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Journal of Chromatography B, 853 (2007) 254–259
JOURNAL OF CHROMATOGRAPHY B
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Development of a RP-HPLC method for screening potentially counterfeit anti-diabetic drugs

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Received 19 October 2006; accepted 18 March 2007
Available online 25 March 2007

Abstract

Pharmaceutical counterfeiting is becoming a serious problem in the world, especially in developing countries including China. Herein an isocratic reversed-phase high performance liquid chromatography (RP-HPLC) method was developed for screening counterfeit medicines and adulterated dietary supplement products. The developed method could be employed to separate and determine simultaneously six anti-diabetic drugs (glipizide, gliclazide, glibenclamide, glimepiride, gliquidone, repaglinide) on an isocratic solvent system using an Alltima C18 column (5 μm, 150 mm × 4.6 mm) with an isocratic mobile phase of methanol–phosphate buffer (pH 3.0; 0.01 mol/L) (70:30, v/v), at a flow rate of 1.0 mL/min and at a wavelength of 230 nm. The proposed method was successfully applied to the analysis of medicinal and dietary supplement samples purchased from the local market in China.
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Keywords: Anti-diabetic drugs; Counterfeit drugs; Substandard drugs; RP-HPLC; Screening analysis

1. Introduction

Pharmaceutical counterfeiting is becoming a serious problem in the world, especially in developing countries including China. No country is free of counterfeit and substandard drugs. Although it is difficult to obtain precise figures, estimates put counterfeits at more than 10% of the global medicines market [1]. Counterfeiting can apply to both branded and generic products with the correct ingredients or with the wrong active ingredients, without active ingredients, with insufficient active ingredients or with fake packaging [2]. China has implemented a crackdown on the manufacture and sale of counterfeit medicines, a problem which is widespread on the mainland since 1999 [3]. A "Fast Drug Identification System" which includes a near infrared (NIR) pre-screening identification system and a fast chemical identification system equipped in a mobile vehicle put into use gradually from 2005 [4]. HPLC identification and quantitation system is being developed from 2005 and this paper is a part of the work.

In recent years, diabetes mellitus has become a common disease affecting human health seriously. There are about 30 million diabetes patients in China, and the incidence appears to be increased [5]. About 90% of diabetes patients are found to be type II (non-insulin-dependent) diabetes mellitus. Therefore, it is especially important to ensure the quality of anti-diabetic drugs for type II. All six anti-diabetic drugs (glipizide, glibenclamide, gliclazide, glimepiride, gliquidone and repaglinide) chosen in this study are commonly used in clinic for type II diabetes mellitus patients. Their structures are shown in Fig. 1. They decrease the amount of glucose by stimulating the pancreas to release insulin. Glipizide, gliclazide, glimepiride, gliquidone and glibenclamide are sulfonylurea oral hypoglycemic drugs, and the first four of them have been widely used because of their acceptable price and good curative effect. Glibenclamide is very cheap, which is mostly possible to be used to substitute expensive ones in counterfeit products. Repaglinide, a non-sulfonylurea oral hypoglycemic drug, is relatively expensive, so it potentially to be counterfeited by cheaper ones. On the contrary, to overstate health care

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doi:10.1016/j.jchromb.2007.03.022

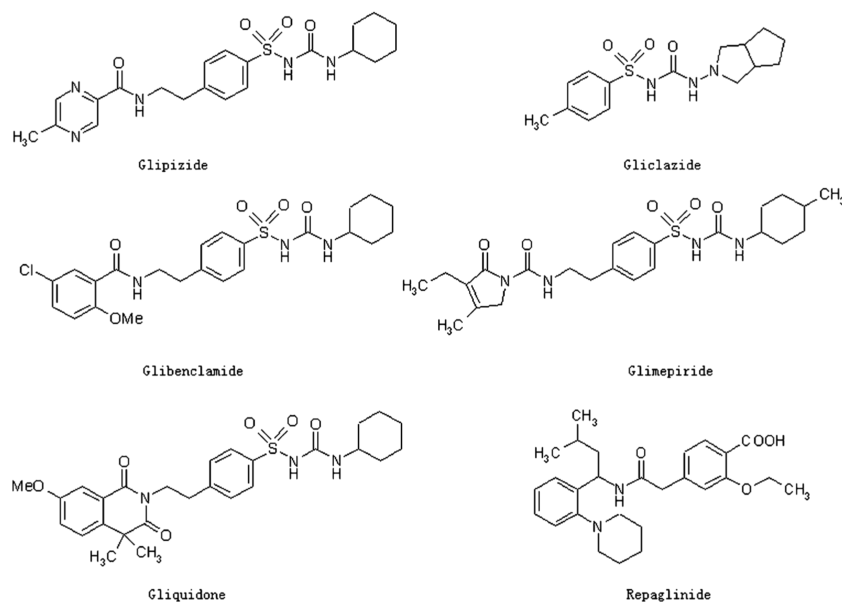

Fig. 1. Chemical structures of the anti-diabetic drugs chosen in this study.

efficacy, chemical anti-diabetic drugs might be added into dietary supplement products. Considering counterfeit products mainly present in rural areas, a simple and accurate method for screening medicines should be developed.

