	101.9
		500	3.50	5.40	98.2
Cefazolin sodium	Milk	10	5.00	6.75	91.9
		50	6.91	7.29	96.4
		500	2.30	4.38	98.3
	River water	10	5.78	6.77	99.9
		50	3.56	4.08	97.6
		500	3.15	6.53	97.2

Table 3
Recovery of amoxicillin, cephradine, and cefazolin sodium from milk and river water samples.

Analyte	*Samples solution*	*Concentration* (ng/mL)	*Absolute recovery* (%)	*Method recovery* (%)
Amoxicillin	Milk	10	99.9 ± 3.6	92.7 ± 4.4
		50	99.9 ± 3.2	92.7 ± 4.3
		500	100.6 ± 6.8	100.3 ± 6.3
	River water	10	99.8 ± 4.7	98.6 ± 2.7
		50	99.3 ± 3.5	90.4 ± 5.2
		500	101.4 ± 7.5	99.7 ± 5.4
Cephalexin	Milk	10	99.9 ± 5.14	98.9 ± 2.6
		50	91.2 ± 3.7	99.7 ± 3.6
		500	101.2 ± 4.3	99.7 ± 5.6
	River water	10	99.4 ± 2.3	99.2 ± 2.6
		50	99.9 ± 7.6	99.7 ± 2.1
		500	96.5 ± 8.3	99.7 ± 2.8
Cefazolin sodium	Milk	10	90.5 ± 4.9	98.7 ± 3.6
		50	100.2 ± 6.4	99.7 ± 3.2
		500	91.3 ± 7.2	99.7 ± 4.1
	River water	10	99.6 ± 3.4	99.8 ± 2.6
		50	95.3 ± 2.8	99.7 ± 1.2
		500	104.1 ± 7.6	99.7 ± 2.9

4. Conclusions

The epoxy-based monolithic column was used as SPE sorbent to simultaneously monitor three amoxicillin, cephradine and cefazolin sodium in milk and river water. In such approach, the sample pretreatment step was embedded into the LC chromatographic system and manual intervention was minimized. The resultant monolith possessed unique advantages of easy preparation, high permeability and selectivity for specific analytes. The good linearity, precision, accuracy and recovery were also achieved. The study provided a re-confirmation in the application of polymer monolith in bioanalysis, particularly for the sample pretreatment of complex biological matrices like milk and river water.

Acknowledgments

This work was supported by the Natural Science Foundation of Hebei University (no. 2009-165), the Key Project Foundation of Hebei Province Higher Education (no. ZD2010234), the Key Basic Research Foundation of Hebei Province (no. 11966411D), the National Natural Science Foundation of China (no. 21175031), the Pharmaceutical Joint Research Foundation of the Natural Science Foundation of Hebei Province and China Shiyao Pharmaceutical Group Co., Ltd. (no. B2011201174) and the issue of second batch of "twelfth Five-year Plan" of Key Special Project for "significant Drug Discovery" of Ministry of Science and Technology of China (no. 2012ZX09103-101-057).

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