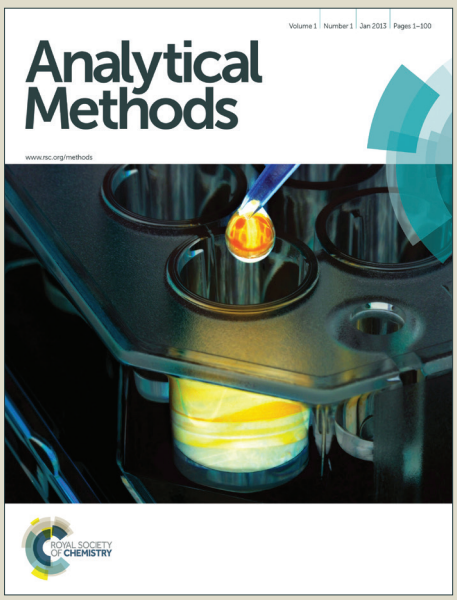
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

A new method to screening aldose reductase inhibitors using ultrahigh performance liquid chromatography-tandem mass spectrometry

Guangyue Hou [a,b], Ruixing Zhang [a,b], Zifeng Pi [a], Fengrui Song [a,*], Zhiqiang Liu [a], Shuying Liu [a]

[a] National Center of Mass Spectrometry in Changchun & Chemical Biology Laboratory, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China

[b] University of Chinese Academy of Sciences, Beijing, 100049, China

*Corresponding author: F. R. Song, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China.

Tel.: (086) 0431-85262044

Fax: (086) 0431-85262044

E-mail address: songfr@ciac.ac.cn

ABSTRACT

In this study, an ultrahigh performance liquid chromatography and tandem mass spectrometry (UPLC-MS/MS) method was developed for the detection of aldose reductase (AR) activity and the screening of AR inhibitors *in vitro*. Glucose was chosen as the substrate of the enzymatic reaction system according to the polyol pathway. In polyol pathway, glucose could be converted into sorbitol. The multiple reaction monitoring (MRM) mode was used to directly detected the level of sorbitol from each analyte and the amount of sorbitol could be reduced via the addition of an inhibitor. The proposed approach had been successfully applied to determine the AR inhibitory activities of epalrestat, flavonoids and natural products extracts. Additionally, we proposed a detailed discussion of the structure-activity relationships of flavonoids according to the inhibitory activities of thirty-two flavonoids. The screening method proposed in our study was similar to the metabolic pathway in diabetic patients and could eliminate the interference of other substances. We could envision that our method could be used for the screening of AR inhibitors as drugs for the treatment of related diabetic complications.

KEYWORDS Aldose reductase, UPLC-MS/MS, Sorbitol, Inhibitor, Screening method

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder disease characterized by chronic hyperglycemia. Diabetic patients usually suffer from complications and the long-term secondary complications are the main cause of morbidity and mortality.[1-4] Because the polyol pathway plays an important role in the development of some diabetic complications, it has been extensively studied.[5,6] Aldose reductase (AR, EC 1.1.1.21), the first and rate-limiting enzyme in the polyol pathway of diabetic patients, reduces glucose to sorbitol in the presence of NADPH.[7-9] It is known that sorbitol does not readily diffuse across cell membranes which results in its intracellular accumulation in insulin-resistant tissues such as small blood vessels, lens, nerves, retina and kidney. As a consequence, the excessive sorbitol seriously blocks the cell membrane pervasion, which ultimately leads to chronic complications of diabetes such as retinopathy, cataract, neuropathy, myocardial ischemic injury and atherosclerosis.[9-11]

AR inhibitors have been shown to be able to delay or substantially prevent the progression of certain long-term diabetic disorders. Until now, epalrestat is currently marketed for use in the treatment of diabetic neuropathy in Japan.[12] Additionally, a great deal of AR inhibitors obtained from natural sources such as flavonoids, stilbenes, coumarins, monoterpenes and related aromatic compounds have been reported.[13-15] Flavonoids are a group of about 5000 known compounds that are widely distributed as polyphenolic compounds in natural plants. These compounds generally possess the character of AR inhibitory activity as well as inherent antioxidant activity.

Consequently, flavonoids are considered as the promising candidates of drug in the treatment of diabetic complications.[15-17]

The traditional spectrometric method, developed by Hayman and Kinoshita, assessed AR activity by measuring the change in absorbance at 340 nm of NADPH, and the surrogate substrate of glucose was used.[18] However, the likelihood of false-positive results increased because NADPH was extremely unstable.[19,20] In view of the above-mentioned facts, the aim of the present work was to develop a direct and rapid method that could assay AR activity and screen AR inhibitors. In recent years, the liquid chromatography (LC) combining with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) has become a useful tool for screening enzyme inhibitors. The analytical method fulfills key requirements in terms of sensitivity, rapidity and selectivity for the analysis at low concentrations of analytes in the complex matrices.[21-25]

