High performance liquid chromatographic determination and pharmacokinetics study of Glibenclamide in rats after oral administration of *XiaoKe* Pill

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Abstract

A simple and specific high-performance liquid chromatographic (HPLC) method was developed for the pharmacokinetic study of glibenclamide in rats after oral administration of *XiaoKe* pill. The plasma samples were deproteinized with acetonitrile after addition of internal standard (i.s.) glipizide. HPLC analysis was performed on a SinoChrom ODS-BP C18 analytical column, using acetonitrile - 0.1% acetic acid (44:56, v/v) as the mobile phase with UV detection at 230 nm. The standard curve was linear over the range of 10.2 - 306.0 ng/mL in rat plasma. This method is successfully applied for the pharmacokinetic study of glibenclamide in rat after oral administration of three doses of *XiaoKe* pill.

Key words: HPLC; Glibenclamide, *XiaoKe* pill; combination of TCM with chemical medicine, pharmacokinetics.

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Introduction

Type 2 diabetes mellitus is a complex metabolic disease, characterized by elevated plasma glucose levels. It results from defects in both insulin secretion and insulin actions. *XiaoKe* pill, which was approved by the SFDA for commercial use many years ago, is a combination of TCM (Traditional Chinese Medicine) with a chemical medicine. It consists of Chinese herbal drugs, such as *Radix Puerariae*, *Radix Astragali*, *Radix Rehmanniae*, *Radix Trichosanthis*, *Fructus Schisandrae*, *Rhizoma Dioscoreae* and a chemical drug glibenclamide**.** The combination of TCM with chemical medicine can not only reduce the dosage and ameliorate the symptoms, but also extenuation the side effects. Pharmacological and clinical evaluations

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indicated that *XiaoKe* pill had a mild, but significant, blood glucose lowering effect and that the long-term use of these agents may be advantageous over chemical drugs in alleviating some of the chronic diseases and complications caused by diabetes [2].

Glibenclamide (Fig.1) is a potent, second generation oral sulfonylurea antidiabetic agent widely used to lower blood flucose levels. It is rapidly and completely absorbed from the gastrointestinal tract. As there is no significant first pass metabolism, 100% of the oral dose is bioavailable[1]. Combination of TCM with chemical medicine is a complex system. The study of its pharmacokinetic has great significance of clarifying the principles of its combination, the mechanism of its action and reforming of dosage. To date, there are no published reports of glibenclamide in rat plasma after oral administration of *XiaoKe* pill or any other combinations with TCM, although some papers have appeared for glabenclamide analysis in biological fluids in recent years [3, 4]. It is necessary to develop an assay to fully evaluate the pharmacokinetics of glibenclamide in such a combination with TCM.
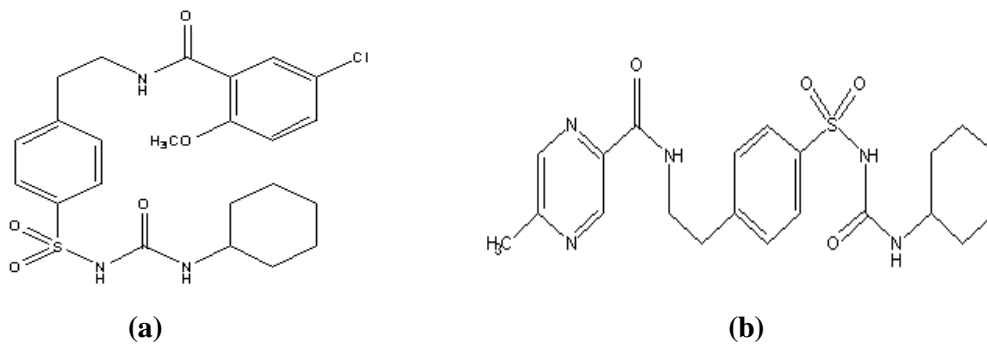

(a)   (b)

Fig.1. Chemical structure of glibenclamide (a) and glipizide (b)

Results and Discussion

Chromatography and extraction procedure

Under optimized HPLC condition, 0.1% acetic acid was added to the mobile phase consisting of a mixture of acetonitrile - water (44:56, v/v) to improve the peak shape. Glibenclamide had a maximum at 230 nm in the DAD spectrum. The i.s. had two maximum at 222 nm and 275 nm, respectively. Therefore, the detection wavelength of 230 nm proved to be more suitable and was selected for the assay.