Several analytical methods have been reported for the detection of anti-diabetic drugs in plasma or urine, including liquid chromatography–tandem mass spectrometry (LC-MS/MS) [6,7], high performance liquid chromatography (HPLC) with ultraviolet (UV) [8-9]. Ho et al. [6] developed an LC–MS/MS for simultaneous detection of 10 anti-diabetics in equine plasma and urine. Lin et al. [7] developed an LC–MS/MS for simultaneous detection of glipizide and rosiglitazone. The two methods were not suitable for undeveloped area because of LC–MS/MS. Aburuz et al. [8] developed LC-UV simultaneous determination of melformin, glibenclamide, glimperide (method1) and melformin, glipizide, gliclazide (method2) in plasma, respectively. Venkatesh et al. [9] developed ternary gradient elution for simultaneous estimation of glibenclamide, gliclazide, glipizide, pioglitazone, repaglinide and rosiglitazone of pharmaceutical formulations. All these methods need expensive instrument or gradient elution so that rural areas where experiment conditions are not well equipped cannot employ them to screen potentially counterfeit drugs. Furthermore, there is no published method for simultaneous determination of these six drugs. It is the objective of this research to develop and validate a simple HPLC method for the separation and simultaneous determination of the six different anti-diabetic drugs.

The major advantage of the proposed method is that six commonly used anti-diabetic drugs can be separated on an isocratic solvent system. Although a combination of two or more anti-diabetic drugs active ingredients would not normally be present in the same tablet formulation, it could provide a useful method for screening potentially counterfeit drugs. The method could also be used to screening dietary supplements that potentially contain chemical anti-diabetic drugs. It has been successfully applied to the analysis of medicinal and dietary supplement samples obtained from the local market in China.

2. Experimental

2.1. Chemicals

The reference standards of glipizide, gliclazide, glibenclamide, glimepiride, gliquidone and repaglinide were obtained from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). Samples of anti-diabetic drugs and dietary supplements were purchased from China market. HPLC grade methanol was purchased from Dima Technology Inc. (Mushkegon, MI, USA). Analytical grade potassium dihydrogen phosphate (KH_2PO_4) and phosphoric acid were purchased from Beijing Chemical Reagents Company (Beijing, China). The water used for chromatography was purified by a Millipore Simplicity^{TM} 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).

2.2. Chromatography

The chromatographic system consisted of waters 2695 separations module and a 2996 photodiode array detector (Waters Co., Milford, MA, USA). UV chromatograms were extracted at a wavelength of 230 nm. Data acquisition and processing was performed using Empower automation system software (Waters Co.). Anti-diabetic drug separation was performed at 25 °C on an Alltima C18 column (150 mm × 4.6 mm, 5 μm) (Alltech Associates, Inc., Deerfield, IL, USA). The mobile phase consisted of methanol–0.01 mol/L phosphate buffer (70:30, v/v). The pH of phosphate buffer was adjusted to 3.0 with phosphoric acid. The injection volume was 10 μL. The mobile phase was delivered at 1.0 mL/min.

2.3. Preparation of standard and sample solutions

Stock solutions of anti-diabetics were prepared at a concentration of 100 μg/mL separately by dissolving the appropriate amount of the reference standards in methanol. The standard solutions of 50 μg/mL were prepared from these stocks by the appropriate dilution. These solutions were stable for 1 week when stored at 2–8 °C. For System Suitability Test, a standard mixture of the six anti-diabetics at 100 μg/mL was prepared in methanol.

Ten tablets of each anti-diabetic drug sample were respectively weighed and triturated to obtain a homogeneous mixture. Sample solutions were prepared by dissolving the suitable amount of each powder in methanol to obtain 50 μg/mL concentration of active substance. Zhiwuyidaosu and pingtangan were dietary supplements which were labeled "adjusting blood sugar for the patients with diabetes mellitus". The main material of the former was bitter melon. The main material of the latter was ganoderma, radix puerariae, medlar, pollen and bitter melon. The contents of 10 capsules of each dietary supplement sample were transferred as completely as possible to a suitable tared container and weighed accurately. An amount of powdered mass equivalent to one capsule was transferred into a 25 mL volumetric flask and dissolved with methanol. All the sample solutions were sonicated for 10 min and filtered through a 0.45 μm filter.

