Development and validation of HPLC-UV-MS method for the control of four anti-diabetic drugs in suspected counterfeit products

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Abstract: An HPLC-UV method has been developed for the determination of valibose, miglitol, voglibose and acarbose, the four anti-diabetic drugs. The separation was accomplished successfully by using reversed phase chromatography (Prevail carbohydrate column, 250 mm × 4.6 mm, 5 μm) with a gradient acetonitrile-phosphate buffer solution (pH 8.0) at a wavelength of 210 nm. Furthermore, the method of a high-performance liquid chromatography coupled with ESI-MS in positive ionization mode has been established. These two methods were successfully applied to the assay and qualitative detection of four *α*-glucosidase inhibitors in the potential counterfeit anti-diabetic drugs.

Key words: anti-diabetic drug; *α*-glucosidases inhibitor; counterfeit drug; HPLC-UV; UPLC-ESI-MS

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HPLC-UV-MS 方法对治疗糖尿病药品或保健品中可能非法添加的 4 种 *α*-葡糖苷酶抑制剂成分的定性与定量分析

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摘要: 本文建立了一种反相高效液相色谱-紫外光谱检测法, 可同时定量检测维力波糖、米格列醇、维格列波糖和阿卡波糖这 4 种 *α*-葡糖苷酶抑制剂。采用 Prevail carbohydrate (250 mm × 4.6 mm, 5 μm) 色谱柱, 以乙腈-磷酸盐缓冲液 (pH 8.0) 进行梯度洗脱, 在 210 nm 下进行检测, 结果维力波糖、米格列醇、维格列波糖和阿卡波糖的线性范围分别为 58.2～932.0 μg·mL^{-1} (r = 0.9999, n = 5)、23.5～376.0 μg·mL^{-1} (r = 0.999 9, n = 5)、0.128～2.050 mg·mL^{-1} (r = 0.999 9, n = 5)、38.2～612.0 μg·mL^{-1} (r = 0.999 9, n = 5); 平均回收率分别为 97.0%、103.6%、96.4% 和 100.4% (n = 9)。与此同时, 本文还建立高相液相色谱-质谱检测方法, 用于这 4 种化合物的定性分析。以上两种方法简便快捷, 结果准确可靠、重复性好, 可用于可疑的糖尿病药品中非法添加 *α*-葡糖苷酶抑制剂的定性定量检测。

关键词: 降糖药; *α*-葡糖苷酶抑制剂; 非法添加药物; HPLC-UV; UPLC-ESI-MS

A counterfeit pharmaceutical product is defined as a product that is mislabeled deliberately and fraudulently with respect to its identity, source or both. Counterfeiting can apply to both branded and generic products and counterfeit products could include products with the correct ingredients or with the wrong ingredients, without active ingredients, with insufficient active ingredient, or with fake packaging[1]. Such a serious problem threatens the whole world, especially in developing countries.

In recent years, diabetes mellitus has become a common disease affecting human health significantly. There are about 40 million diabetes patients in China,

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and the incidence appears to be increasing[2]. About 90% of diabetes patients are found to be non-insulin-dependent diabetes mellitus (NIDDM). Therefore, it is crucial to ensure the quality of anti-diabetic drugs for NIDDM and ascertain that synthetic active substances potentially administered in traditional Chinese medicines or health food products were not added illegally in these anti-diabetic products. The α-glucosidase inhibitors (AGIs) increase gastro-intestinal motility, which could affect absorption of other concurrently administered anti-diabetic drugs[3]. They were considered generally safe, effective, well tolerated and used increasingly in the treatment of NIDDM. This study aims at establishing a method to simultaneously detect valibose, miglitol, voglibose and acarbose, four AGIs in suspected counterfeit drugs.

Valibose, miglitol and voglibose are pseudo-monosaccharides AGIs and acarbose is a pseudo-oligosaccharide AGI. Their structures are shown in Figure 1. Several analytical methods have been reported for the detection of AGIs. Voglibose was analyzed using HPLC with post column derivation fluorescencemetry[4, 5] or by LC-MS[5]. Acarbose was analyzed by HPLC-RID[6] or HPLC-UV[7, 8]. Miglitol was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS)[9]. It is in this paper to report at the first time the development and validation of an HPLC-UV method for the simultaneous separation and determination of these AGIs and to establish an HPLC-MS/MS confirmation method in this paper.