Method validation

Typical chromatograms of blank, spiked plasma and plasma sample are given in Fig. 2, which showed no interfering peaks in the region of the location of the peaks of the analyte and i.s.. The retension times of glibenclamide and i.s. were approximately 5.3 and 13.5 min, respectively, and the total run time was 18.0 min.

The evaluation of the linearity was performed with an eight-point calibration curve over the concentration range of 10.2 ng/mL - 306.0 ng/mL. The regression equation of the calibration curves was: y = 0.0513x + 0.0109, and r was 0.9985. The lower limit of quantification defined as the lowest concentration on the calibration curve, was 10.2 ng/mL, with the

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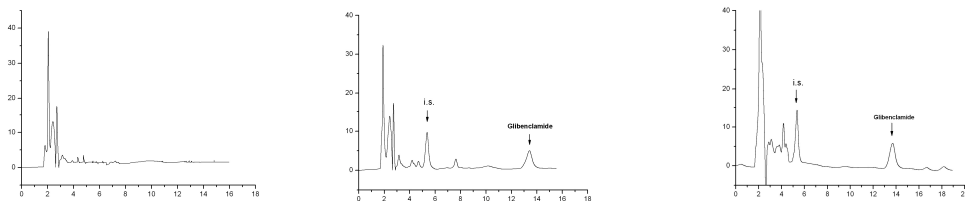precision and accuracy verified by repeated analysis.

Fig.2 .Representative chromatograms of blank plasma (a), plasma spiked with glipizide and glibenclamide (b) and plasma sample after oral administration of *XiaoKe* pill (c)

The accuracy and precision of the method were evaluated with QC samples at three concentrations and using five replicates. The results are shown in Table 1.

Table 1 Precision and accuracy of glibenclamide of xiaoke tablet in rat plasma

Added concentration	Intra-day			Inter-day		
(ng/mL)	Mean detected concentration (ng/mL)	R.S.D.(%)	RE(%)	Mean detected concentration (ng/mL)	R.S.D.(%)	RE(%)
20.4	20.26±2.92	12.36	-11.37	19.23±2.33	12.14	-5.74
102	90..39±8.13	6.12	-9..07	92..83±6.68	7.19	- 8.99
255	228.78±11.64	6.79	-8.29	230.38±13.58	5.89	-9.65

(intra-day:n=5;inter-day:n=3 days with 5 replicates per day)

The extraction recoveries of glibenclimide at three concentrationsare shown in Table 2. The average extraction recovery of glibenclimide and i.s. suggested that was little loss during extraction. It could be attributed to the solubility of glibenclimide in acetonitrile and the one-step protein precipitation used in the sample preparation.

Table 2 Recovery of glibenclamide of xiaoke tablet in rat plasma(n=6)

Added concentration (ng/mL)	Recovery (%)	R.S.D. (%)
20.4	90.7±10.93	9.70
102	90.51±5.98	5.41
255	88.51±4.56	5.15

The results of the stability study involving QC samples at two levels (20.4 ng/mL and 255.0 ng/mL, n=5) are presented in Table 3. It confirmed the high stability of glibenclimide throughout the determination.