In the present study, glucose was chosen as the substrate of the enzymatic reaction *in vitro*. The reaction can be described as follows: glucose + NADPH → sorbitol + $NADP^+$. Then sorbitol was directly detected using ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method and the AR inhibitory activities of compounds were evaluated. The multiple reaction monitoring (MRM) mode, a sensitive and specific quantitative mode of MS/MS analysis, was used to monitor the mass-to-charge ratio (*m/z*) of parent ions and diagnostic fragment ions for the quantification of sorbitol. The experimental conditions including pH value, temperature, concentrations of glucose and NADPH,

and reaction time were firstly optimized. Furthermore, the validated approach had been successfully used to evaluate the AR inhibitory activities of epalrestat, flavonoids and natural products extracts. A detailed discussion of the structure-activity relationships of flavonoids was proposed according to the inhibitory activities of thirty-two flavonoids. The proposed approach to directly detect sorbitol was similar to the biological pathway in diabetic patients and the UPLC-MS/MS approach could eliminate the interference of other substances. Our detection method would have a good potential to be used for the detection of AR activity and the screening of AR inhibitors *in vitro*.

MATERIALS AND METHODS

Reagents and Materials

Sorbitol, xylitol, DL-glyceraldehyde, ammonium acetate, Tris (hydroxymethyl) metyl aminomethane, bis-acrylamide, bromophenol blue, sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylethylenediamine, acrylamide, glycine and commassie blue G-250 were purchased from Sigma Chem. Co. (St. Louis, MO, USA). NADPH-Na_4 was obtained from F. Hoffmann-La Roche Ltd (Basel, Switzerland). β-mercaptoethanol was purchased from Dingguo Biotec. Co. (Beijing, China). Epalrestat was from the Yangtze River Pharmaceutical Group (Taizhou, China). Flavonoids standards were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol of HPLC grade was supplied by Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was

purified using a Milli-Q plus water purification apparatus (Bedford, MA, USA). All other chemicals and reagents were at least of analytical grade.

Preparation of AR

According to the previously described methods, AR was partially purified from bovine lenses.[18,26] After homogenization, centrifugation, $(NH_4)_2SO_4$ fractionation (40-75%) and ultra-filtration, the crude AR was obtained. The whole preparation procedure was implemented with 2 mmol L^{-1} β-mercaptoethanol at 4 ℃. Furthermore, the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) approach was used to analyze the crude enzyme. According to the description in the book,[27] the discontinuous electrophoresis was employed in the experiment. The separating gel composed of 12% acrylamide was selected, while 5% acrylamide was applied in the stacking gel. A protein molecular weight marker (low) obtained from TaKaRa Biotechology Co. (Dalian, China) was used and the molecular weight range of the marker was 14.3-97.2 kDa. And finally, the activity of AR was performed as previously described[26] but with minor modifications. The AR activity was determined by measuring the decrease of NADPH absorption at 340 nm over 5 minutes.

Natural Products Extract Preparation

The natural products were obtained from Tongrentang Pharmacy (Changchun, China) and authenticated by Professor Shumin Wang (Changchun University of Traditional Chinese Medicine, China). Two grams of natural product powder through 100 mesh sieve were weighed accurately. 10 mL of 60% ethanol as the extracting solution was

added to the powder and then ultrasound-assisted extraction process was used for the extraction of bioactive valuable compounds from natural products at 30 ℃ for 40 min. Then, the extracts were centrifuged at 4000 rpm for 10 min and the supernatants were filtered through a 0.22 μm membrane filter. Afterward, the filtrates were quantified to 10 mL with the same solvent and the obtained stock solutions were stored at 4 ℃.

Reaction System for Screening AR Inhibitors

The enzyme reaction was performed in a total volume of 200 μL containing 100 mmol L^{-1} ammonium acetate buffer (pH 6.2), 5 mmol L^{-1} β-mercaptoethanol, optimized amount of enzyme, 0.45 mmol L^{-1} NADPH, 5 mmol L^{-1} glucose and different concentrations of samples (or corresponding solutions), and then the reaction was started by adding glucose. The reaction was terminated by adding 800 μL of methanol after 25 minutes at 37 ℃. Then, xylitol, the internal standard, was added to the reaction system with a final concentration of 1 μmol L^{-1}. Epalrestat was used as positive control in this study. The inhibiting capacity of analytes was evaluated according to the concentration of sorbitol in the enzymatic reaction system using the UPLC-MS/MS analysis. All the screening assays were performed in triplicate.

UPLC-MS/MS Analysis

UPLC-MS/MS analyses were performed on an ACQUITY^{TM} UPLC system (Waters, Milford, MA, USA) combined with a Xevo^{TM} TQ mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) interface Z-spray. UPLC system equipped with a binary solvent delivery system, an

auto-sampler and column heater. The chromatographic separation was achieved using a Waters UPLC® BEH Shield RP 18 column (50 mm×2.1 mm, 1.7 μm, Waters Corp., Milford, MA, USA) with pre-column 31PK, BEH C18 (5 mm×2.1 mm, 1.7 μm, Waters Corp., Milford, MA, USA). The mobile phase consisted of 20% solvent A (methanol) and 80% solvent B (water), delivered at a flow rate of 0.2 mL min^{-1}. The temperatures of sample manager and separation column heater were set at 4 and 35 ℃, respectively. The injection volume of sample was 3 μL. The ion source temperature and desolvation temperature were maintained at 150 and 350 ℃, respectively. The spray voltage was set at 3.0 kV. Nitrogen was supplied as the cone gas and desolvation gas at flows of 60 and 800 L h^{-1}, respectively, while argon was used as collision gas at a pressure of 0.1 MPa and at a flow of 0.0096 L h^{-1}. The tandem mass spectrometry analyses were operated in the MRM mode for the accurate quantitative analysis in positive mode. The direct flow-injection mode was applied to optimize the cone voltages and the collision energies.