Experimental

Chemical and reagents The reference substance of acarbose was obtained from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). The other two reference substances of miglitol and voglibose were obtained from Zhejiang Medicine Co., Ltd. Xinchang Pharmaceutical Factory. The reference substance of valibose was from Shenzhen TaiTai Pharmaceutical Co., Ltd. as a gift. HPLC grade acetonitrile was purchased from Dima Technology Inc (Mushkegon, MI, USA). Analytical grade dipotassium hydrogen phosphate (K_2HPO_4) and phosphoric acid were purchased from Beijing Chemical Reagents Company (Beijing, China). Water used for chromatography was purified by a Millipore Simplicity™ Personal Ultrapure Water Systems (Molsheim, France). All solvents and sample solutions were filtered through 0.45 μm membrane filters or filtration units (Tianjin Tengda Filter Equipment Plant, Tianjin, China).

HPLC-UV The chromatographic system consisted of Waters 2695 separations module and a 2996 photodiode array detector (Waters Co., Milford, MA, USA). UV chromatograms were generated at a wavelength of 210 nm. Data acquisition and processing was performed using Empower automation system software (Waters Co.). Anti-diabetic drug separation was performed at 35 ℃ on a Prevail carbohydrate column (250 mm × 4.6 mm, 5 μm) (Alltech Associates, Inc., Deerfield, IL, USA). The mobile phase A was acetonitrile, mobile phase B phosphate buffer (pH 8.0; 2.2 g dipotassium hydrogen phosphate dissolved in 1 000 mL water, pH value adjusted to 8.0 using phosphate acid). The gradient eluting system was shown in Table 1. The injection volume was 20 μL.

Preparation of reference solution and sample solution Stock solutions of valibose, miglitol, voglibose and acarbose were prepared at a concentration of 932, 376, 2 050 and 612 $\mu g \cdot mL^{-1}$ respectively by

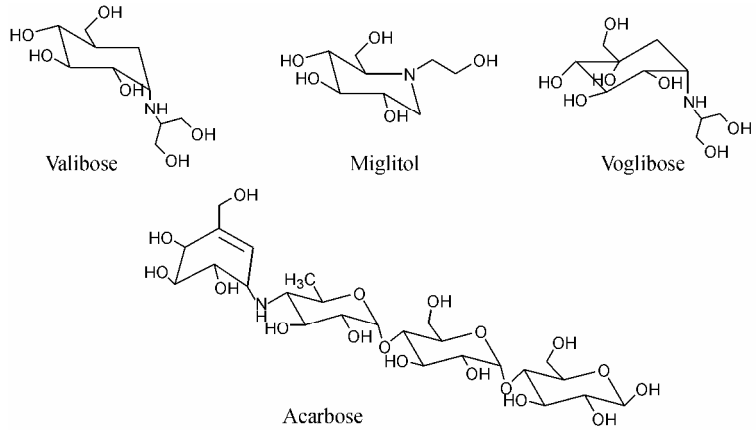

Figure 1 Structures of four inhibitors of α-glucosidases

Table 1 Gradient elution system in HPLC-UV detection

Time	Flow/mL·min^{-1}	Mobile phase/%	
		A	B
Intial	0.5	80	20
30	0.5	50	50
35	0.5	80	20
58	0.5	80	20

dissolving the appropriate amounts of the reference substances in diluting solution (acetonitrile-water 80∶20). The reference solutions for the system suitability test with concentrations of 466 μg·mL^{-1} valibose, 188 μg·mL^{-1} miglitol, 1 025 μg·mL^{-1} voglibose and 306 μg·mL^{-1} acarbose were prepared by dilution of the respective stock solutions. These solutions were stable for 24 hours when stored at 10 − 30 ℃.

The contents of 10 capsules or tablets of each health food sample or traditional Chinese medicine were transferred as completely as possible to a suitable tarred container and weighed accurately. An amount of powdered mass equivalent to one capsule or one tablet was transferred into a 50 mL volumetric flask and dissolved with diluting solution. All the sample solutions were sonicated for 15 min and filtered through a 0.45 μm filter for detection.