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Table 3 Stability of glibenclamide of xiaoke tablet in rat plasma (n=6)

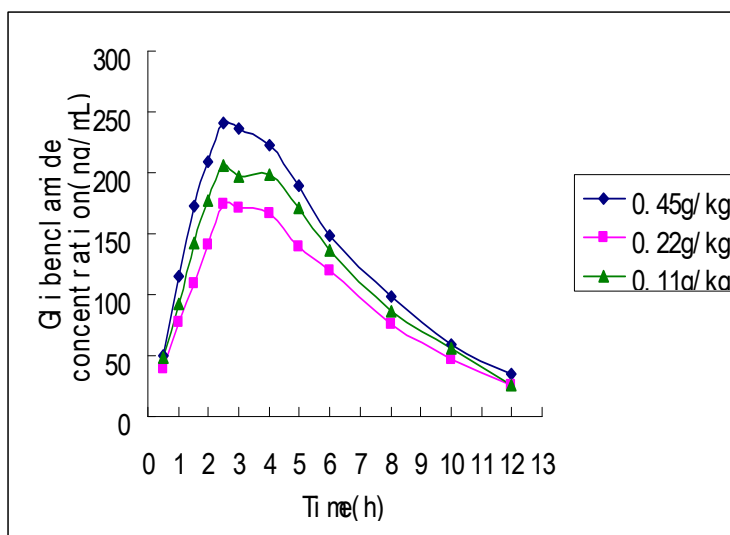| Stability	Added concentration (ng/mL)	Mean detected concentration (ng/mL)	R.S.D.(%)	RE(%)
Freeze-thaw	20.4	20.82±1.58	7.61	4.08
	255	226.52±11.85	5.23	-11.17
Long-term	20.4	20.66±1.50	7.27	10.97
	255	231.42±10.94	4.73	-9.25
Post-preparative	20.4	20.67±1.89	9.14	11.15
	255	230.09±10.12	4.40	-9.77

Fig.3. Mean plasma concentration-time curves of glibenclamide of *XiaoKe* pill in rats (mean ± S.D., n=5) after oral administration of doses of 0.11, 0.22, 0.45 g/kg

Table 4 Pharmacokinetic parameters of glibenclamide of *XiaoKe* tablet in rats(mean±S.D.,n=5) after oral administration of doses of   0.11,0.22,0.45g/kg

Parameter	0.11g/kg	0.22g/kg	0.45g/kg
$T_{1/2}$(h)	2.246±0.297	2.760±0.219	2.832±0.158
T_{max}(h)	2.698±0.211	3.064±0.172	3.210±0.187
C_{max}(ng/ mL)	95.46±13.09	158.40±3.41	194.4±6.54
Cl(mL/min)	2.766±0.188	3.066±0.132	4.958±0.266
AUC_{0-t}(ng/mL*h)	661.400±24.915	1151.408±27.377	1499.962±91.805
$AUC_{0-\infty}$(ng/mL*h)	719.032±50.103	1249.528±41.003	1631.560±102.897
MRT(h)	4.214±0.103	5.042±0.187	5.144±0.181

Pharmacokinetic data were processed by a 3p97 program. The plasma concentration-time

curves of glibenclimide of *XiaoKe* pill in rats following oral administration of 0.11, 0.22 and 0.45g/kg body weight are shown in Fig.3. The plasma concentration of glibenclimide was detectable just up to 30 min in rats using this analytical method. All these pharmacokinetic parameters are given in Table 4.

Materials and Methods

Experimental
Xiaoke pill was provided by the Guangzhou ZhongYi Pharmaceutical Ltd. (*batch* No.H00724). The internal standard (i.s) glipizide, was provided by the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). HPLC grade acetonitrile was purchased from Sinopharm Chemical Reagent Co.Ltd. (Shanghai, China).

Chromatographic system and conditions
The analysis was carried out on Elite HPLC system (Dalian, China) consisting of two P230 pumps and a DAD detector. The analytes were determined at room temperature on an analytical column (SinoChrom ODS-BP C18, 200 mm × 4.6 mm, i.d., 5 μm). The mobile phase consisted of a mixture of acetonitrile - 0.1% acetic acid (44:56, v/v). The analysis was carried out at a flow rate of 1.0 mL/min with the detection wavelength set at 230 nm.