Validation of the Method

In order to ensure the obtained results were reliable, the suitability of the approach was properly verified prior to its application in real samples. Method validation was implemented via evaluating performance characteristics including linearity, accuracy, precision, matrix effects, limit of quantification (LOQ) and limit of detection (LOD).

The precision of the method was determined by the replicate analyses of analytes containing sorbitol at all concentrations utilized to construct calibration curve. A

series of samples with different concentration levels dissolved in enzymatic reaction buffer solution were utilized for the construction of calibration curves. LOD and LOQ are used to describe the lowest concentrations of an analyte that can be reliably measured by an analytical procedure. In cases where noise was present in the chromatogram of the analyte, the signal-to-noise values of at least three at LOD and ten at LOQ, were required. The intra-day and inter-day precision and accuracy were investigated at three levels (five parallels of each concentration). The accuracy of the method was expressed by relative error (RE), while the precision was estimated as the relative standard deviation (RSD). The suitability of the precision and accuracy was assessed by the following criteria: the RSD should not exceed 15% and the accuracy should be within 15% of the actual values. The matrix effects occurring during electrospray ionization (ESI) process of natural products extracts were quantitatively assessed using post-extraction spike method in this study. The matrix effect was evaluated by comparing the response of an analyte in neat solution to the response of the analyte spiked into a blank matrix sample that has been carried through the sample preparation process.[28,29]

RESULTS AND DISCUSSION

Verification of AR

An apparent strip of the crude enzyme appeared at relative molecular mass of 36 kDa in comparison with the bands of electrophoretic marker protein. An appropriate blank, without substrate, was employed for corrections in the AR activity assay

process. In our experiment, one enzyme unit was defined as the amount of the enzyme that catalyzes the conversion of one micro mole of NADPH per minute, and the temperature was usually specified at 25 ℃. The result indicated that the enzyme activity unit of crude AR was 14.3. Therefore, the crude enzyme could be applied to screening AR inhibitors *in vitro*.

UPLC-MS/MS Approach Development

In our experiment, to develop a suitable and robust LC approach, in the preliminary experiments several experimental parameters were investigated, including the flow rate, the column temperature, the mobile phase and the gradient elution program. Because of the high selectivity achieved in MRM detection mode, a complete chromatographic resolution between sorbitol and xylitol is not necessary. In the experiment, the main function of the chromatographic column was to eliminate interference and thereby increase the sensitivity of analytes. Under those conditions, an isocratic separation elution was applied for the chromatographic separation and the binary mobile phase was comprised of 20% methanol and 80% water. Furthermore, other parameters such as flow rate and column temperature were ascertained in order to stabilize the retention times.

The IntelliStart function was utilized to optimize the cone voltages and the collision energies prior to the UPLC-MS/MS analysis. The appropriate MS parameters were obtained via the combined mode, which was more consistent with the injection status of real samples. To collect sufficient data for the integrative research, two precursor/product ion transitions of the target were chosen to create

MS/MS approach. The highest sensitive ion transition was selected as the quantitative ion pair, while another one was chosen for qualitative analysis for the confirmatory analysis. The product ion spectra and the UPLC-MS/MS chromatograms of the quantitative transitions of sorbitol and xylitol in positive ion mode were shown in Figure 1. The ions marked with asterisk (*) were used for the quantitative analysis, while the confirmatory ions were marked with hash (#). The cone voltages of quantitative ions were both of 16 V, while the collision energy was 10 eV. The data were acquired via MassLynx4.1 software (Waters), and the MassLynx4.1 with TargetLynx was applied to data processing.

The AR inhibition (%) was calculated as follows: AR inhibition (%) = $[(C_b - C_a) / C_b] \times 100\%$ Eq. (1). Where, C_a and C_b denoted the sorbitol concentration of the enzymatic reaction with and without the screening compounds via UPLC-MS/MS analysis, respectively. The inhibiting capacity of compounds was indicated via the AR inhibition ratio. The inhibiting degree was calculated using Eq. (1).