Specificity The method specificity was assessed by comparing the chromatograms obtained from the drug and the most commonly used excipients mixture with those obtained from blank solution of excipients diluting without active drug component. The excipients such as silica, starch, talc, magnesium stearate, microcrystalline cellulose and etc. were used to check the interference.

Linearity, range, limit of quantitation and limit of detection The reference solutions of 466 μg·mL^{-1} valibose, 188 μg·mL^{-1} miglitol, 1 025 μg·mL^{-1} voglibose and 306 μg·mL^{-1} acarbose were considered to be 100% solution and five different concentration levels (12.5%, 25%, 50%, 100%, 150% solutions) were prepared from each stock solution and diluted with acetonitrile-water (80∶20). Linear relationship was obtained between the peak areas and the corresponding concentrations. The equations of linear regression were performed using least-squares method.

The limit of quantitation (LOQ) was the lowest concentration of the sample assayed when the signal/noise ratio was at least 10∶1. The limit of detection (LOD) was defined as a signal/noise ratio of 3∶1.

Precision and recovery The precision of the method were evaluated by analyzing the reference solution of 466 μg·mL^{-1} valibose, 188 μg·mL^{-1} miglitol, 1 025 μg·mL^{-1} voglibose and 306 μg·mL^{-1} acarbose (100% solution) with five replicates.

The recovery tests were conducted to evaluate the extraction method described above. They were performed by adding known amounts of stock solutions to the blank samples and preparing solutions with diluting solution. The percentage of recovery was calculated by comparing the determined amount of these standards with the added amount.

LC-MS analysis An Acquity^{TM} UPLC separation module was used to deliver samples into the Quattro Premier XE (Micromass, Waters, USA) triple-quadrupole mass analyzer, controlled by Masslynx software.

Chromatographic separation was modified based on the HPLC condition. The same column was used and the mobile phase A was acetonitrile and mobile phase B was 10 mmol ammonium formate buffer. The gradient eluting system was shown in Table 2. The injection volume was 5 μL.

Table 2 Gradient elution system in LC-MS/MS detection

Time	Flow/mL·min^{-1}	Mobile phase/%	
		A	B
Initial	0.5	80	20
25	0.5	50	50
28	0.5	80	20
45	0.5	80	20

The parameters of mass spectrometer were as follows: ESI+, capillary (3.0 kV), cone (valibose: 30 V, miglitol: 30 V, voglibose: 40 V, acarbose: 30 V), collision energy (valibose: 30 eV, miglitol: 25 eV, voglibose: 35 eV, acarbose: 25 eV), collision gas flow (0.12 L·h^{-1}), source temperature (105 ℃) and desolvation temperature (350 ℃), desolvation gas flow (350 L·h^{-1}).

Results

1 Method development

To optimize the conditions of separation, preliminary tests were performed with the four AGIs. Separation was tried on four kinds of columns: ODS, HILIC silica, amino-bonded silica and carbohydrate-bonded silica four kinds of columns. The Prevail carbohydrates column performed a better separation. Since the carbohydrates-bonded column is delicate which could only allow the pressure under 2 000 psi. The flow rate is set at 0.5 mL·min^{-1} to ensure the highest pressure of

the column around 1 500 psi. The wavelengths of maximal absorption for four AGIs were around 200 nm. In order to get the best signal-noise ratio, acetonitrile and phosphate buffer were chosen as mobile phase and 210 nm was set for the quantification analysis by HPLC-UV. A gradient eluting system was established for the better resolutions. Under this experimental condition, the chromatogram was shown in Figure 2A. The method was suitable for the separation and simultaneous determination of 4 drugs.