Preparation of calibration standards and quality control samples
Stock standard solutions of glibenclamide and i.s. were prepared with acetonitrile. Eight calibrators were prepared by dilution of stock solutions followed by spiking with blank plasma. The calibration range was 10.2 ng/mL - 306.0 ng/mL. Quality control (QC) samples were prepared at low (20.4 ng/mL), medium (102.0ng/mL), and high (255.0 ng/mL) concentrations in bulk and aliquots were stored frozen before use.

Plasma sample preparation
To 400 μL plasma, 100 μL i.s. (8.7μg/mL), and 1.5mL acetonitrile were added, followed by vortex mixing for 1 min and centrifuging at 3000×g for 10 min. The supernatant was collected and evaporated to dryness at 50℃ under a gentle stream of nitrogen. The residue was reconstituted with 50 μL acetonitrile, and centrifuged at 10,000×g for 5 min, and an aliquot (20 μL) of the supernatant was injected into the HPLC system.

Method validation
To determine the selectivity of this method, blank rat plasma, plasma spiked with known amounts of glibenclamide and i.s. (8.7 μg/mL) and plasma samples from rats after oral doses of glibenclamide were analyzed.

The linearity was evaluated over the concentration range of 10.2 ng/mL - 306.0 ng/mL at eight levels (10.2, 20.4, 51.0, 102.0, 153.0, 204.0, 255.0 and 306.0 ng/mL). The calibration curves for glibenclamide in plasma were generated by plotting the peak area ratio of

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glibenclamide to i.s. versus the nominal concentrations in the standard plasma samples.

The intra- and inter- assay precisions were evaluated by analyzing the quality control samples at three concentration levels of glibenclamide (20.4, 102.0 and 255.0 ng/mL). For the intra-day validation, five replicates of the QC plasma samples were analyzed on the same day. For the inter-day validation, five replicates of the QC plasma samples were analyzed on three different days. The accuracy of the assay was determined by comparing the means of the determined Glibenclamide concentrations with the nominal values expressed.

The extraction recovery was determined by comparing the peak areas of glibenclamide obtained for the QC samples (20.4, 102.0 and 255.0 ng/mL, n=5) that were subjected to the extraction procedure with those obtained from blank plasma extracts that were spiked post-extraction at the corresponding concentrations.

The freeze-thaw stability was tested after three freeze (24h storage, -20℃) and thaw (room temperature for 2-3 h) cycles and the long-term stability of glibenclamide in plasma was studied using QC samples at two concentration levels (20.4 ng/mL and 255.0 ng/mL) stored at -20℃ for two weeks. The post-preparation stability was assessed by analyzing six replicates of the reconstituted QC samples stored at 25℃.

Animals and pharmacokinetic study

Male Wistar rats, weighing 200-250g, were obtained from the Centre of experimental animal, China Medical University (Shenyang, China). They were kept in an environmentally controlled breeding room for 1 week before the experiment and fed with standard laboratory food and water ad libitum and fasted overnight before the experiment. Three groups (5 rats/group) were randomly assigned to receive *XiaoKe* pill solution via oral administration of 0.11, 0.22 and 0.45 g/kg, respectively. Blood samples (1mL) were collected into heparinized tubes from the ophthalmic vein at times of 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 and 12h after oral administration and then centrifuged at 4000×g for 10 min. The obtained plasma was stored at -20℃ until analysis. Physiological saline (1mL) was administration to compensate for the blood loss after each blood was withdrawn.

Conclusion

The method developed in this study is specific, simple and fast for the pharmacokinetic study of glibenclamide in rat after oral administration of *XiaoKe* pill. The standard curve was linear over the range of 10.2 ng/mL - 306.0 ng/mL in rat plasma. The average extraction recovery of glibenclamide was 90.57 ± 7.40%, The method was successively applied to investigate the pharmacokinetics of glibenclamide in a combination with TCM for the first time.

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