2.4. 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 (excipients solution in methanol without drug). The excipients such as silica, starch, talc, magnesium stearate, microcrystalline cellulose, polyvidone, carboxymethyl starch sodium, croscarmellose sodium, hypromellose and amylum pregelatinisatum were used to check the interference.

2.5. Linearity, range, limit of quantitation and limit of detection

Seven different concentration levels (1, 5, 10, 20, 50, 80, 100 μg/mL) were obtained from each stock solution and diluted with methanol. Each concentration solution was prepared in triplicate. Linear relationship was obtained between the peak area and the corresponding concentrations. The equations of linear regression were performed using least-squares method.

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

2.6. Accuracy, precision and recovery

The quality control samples were prepared by spiking reference standards in blank excipients mixture which was the same as in Section 2.4. Three replicates of three different concentrations (10, 50, 90 μg/mL) of quality control samples were analyzed on three different days in order to determine the accuracy and precision. One-way analysis of variance (ANOVA) was used to calculate the intra- and inter-day variation in these parameters.

Recovery tests were performed by adding known amounts of stock solutions to the samples with known content and preparing solutions with methanol. The percentage of recovery was calculated by comparing the determined amount of these standards with the added amount.

2.7. Robustness

The robustness was evaluated by deliberate variations of the method parameters. The factors selected to examine were flow rate (mL/min), pH of mobile phase, temperature (°C). One factor at a time was changed to estimate the effect.

3. Results

3.1. Method development

In order to select optimum conditions of separation, preliminary tests were performed with the six anti-diabetic drugs. Parameters such as detection wavelength, optimal mobile phase and their proportions, optimum pH and concentration of the buffer were studied. The wavelength of maximal absorption for glipizide, gliclazide, glibenclamide, glimepiride, gliquidone and repaglinide are 228, 229, 230, 230, 224, 244 nm, respectively, 230 nm was set for the quantification by HPLC, since all the drugs have strong absorbance at this wavelength and it was suitable for simultaneous determination of six different anti-diabetic drugs.

Methanol and phosphate buffer pH 3.0 were chosen as mobile phase. Different ratios of methanol–phosphate buffer were tried. Retention factors (k) of the six anti-diabetic drugs were plotted against proportion of organic modifier (Fig. 2). It showed that the optimal proportion of organic phase was 70%, where all the anti-diabetic drugs were separated and had suitable retention time. Considering pK_a values of these drugs, pH 3.0 was selected where the compounds were un-ionized and symmetric peaks could be obtained. At pH 3.0 all the anti-diabetics were

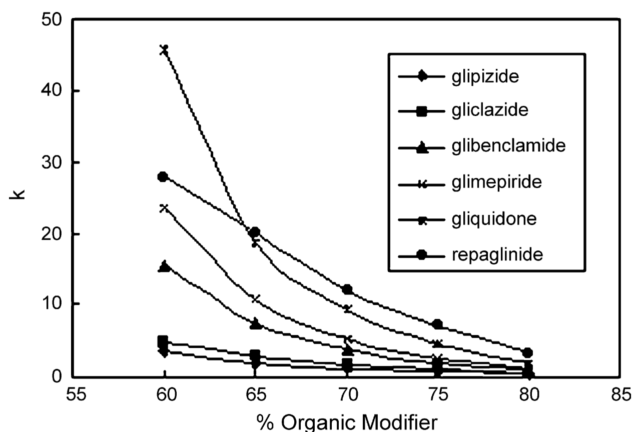

Fig. 2. Effect of change in composition of mobile phase on retention factor of six anti-diabetic drugs.

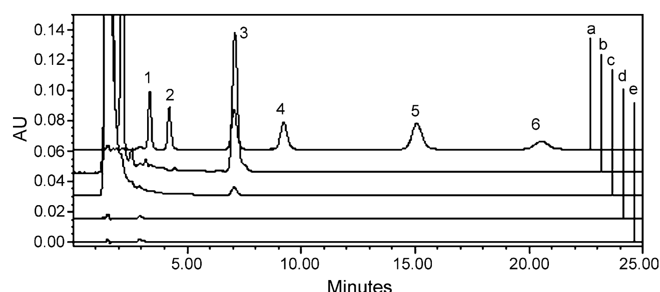

Fig. 3. HPLC chromatograms of (a) topical chromatogram of mixture: 1, glipizide; 2, gliclazide; 3, glibenclamide; 4, glimepiride; 5, gliquidone; 6, repaglinide (each at 17 μg/mL). (b) Pingtangan capsules. (c) Zhiwuyidaosu capsules. (d) Gliclazide tablets of 051016. (e) Blank.