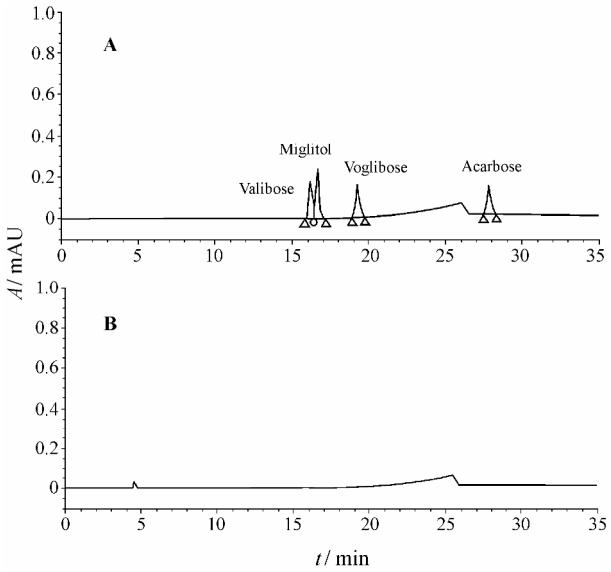

Figure 2 HPLC chromatograms of mixture reference solution (A) and blank (B)

2 Method validation

The analytical performance parameters such as specificity, linearity, range, precision, limit of detection and limit of quantification were validated. Specificity was investigated by using different excipients without active substance and verifying the absence of interferences. LC-MS/MS application is preferred in the health food analysis where a mass confirmation is necessary due to the interference from the presence of related substances of natural origin. The linearity of the method used for each anti-diabetic drug was evaluated on a standard curve of the peak area (y, mV·s) versus the concentration of the analyte (x, μg·mL^{-1}). A six-point calibration curve was constructed with working standards and was found linear ($r^2 \geqslant 0.999\ 9$) for each of the analytes over their curve ranges. The linear equation, correlation coefficient, LOQ and LOD were shown in Table 3.

Precision was calculated on extracted samples and expressed as the relative standard deviation (RSD, %) of replicate measurements (n = 6). Good RSD (%) values were obtained for valibose (1.1%), miglitol (1.3%), voglibose (1.5%) and acarbose (1.5%).

For the recovery, unspiked samples were used as blanks, prepared as reported for spiked samples without the addition of standards. The test was performed at three level concentrations separately. 0.437 mg·mL^{-1} valibose, 0.201 mg·mL^{-1} miglitol, 1.173 mg·mL^{-1} voglibose and 0.315 mg·mL^{-1} acarbose were added as 100% solutions; 0.368 mg·mL^{-1} valibose, 0.148 mg·mL^{-1} miglitol, 0.921 mg·mL^{-1} voglibose and 0.253 mg·mL^{-1} acarbose were considered to be 80% solutions while as 0.574 mg·mL^{-1} valibose, 0.254 mg·mL^{-1} miglitol, 1.347 mg·mL^{-1} voglibose and 0.413 mg·mL^{-1} acarbose to be 120% ones. Triplicate samples of each concentration level (n = 3) were prepared and the recovery at each level (n = 3) and mean recovery (n = 9) were determined. Table 4 showed the results from recovery studies.

The stability of the reference solutions was considered at 0, 2, 4, 6, 8, 12, 24 and 36 h. They were stable at the 10−30 ℃ for up to 24 hours.

Table 3 Results of the linearity, LOD and LOQ

Analyte	Equation	r^2	LOD/ng	LOQ/ng	Range/μg·mL^{-1}
Valibose	y = 3E+06x−7 810.5	0.999 9	34.8	116	58.2−932.0
Miglitol	y = 1E+07x−28 640	0.999 9	284.1	470	23.5−376.0
Voglibose	y = 1E+06x−31 107	0.999 9	768.0	2 560	128.0−2 050.0
Acarbose	y = 4E+06x−58 352	0.999 9	22.8	76	38.2−612.0

Table 4 Results from recovery studies for the four AGIs (mean±SD)

Compounds	Levels in spiked samples /mg·mL^{-1}			
	80% (n = 3)	100% (n = 3)	120% (n = 3)	Mean (n = 9)
Valibose	98.0 ± 1.2	95.5 ± 0.2	97.6 ± 1.1	97.0 ± 1.4
Miglitol	103.2 ± 1.1	104.7 ± 0.4	103.29 ± 1.5	103.6 ± 0.9
Voglibose	96.6 ± 0.7	97.4 ± 0.3	95.1 ± 0.8	96.4 ± 1.2
Acarbose	100.4 ± 0.5	99.7 ± 0.2	101.0 ± 0.8	100.4 ± 0.6