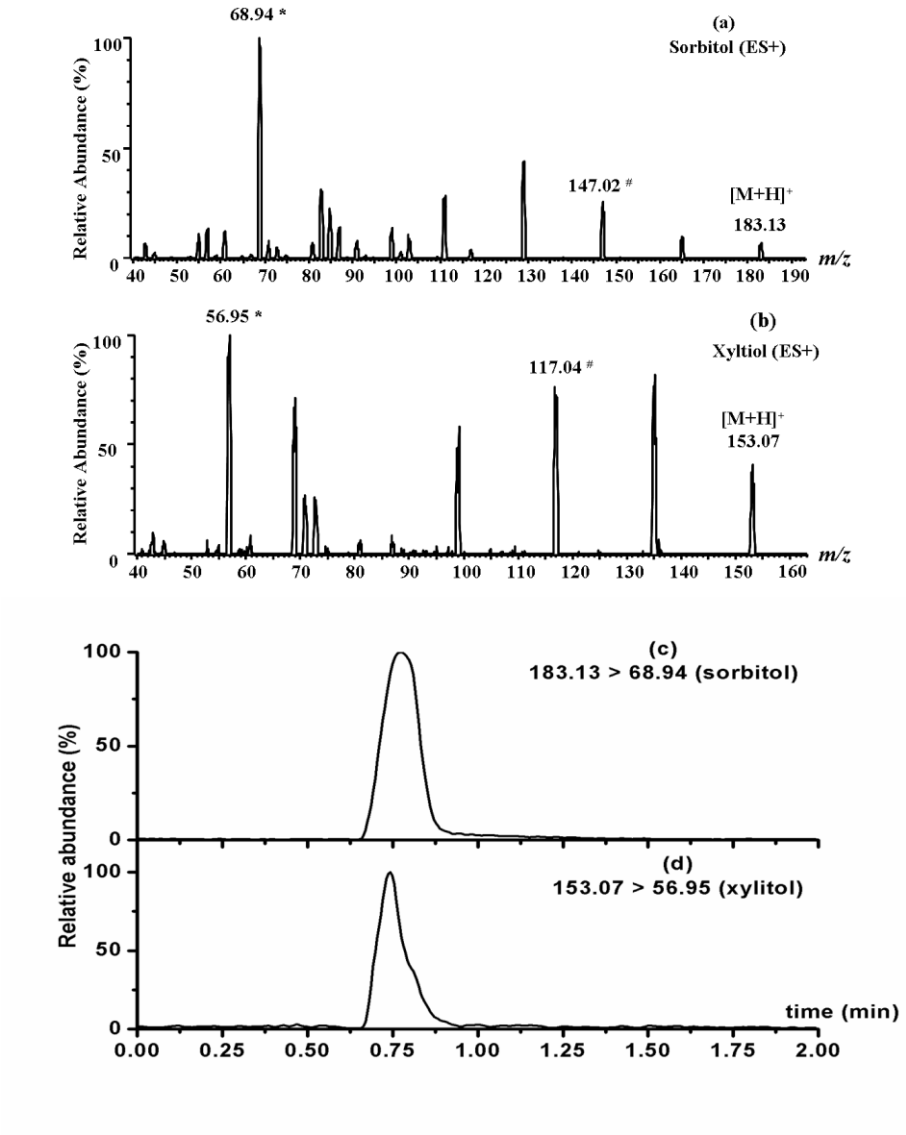


Figure 1 The product ion spectra ((a) and (b)) and chromatograms ((c) and (d)) of sorbitol and xylitol

Ions marked with asterisk (*) were the quantitative ions, Ions marked with hash (#) were the qualitative ions.

Validation of UPLC-MS/MS Approach

Peak area ratios (sorbitol/xylitol) of the analytes were utilized for the construction of linear calibration curve using UPLC-MS/MS method. Calibration (Table 1) was corrected by weighting coefficient 1/x, and the concentration range of

sorbitol was between 0.1 and 50 μmol L^{-1}. The standard curve, which was not forced through the origin, presented excellent linearity with a correlation coefficient of 0.996. Meanwhile the LOD was appropriate for quantitative detection of analytes in the enzymic reaction systems. The precision and accuracy results are shown in Table 2, which met the requirements of an analytical assay. The matrix effect was evaluated by comparing the response of an analyte in neat solution to the response of the analyte spiked into a blank matrix sample (*Radix Scutellariae*, *Folium Ginkgo*, *Fructus Evodiae*, *Cortex Phellodendri*, *Rhizoma Anemarrhenae* and *Panax notoginseng*). The assessment of matrix effect indicated that the proposed method was free from matrix effect contributions because the value is greater than 85% and less than 110%.

Table 1 The regression data, LOQ and LOD of sorbitol in our experiment.

compound	Linear range (μmol L^{-1})	Linear regression equation	correlation coefficient	LOQ (μmol L^{-1})	LOD (μmol L^{-1})
sorbitol	0.1-50	y=0.74x + 0.097	0.996	0.10	0.03

Table 2 Precision and accuracy of sorbitol of the UPLC-MS/MS method (n=5).

concentration added (μmol L^{-1})	Intra-day concentration measured (μmol L^{-1})	Precision (%, RSD)	Accuracy (%, RE)	Inter-day concentration measured (μmol L^{-1})	Precision (%, RSD)	Accuracy (%, RE)
0.20	0.19 ± 0.01	4.67	3.43	0.21 ± 0.01	4.09	1.34
2.00	2.04 ± 0.11	3.07	1.82	2.04 ± 0.04	1.79	1.92
20.00	20.33 ± 0.73	2.41	1.65	19.80 ± 0.59	2.84	0.99

relative standard deviation (RSD); relative error (RE)