separated and phosphate buffer ($pk_{a1} = 2.12$) has buffer capacity in this area. The concentrations of buffer which were 0.01, 0.025, 0.05 mol/L had no obvious effect on the resolution of the six anti-diabetic drugs. Therefore, the mobile phase composition was methanol–0.01 mol/L KH_2PO_4 (70:30, v/v) with pH adjusted to 3.0. Under this experimental condition, the mixture chromatogram was shown in Fig. 3(a). The method was suitable for the separation and simultaneous determination of six drugs.

3.2. Method validation

The analytical performance parameters such as specificity, linearity, range, precision, accuracy, limit of detection and limit of quantification were validated according to International Conference on Harmonization ICH Q2B guidelines. Specificity was investigated by using different excipients without active substance and verifying the absence of interferences. 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). A seven-point calibration curve was constructed with working standards and was found linear ($r^2 \geq 0.9999$) for each of the analytes over their curve ranges. The slopes were calculated using the plot of drug concentration versus area of the chromatogram. The linear equation, R.S.D. of slope, correlation coefficient, LOQ and LOD were shown in Table 1.

The results of the determination of accuracy and precision of the assay are presented in Table 2. These results showed that the method was accurate (accuracy from 99.12% to 101.75%) and precise (intra-day precision from 0.05% to 0.70% and inter-day precision from 0.22% to 1.55%). Recovery test was performed at

Table 1
Results of the linearity, LOD and LOQ

Analyte	Equation	R.S.D. of slope (%) ($n=3$)	r^2	LOD (μg/mL)	LOQ (μg/mL)	Range (μg/mL)
Glipizide	$y = 29.9068x - 0.0398$	0.13	0.99997	0.10	0.34	1–100
Gliclazide	$y = 24.7202x + 0.0022$	0.48	0.99996	0.12	0.38	1–100
Glibenclamide	$y = 31.2421x - 1.3877$	0.16	0.99993	0.24	0.77	1–100
Glimepiride	$y = 30.2891x + 5.8261$	0.20	0.99992	0.26	0.88	1–100
Gliquidone	$y = 35.3490x - 1.9774$	0.46	0.99992	0.35	1.05	1–100
Repaglinide	$y = 11.3069x + 0.0238$	0.21	0.99990	1.05	3.95	5–100

Table 2
Accuracy and precision of the determination of the six anti-diabetics

Analyte	Spiked concentration (μg/mL)	Mean-measured concentration (μg/mL)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
Glipizide	11.14	11.09	99.55	0.16	0.42
	55.70	55.72	100.03	0.20	0.26
	89.12	89.39	100.30	0.10	0.32
Gliclazide	10.91	10.86	99.50	0.16	0.43
	54.55	54.77	100.40	0.21	0.35
	87.28	86.51	99.12	0.29	0.45
Glibenclamide	10.31	10.32	100.10	0.68	0.91
	51.55	51.19	99.29	0.14	0.37
	103.10	103.56	100.44	0.64	0.65
Glimepiride	10.44	10.47	100.25	0.70	0.61
	52.20	52.00	99.62	0.05	0.26
	83.52	83.85	100.39	0.12	0.29
Gliquidone	11.39	11.42	100.26	0.38	0.33
	56.95	56.96	100.02	0.32	0.22
	91.12	91.12	100.00	0.61	0.44
Repaglinide	11.60	11.80	101.75	0.31	1.55
	58.00	57.90	99.83	0.68	0.61
	92.80	92.58	99.76	0.54	0.82

Table 3
Results from recovery studies for the six anti-diabetic drugs

Compounds	Levels in spiked samples (μg/mL)			
	45, mean ± S.D. ($n = 3$)	50, mean ± S.D. ($n = 3$)	55, mean ± S.D. ($n = 3$)	Mean, mean ± S.D. ($n = 9$)
Glipizide	99.70 ± 1.08	101.39 ± 1.15	100.30 ± 0.85	100.49 ± 0.46
Gliclazide	100.25 ± 0.35	100.50 ± 0.75	100.39 ± 0.43	100.40 ± 0.48
Glibenclamide	99.79 ± 0.47	100.42 ± 1.41	100.87 ± 1.80	100.36 ± 1.26
Glimepiride	101.65 ± 0.25	100.18 ± 0.47	101.21 ± 0.66	101.01 ± 0.78
Gliquidone	100.13 ± 1.49	101.43 ± 0.77	99.06 ± 1.76	100.21 ± 1.59
Repaglinide	99.07 ± 1.38	99.61 ± 1.36	98.88 ± 1.44	99.18 ± 1.25

three levels that concentrations were 45, 50, 55 μg/mL, respectively. 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 3 showed the results from recovery studies.