3 HPLC-MS/MS analysis

The ions were monitored in selected ion-recording (SIR) mode and the mass operating parameters were optimized by manual injection experiments. The four AGIs were all ionized in the positive ion mode (Table 5). The SIR chromatogram was shown in Figure 3. The method was suitable and highly sensitive. The LODs of the valibose, miglitol, voglibose and acarbose were 65, 50, 60 and 755 pg, respectively. The parent ion and daughter ion mass spectra were shown in Figure 4 and 5.

4 Applications

The proposed method was applied to screen AGIs

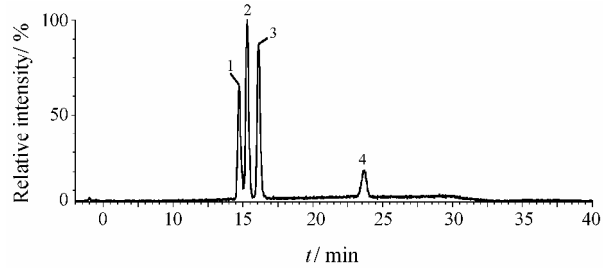

Figure 3 SIR chromatograms of four AGIs reference solution. 1: Valibose; 2: Miglitol; 3: Voglibose; 4: Acarbose

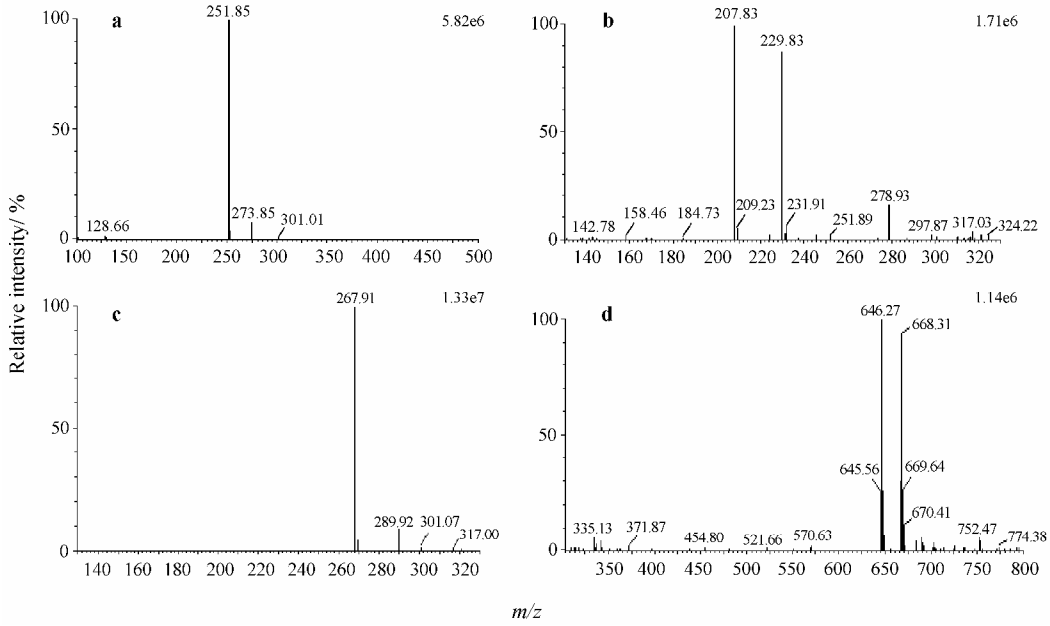

Figure 4 Full-scan parent ion spectra for valibose (a), miglitol (b), voglibose (c) and acarbose (d)