Optimization of Enzymatic Reaction Conditions

Effect of pH Value and Temperature on the Enzymatic Reaction

Previous studies have indicated that enzymatic reaction should be carried out under acidic conditions,[26] thus the ammonium acetate buffer solution was elected as the reaction medium because it can provide an acidic reaction conditions and could be compatible with MS. The effect of pH value on the enzymatic reaction was investigated at different pH values (pH5.6, pH6.0, pH6.2 and pH6.4). The level of sorbitol was increased firstly with increasing pH values. Finally, pH 6.2 was chosen as the optimum pH value because the amount of sorbitol achieved a maximum at pH 6.2 (Figure 2-a). The result might be attributed to that the optimum pH was 6.2 for AR in our experiment. The effect of temperature was studied in the temperature range of 25-40 ℃ (Figure 2-b). Finally, 37 ℃ was selected as the reaction temperature in this experiment.

Optimization of Concentrations of Glucose, NADPH and AR

The influence of glucose concentration was investigated by varying the concentrations in the range from 0.2 mmol L^{-1} to 10 mmol L^{-1}. In order to figure out the effect of glucose concentration, 10 μmol L^{-1} epalrestat as the positive control was added into the reaction system and the AR inhibition (%) was calculated using Eq. (1). Figure 2-c showed that the AR inhibition of epalrestat was changed as the glucose concentration changed. According to the above result, 5 mmol L^{-1} of glucose was employed in this study. Under the same experimental conditions, the effect of NADPH concentration was explored in the concentration range of 30-600 μmol L^{-1}

(Figure 2-d). Accordingly, 450 μmol L^{-1} was chosen as the optimum concentration of NADPH. The influence of crude AR concentration was also investigated in this study. Both detection sensitivity and cost-effective were considered in this procedure. Based on the above analysis and the results (Figure 2-e), 30 μL of crude AR was applied into the further research.

Effect of Reaction Time and Quenching

The influence of reaction time was investigated at different reaction time points (from 2 to 50 minute). Figure 2-f shows that the amount of sorbitol at different reaction time. Considering the screening cycles and detection sensitivity, 25 minute was chosen as the optimum reaction time. After a defined reaction time, the enzymatic reaction was terminated by addition of methanol, which can inactivate the enzyme. Then, the different amount of methanol (400 μL, 600 μL and 800 μL) were investigated. The results indicated that 800 μL of methanol could completely terminate the enzymatic reaction.

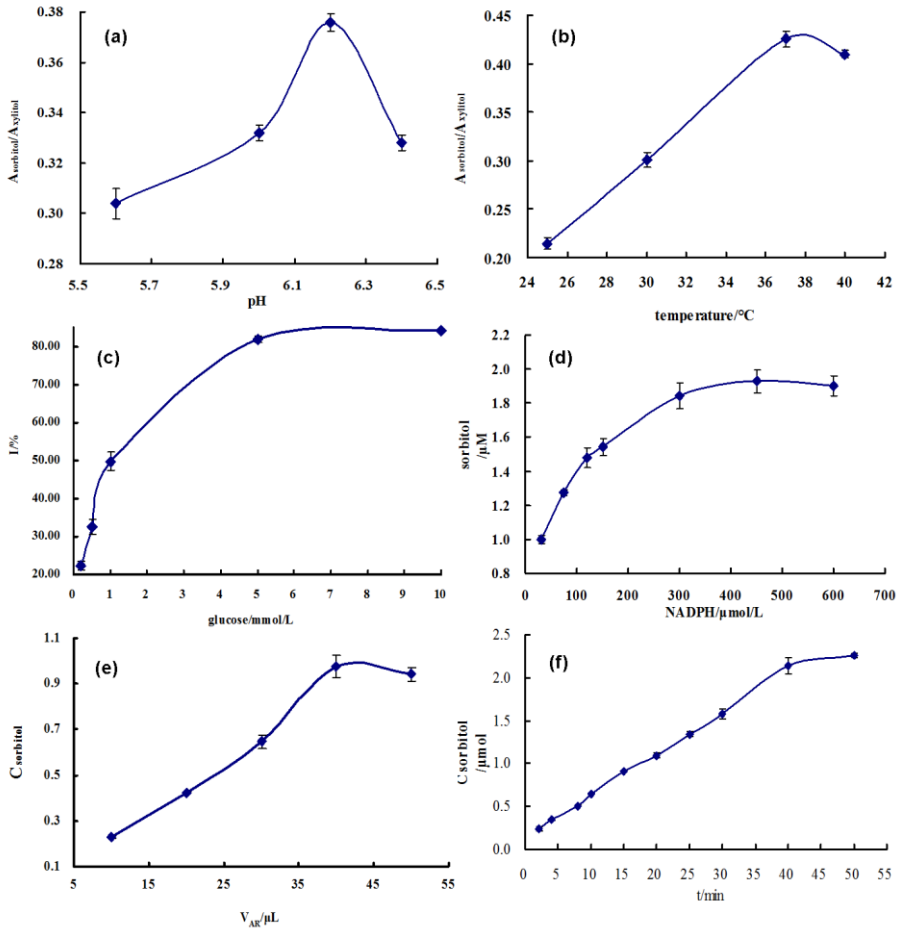


Figure 2 The effect of enzymatic reaction conditions (a) pH value, (b) reaction temperature, (c) glucose concentration, (d) concentration of NADPH, (e) crude AR concentrtion, (f) reaction time on the enzymatic reaction.