The robustness of the proposed method was evaluated after the following parameters had been altered deliberately: the mobile phase flow rate in the variants: 0.9 and 1.1 mL/min, the pH of buffer in the variants: 2.9 and 3.1 and the temperature in the variants: 27, 30 and 33 °C. The retention time of each compound was evaluated. The resolution had no significant changes when the parameters were modified. The resolution between glipizide and gliclazide was 3.58 ± 0.10, which was nearest among six peaks. Method robustness shows that the minor changes of the operational parameters do not lead to essential changes of the chromatographic separation (resolution and retention time). But retention time of analytes will increase or decrease when the composition of the mobile phase has been changed from Fig. 2.

A System Suitability Test was performed by six replicate injections of standard mixture verifying the following parameters: resolution of neighbor peak is more than 3, %R.S.D. of each peak retention time is less than 0.3% and %R.S.D. of each peak area is less than 1.5%.

3.3. Applications

The proposed method was applied to screen anti-diabetic drug and dietary supplement preparations available in China market. Each sample was tested in duplicate and the average results were shown in Table 4. For chemical anti-diabetic drugs, the retention time and UV spectrum of the sample must be the same as reference standard and the content of active ingredient must be in the range of legal criterion. There was no peak on the chromatogram of gliclazide tablets that batch was 051016 and the chromatogram was shown in Fig. 3(d). Therefore gliclazide tablets of 051016 were counterfeit, which contained no active substance. For the dietary supplements which were not allowed to contain any chemical anti-diabetic drug, there should be no peaks at the retention time of the six anti-diabetic drugs. But a peak at 7.1 min was found in both zhiwuyidaosu and pingtangan. The retention time and UV spectrum accorded with glibenclamide RS. So glibenclamide was adulterated in zhiwuyidaosu capsules and pingtangan capsules. The chromatograms from analyses of zhiwuyidaosu and pingtangan were shown in Fig. 3.

4. Discussion

This paper described the development and validation of an HPLC method for the simultaneous determination of six anti-diabetic drugs. The reagents of the method were inexpensive and instrument was simple, therefore, the proposed method would be used in rural area of China and developing country for screening counterfeit or substandard anti-diabetic drugs.

When we started method development, the assay of Ho et al. [6] and Lin et al. [7] was available. Since then Aburuz et al. [8] and Venkatesh et al. [9] published HPLC-UV methods for the simultaneous determination of anti-diabetics. The proposed

Table 4
Formulation assay results

Samples	Pharmaceutical dosage form	Batch	Content
Glipizide	Tablets (2 mg)	20050901	99.55%
Glipizide	Tablets (5 mg)	05101401	101.32%
Gliclazide	Tablets (80 mg)	0509092	94.61%
Gliclazide	Tablets (80 mg)	060313	103.75%
Gliclazide	Tablets (80 mg)	051016	0
Glibenclamide	Tablets (2.5 mg)	060204	98.88%
Glibenclamide	Tablets (2.5 mg)	060504	92.35%
Glimepiride	Tablets (2 mg)	050901	91.62%
Gliquidone	Tablets (30 mg)	060103	99.11%
Repaglinide	Tablets (2 mg)	5B023	99.72%
Zhiwuyidaosu	Capsules (0.5 g/capsule)	20040418	0.057 mg glibenclamide/capsule
Pingtangan	Capsules (0.4 g/capsule)	040316	1.12 mg glibenclamide/capsule

method used external standard method to quantify under the isocratic elution and operated conveniently. Furthermore, method validation showed good recovery and precision.

5. Conclusions

A simple and accurate isocratic reversed-phase HPLC method has been developed herein for the separation and simultaneous determination of six drugs commonly used to treat type II diabetes mellitus. The proposed method has been validated by good linearity, precision, accuracy and robustness. It is suitable for the screening of counterfeit or substandard preparations of anti-diabetic drugs.

Acknowledgements

The authors wish to thank Prof. Qiming Zhang and Chanqin Hu (NICPBP) for technical support, Huamin An and Fei Yin (Henan Institute of Drug Control) for their help in collecting part of anti-diabetic drugs from Henan market.

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