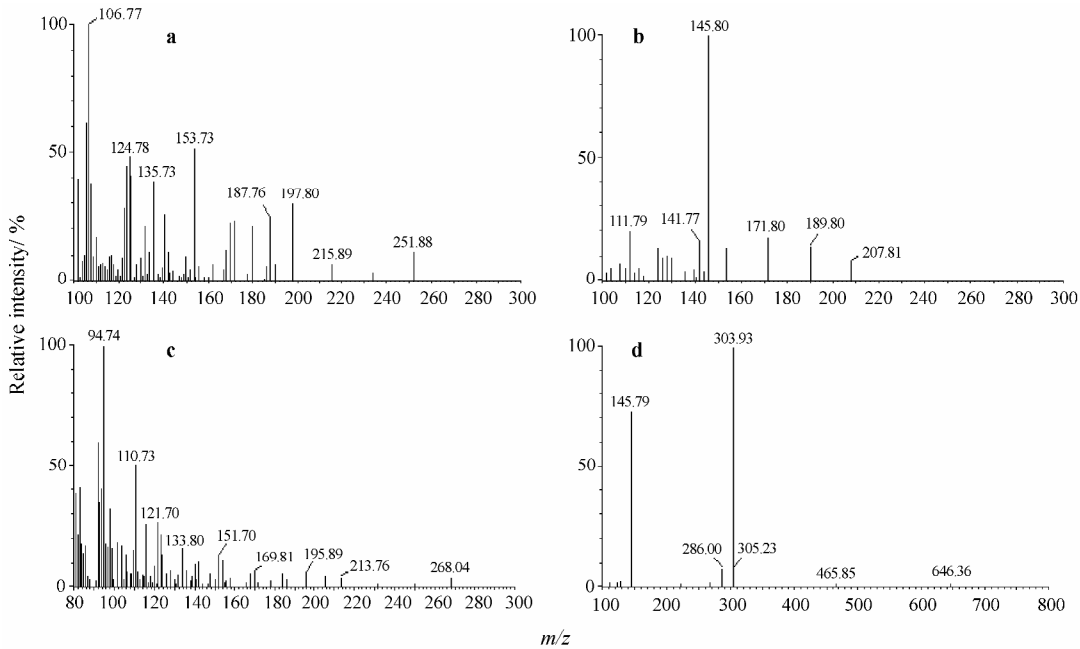

Figure 5 Daughter ion spectra for valibose (a), miglitol (b), voglibose (c) and acarbose (d)

Table 5 Name and chemical formula, relative molecular mass (M_r), retention time t_R, base peak for each AGIs

	Formula	M_r	t_R/min	$[M+H]^+$ (m/z)
Valibose	$C_8H_{17}NO_4$	251.28	14.71	251.85
Miglitol	$C_8H_{17}NO_5$	207.22	15.30	207.83
				229.83$[M+Na]^+$
Voglibose	$C_{10}H_{21}NO_7$	267.28	16.12	267.91
Acarbose	$C_{25}H_{43}NO_{18}$	645.60	23.69	646.27
				668.31$[M+Na]^+$

in health food preparations and traditional Chinese medicines available in Chinese market, which were not allowed to contain any chemical anti-diabetic drug.

We hypothesized that in order to produce a pharmacological effect, the amount of active substance added illegally should be comparable to the daily minimum amount administered in health food preparations and traditional Chinese medicines. The minimum daily dosages for valibose, miglitol, voglibose and acarbose were 1, 75, 0.6 and 150 mg respectively. Consequently, an illegal preparation could be tested using UV detector except voglibose determination. For voglibose, LC-MS/MS detection was effective and a semiquantitative test could be done. The further research of quantitative test of voglibose using LC-MS would be studied in the future.

In the analysis, it is supposed to be no peaks detected at the retention times of the four AGIs. For the suspicious peak with the same retention time, once UV spectrum, parent ion and daughter ion mass spectrum are the same as reference standard, then the adding content of active ingredient should be reported. Among the samples we tested (tablets, capsules, total 6 batches) none was reported positive with containing the four AGIs.

Conclusions

The main advantage of the proposed method is that the four popular administered AGIs can be detected simultaneously within one injection analysis. An advanced UPLC-MS/MS method was further developed which is preferred owing to its great specificity and high sensitivity. Generally a combination of two or more anti-diabetic active ingredients would not present in one tablet formulation, it could be a universal method for screening potentially counterfeit drugs, especially screening health foods and traditional Chinese medicines in order to investigate the illegal presence of AGIs.

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