Validation of the Proposed Screening Method

IC_{50} value refers to the micromolar concentration of the inhibitor required to produce a 50% inhibition of the enzyme-catalyzed reaction. The IC_{50} value of epalrestat was evaluated for the validation of the proposed approach. In the procedure, IC_{50} was calculated using non-linear regression analysis of the plot of percent inhibition versus log inhibitor concentration (GraphPad Prism software). The results showed that the IC_{50} value of epalrestat was (0.771 ± 0.004) μmol L^{-1} which was

consistent with the result reported in previous research,[30] and this could validate the accuracy and feasibility of the screening method.

Application to Flavonoids

The AR inhibitory activities of flavonoids were determined by the proposed method. The AR inhibitory activities of flavonols investigated in this study were expressed as IC_{50} value and a higher IC_{50} value indicated lower effectiveness in inhibition of AR activity. Here, the IC_{50} values of myricitrin, quercitrin, morin and rutin were (5.82 ± 0.08) μmol L^{-1}, (16.48 ± 1.19) μmol L^{-1}, (15.88 ± 0.08) μmol L^{-1} and (18.39 ± 0.74) μmol L^{-1}, respectively. Additionally, the AR inhibitory activities of flavones, flavonols, flavavones and isoflavones were illustrated by their percentage inhibition (%) in the same final concentration (50 μmol L^{-1}). The screening results shown in Table 3 indicated that the capacity of flavonoids as AR inhibitors depended upon their molecular structures.

Table 3 The aldose reductase (AR) inhibition ratios of flavonoids analyzed using UPLC-MS/MS method.

flavonoids		AR inhibition (%, ± SD, n=3)	flavonoids		AR inhibition (%, ± SD, n=3)
flavone	luteolin	47.09 ± 0.32	flavonol	myricitrin	66.09 ± 1.28
	baicalin	29.97 ± 0.11		quercitrin	61.08 ± 0.20
	baicalein	29.37 ± 0.44		morin	59.53 ± 0.06
	luteoloside	24.56 ± 0.18		rutin	51.00 ± 0.16
	vitexin	22.35 ± 0.28		hyperin	43.67 ± 0.36
	isovitexin	20.63 ± 0.26		myricetin	42.94 ± 0.14
	apigenin	18.53 ± 0.50		quercetin	26.02 ± 0.64
	acacetin	6.55 ± 0.56		isorhamnetin	12.19 ± 0.94
	wogonin	4.96 ± 0.32		kaempferol	1.79 ± 0.48
isoflavone	genistein	47.87 ± 0.44	flavavone	liquiritigenin	31.11 ± 0.37
	daidzein	27.42 ± 0.79		naringenin	26.98 ± 0.52
	puerarin	23.92 ± 0.11		liquiritin	26.06 ± 1.27
	chrysin	23.87 ± 0.31		naringin	15.51 ± 0.46
	daidzin	13.38 ± 0.26		hesperetin	14.67 ± 0.35
	formononetin	12.86 ± 0.26		neohesperidin	11.89 ± 1.57
	genistin	12.55 ± 0.39		hesperidin	4.14 ± 0.52

The final concentration of flavonoids was 50 μmol L^{-1} in the enzymatic reaction; Values are presented as means ± SD (standard deviation).

Myricitrin and quercitrin are 3-O-rhamnoside of myricetin and quercetin, respectively (Table 4). The main difference of structures between quercetin and myricetin is that the groups at C-5' position in ring B are H and OH, respectively. The inhibitory activities of the above-mentioned four compounds demonstrated that the presence of 5'-OH and rhamnoside residues could enhance the inhibitory activities of certain flavones. A comparision of the AR inhibitory activities between quercetin and morin showed that 2'-OH made a greater impact than 3'-OH in enhancing the AR inhibitory activity. For quercitrin, hyperin and rutin, whose aglycone is quercetin, their screening results revealed that the inhibitory activity of compounds varied

considerably when the saccharide residues were different. The above results demonstrated that the activity strength order of the saccharide residues was rhamnoside residue > rhamnosyl-glucoside residue > galactoside residue. The presence of 3'-OH could enhance the AR inhibitory activity of flavonoids through the comparision of quercetin and kaempferol.

The prime difference between apigenin and baicalein as aglycone was the position of hydroxyl group, which attached to C-4' and C-6, respectively. In view of the above mentioned description, the activity of compound containing 6-OH was better than that containing 4'-OH. Based on the structure characteristics and the inhibitory intensity of baicalin, baicalein, luteoloside and luteolin, the substitution of OH with glucuronic acid retained the inhibitory activity, while the introduction of glucoside residue greatly decreased the AR inhibitory activity. According to the inhibitory activity of apigenin, acacetin, vitexin and isovitexin, the presence of glucosyl at C-6 and C-8 was advantageous to the AR inhibitory potency, whereas the substitution of OH with methoxy group at C-4' was adverse to the inhibitory activity. Furthermore, the results of daidzein and formononetin also revealed that the introduction of methoxy group at C-4' could decrease the inhibitory capability.

The results of inhibitory activity of daidzein and chrysin showed that the existence of OH at C-4' could enhance inhibitory activity more than that at C-5. The AR inhibitory potency of daidzein, daidzin, puerarin, genistein and genistin revealed that the inhibitory strength of compound decreased with the presence of 7-O-glucoside and 8-C-glucoside residues. The inhibitory capability decreased with

the introduction of 5-OH and 4'-OCH_3 residues by comparison of the results of liquiritigenin, naringenin and hesperetin. According to the results of liquiritigenin, liquiritin, naringenin and naringin, we deduced that 4'-OH and 7-OH were substituted by the glucoside and rhamnosylglucoside residues, respectively, resulting in the decrease of inhibitory activity.

In view of the above-mentioned results, we speculated that the capacity of flavonoids as AR inhibitors was related to their chemical structures. The presence of two hydroxyl groups in catechol orientation of ring B (3'-OH and 4'-OH) plays a major role in AR inhibitory activity. The more hydroxyl groups there are in the structures of flavonoids, the better AR inhibitory potency they have. The substitution of hydroxyl group with methoxy group was a very adverse effect on the AR inhibitory activity of flavonoids. Moreover, the majority of aglycones showed a stronger inhibitory activity than their corresponding glycosides in the present study.

Application to Natural Products Extracts

The AR inhibition ratios of *Fructus Evodiae*, *Cortex Phellodendri*, *Rhizoma Anemarrhenae* and *Panax notoginseng* of same final concentration (2 mg mL^{-1}) were 44.28%, 42.93%, 43.62% and 18.09%, respectively. Additionally, the IC_{50} values of *Radix Scutellariae* and *Folium Ginkgo* were 0.52 mg mL^{-1} and 1.02 mg mL^{-1}, respectively. These results demonstrated that the AR inhibitory activities of natural products rich in flavonoids were better than that rich in alkaloids and saponins in the reaction system.

Table 4 Chemical structures of Epalrestat and flavonoids used in the study.

Structures	Name	Nomenclature
	Epalrestat	2-[(5Z)-5-[(E)-3-phenil-2-methylprop-2-enylidene]-4-oxo-2-thioxo-3-thiazolidinyl]acetic acid
	quercetin	3,5,7,3',4'-pentahydroxyflavone
	quercitrin	quercetin-3-O-rhamnoside
	hyperin	quercetin-3-O-galactoside
	rutin	quercetin-3-O-rhamnosylglucoside
	morin	3,5,7,2',4'-pentahydroxyflavone
	kaempferol	3,5,7,4'-tetrahydroxyflavone
	myricetin	3,5,7,3',4',5'-hexahydroxyflavone
	myricitrin	myricetin-3-O-rhamnoside
flavonol	isorhamnetin	quercetin-4'-methoxy
	apigenin	5,7,4'-trihydroxyflavone
	baicalein	5,6,7-trihydroxyflavone
	baicalin	baicalein-7-O-glucuronic acid
	wogonin	5,7-dihydroxy-8-methoxyflavone
	acacetin	5,7-dihydroxy-4'-methoxyflavone
	luteolin	5,7,3',4'-tetrahydroxyflavone
	luteoloside	luteolin-7-O-glucoside
	vitexin	apigenin-8-C-glucoside
flavone	isovitexin	apigenin-6-C-glucoside
	daidzein	7,4'-dihydroxyisoflavone
	daidzin	daidzein-7-O-glucoside
	puerarin	daidzein-8-C-glucoside
	genistein	5,7,4'-trihydroxyisoflavone
	genistin	genistein-7-O-glucoside
	formononetin	7-hydroxy-4'-methoxyisoflavone
isoflavone	chrysin	5,7-dihydroxyisoflavone
	liquiritigenin	7,4'-dihydroxyflavanone
	liquiritin	liquiritigenin-4'-O-glucoside
	naringenin	5,7,4'-trihydroxyflavanone
	naringin	naringenin-7-O-rhamnosylglucoside
	hesperetin	5,7,3'-trihydroxy-4'-methoxyflavanone
	hesperidin	hesperetin-7-O-rhamnosylglucoside
flavavone	neohesperidin	hesperetin-7-O-neohesperidoside

CONCLUSIONS

In this paper, we demonstrated a new UPLC-MS/MS approach for the detection of AR activity and the screening of AR inhibitors *in vitro*. Glucose was chosen as the substrate of the enzymatic reaction system and the amount of sorbitol was directly detected using UPLC-MS/MS approach. Furthermore, the application of MRM mode, a sensitive and specific quantitative mode, could eliminate the interference of other substances. The proposed screening approach in our study was proved to be robust, accurate and sensitive. The AR inhibitory activities of epalrestat, flavonoids and natural products extracts had been studied using the validated method. Based on the AR inhibitory activities of thirty-two flavonoids, the structure-activity relationships of flavonoids were discussed in detail. Our proposed method simulated the polyol pathway of diabetic patients and it could be used to for determining AR activity and screening AR inhibitors as potential drugs of diabetic complications.

ACKNOWLEDGMENTS

This research was financially supported by the National Natural Science Foundation of China (No. 81373952) and the Innovation Method Fund of China (No. 2012IM030600).

REFERENCES

1. D. J. Porte, M. W. Schwartz, *Science*, 1996, **272,** 699-700.
2. I. M. Stratton, A. I. Adler, H. A. Neil, D. R. Matthews, S. E. Manley, C. A. Cull, D. Hadden, R. C. Turner, R. R. Holman, *BMJ.*, 2000, **321,** 405-412.
3. R. Ramasamy, I. J. Goldberg, *Circ. Res.*, 2010, **106,** 1449-1458.
4. M. Brownlee, *Nature*, 2001, **414,** 813-820.
5. M. Brownlee, *Diabetes*, 2005, **54,** 1615-1625.
6. S. S. Chung, E. C. Ho, K. S. Lam, S. K. Chung, *J. Am. Soc. Nephrol.*, 2003, **14,** S233-S236.
7. D. K. Wilson, K. M. Bohren, K. H. Gabbay, F. A. Quiocho, *Science*, 1992, **257,** 81-84.
8. P. F. Kador, J. H. Kinoshita, N. E. Sharpless, *J. Med. Chem.*, 1985, **28,** 841-849.
9. P. A. Kumar, G. B. Reddy, *Exp. Eye Res.*, 2007, **85,** 739-740.
10. Z. Dagher, Y. S. Park, V. Asnaghi, T. Hoehn, C. Gerhardinger, M. Lorenzi, *Diabetes*, 2004, **53,** 2404-2411.
11. M. A. Pfeifer, M. P. Schumer, D. A. Gelber, *Diabetes*, 1997, **46,** S82-S89.
12. N. Hotta, Y. Akanuma, R. Kawamori, K. Matsuoka, Y. Oka, M. Shichiri, T. Toyota, M. Nakashima, I. Yoshimura, N. Sakamoto, Y. Shigeta, *Diabetes Care*, 2006, **29,** 1538-1544.
13. K. Kawanishi, H. Ueda, M. Moriyasu, *Curr. Med. Chem.*, 2003, **10,** 1353-1374.
14. J. Á. de la Fuente, S. Manzanaro, *Nat. Prod. Rep.*, 2003, **20,** 243-251.
15. H. A. Jung, M. D. Islam, Y. S. Kwon, S. E. Jin, Y. K. Son, J. J. Park, H. S. Sohn, J. S. Choi, *Food Chem. Toxicol.*, 2011, **49,** 376-384.
16. H. L. de Winter, M. von Itzstein, *Biochem.*, 1995, **34,** 8299-8308.
17. T. B. Ng, F. Liu, Z. T. Wang, *Life Sci.*, 2000, **66,** 709-723.
18. S. Hayman, J. H. Kinoshita, *J. Biol. Chem.*, 1965, **240,** 877-882.
19. C.-H.Wong, G. M. Whitesides, *J. Am. Chem. Soc.*, 1981, **103,** 4890-4899.
20. J. T. Wu, L. H. Wu, J. A. Knight, *Clin. Chem.*, 1986, **32,** 314-319.
21. H. Chen, E. Adams, A. V. Schepadael, *Talanta*, 2013, **107,** 88-94.

22. S. Liu, J. Xing, Z. Zheng, F. Song, Z. Liu, S. Liu, *Anal. Chim. Acta*, 2012, **715,** 64-70.

23. K. D. Greis, *Mass Spectrom. Rev.*, 2007, **26,** 324-339.

24. J. Liu, X. Cheng, L. Fu, *Anal. Methods*, 2012, **4,** 1797-1805.

25. T. Cai, L. Zhang, H. Wang, J. Zhang, Y. Guo, *Anal. Chim. Acta*, 2011, **706,** 291-296.

26. C. Nishimura, T. Yamaoka, M. Mizutani, K. Yamashita, T. Akera, T. Tanimoto, *Biochim. Biophys. Acta*, 1991, **1078,** 171-178.

27. J. Sambrook, D. W. Russell, ed., P. Huang P, translated, *Molecular Cloning: A Laboratory Manual*, third ed., Science Press, Beijing, China, 2002.

28. B. K. Matuszewski, M. L. Constanzer, C. M. Chavez-Eng, *Anal. Chem.*, 2003, **75,** 3019-3030.

29. A. Furey, M. Moriarty, V. Bane, B. Kinsella, M. Lehane, *Talanta*, 2013, **115,** 104-122.

30. R. Ottanà, R. Maccari, M. Giglio, A. Del Corso, M. Cappiello, U. Mura, S. Cosconati, L. Marinelli, E. Novellino, S. Sartini, C. La Motta, F. Da Settimo, *Eur. J. Med. Chem.*, 2011, **46,** 2797-2806.