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International Journal of Pharmaceutical Research & Allied Sciences, 2017, 6(4):80-87

Research Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

UPLC-MS/MS Method for Simultaneous Quantification of Glimepiride and Metformin in Human Plasma

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ABSTRACT

Metformin hydrochloride and glimepiride are commonly combined in one tablet for the Management of type 2 diabetic patients. This study offers a tandem mass spectrometric method for the concurrent quantification of the studied drugs in plasma. They were extracted on using n-heptane: propanol (3:2, v/v) and chromatographed on Waters RP-C_{18} column. The mobile phase was water containing 0.1% formic acid: methanol (3: 7, v/v) as a mobile phase. The linearity ranges were 50 - 2000 ng/mL for metformin hydrochloride and 0.1 - 1000 ng/mL for glimepiride. Validation process was fully carried out for the proposed method. This work can be applied for the estimation of the studied drugs for bioavailability and pharmacokinetic studies.

Keywords: *pharmacokinetic; LC-MS/MS; glimepiride, metformin*

INTRODUCTION

Non-insulin diabetic patients are characterized by problems in two aspects, which are insulin secretion and insulin sensitivity [1, 2]. The insulin resistance is found early in non-insulin diabetic patients [1, 2, 3], but later on, there is a marked drop down in ß-cell function [1, 4, 5]. In many cases, insulin resistance is coupled with hypertension, dyslipidemia, and other metabolic disorders [6]. Oral antidiabetic drugs usually act on one or more targets on the pathophysiological pattern and, as soon as the treatment with single drug begins to lose its effect, long-term treatment with a combination of two anti-diabetic agents is often a must [7]. So the combination of glimepiride (GLM) and metformin hydrochloride (MET), as a biguanide derivative, is found very effective treatment of mild and moderate type 2 diabetic cases. GMP does its action through triggering the release of insulin from intact pancreatic cells [8]. MET exhibits its action by lowering the biosynthesis of hepatic glucose and gluconeogenesis. It may give its action by improving insulin sensitivity peripherally [9, 10].

On reviewing the literature, GLM was quantified using tandem mass spectrometry either alone or with other drugs in biological fluids [11-13]. The same technique was also applied for determination of MET either alone or in combination with other medicaments [14-19]. Sengupta et al. determined the studied drugs with pioglitazone [18] but the sensitivity levels were not satisfactory for the aimed drugs, so the target of this paper was to introduce a simple tandem mass spectrometric method with modified experimental conditions and extraction protocol for a highly sensitive quantification of the target drugs in biological fluids (human plasma) in not more than three-minute injection.

1. MATERIALS AND METHODS

2.1. Chemicals and reagents

GLM and MET with purity levels of 99.8 and 99.7%, respectively, were supplied by Sanofi Aventis, Cairo, Egypt. Atorvastatin (IS), with 99.9% purity, was supplied by Pfizer scientific office, Cairo, Egypt. Human plasma was collected from local hospitals, Cairo, Egypt. Formic acid, methanol and distilled water (HPLC grade) were supplied by SIGMA chemicals, Cairo, Egypt. Propanol and n-heptane were purchased from ADWIC, Cairo, Egypt.

2.2. Instrumentation

Waters Acquity^{TM} tandem mass spectrometer together with a quaternary pump and a triple quadrupole detector were used. Mass Lynx 4 software was used for data treatment.

2.3. Chromatographic and mass spectrometric conditions

Chromatographic analysis was done on Waters Acquity^{TM} ultra performance C_{18} column with 15 cm length and 2.1 mm internal diameter. The packing material was 1.7 µm particle size. The developing system was water containing 0.1% formic acid: methanol (3: 7, v/v). The flow rate was 0.2 mL/min. Multiple reactions monitoring (MRM) was used for the tandem mass spectrometer. All drugs were analysed using an electrospray ionization (ESI) interface in positive ionization mode. The optimized working parameters are presented in Table 1.

2.4. Preparation of standard and quality control (QC) samples

2.4.1. Standard solutions

Stock standard solutions (100 µg/mL) of GLM, MET and IS were separately prepared in methanol. Methanol was used for preparation of working solutions by appropriate dilution. Working solutions for calibration were separately prepared in the ranges of 1–10000 ng/mL (GLM) and 500 - 20000 ng/mL (MET).

2.4.2. Preparation of calibration standards and quality control samples

Spiking of 350 µL blank plasma with 50 µL from the working solutions of the studied drugs was done to get plasma standards in concentration range of 0.1 - 1000 ng/mL (GLM) and 50 - 2000 ng/mL (MET). Samples with different concentrations representing LLOQ, QCL, QCM and QCH were prepared to perform validation process.

2.5. Extraction protocol

Fifty microliters of the internal standard working solution was added after spiking the plasma with the studied drugs. The solution was mixed for 30 seconds then, 7 mL from the extraction mixture (n-heptane: propanol, 3:2, v/v) was added. The solution mixture was mixed for 2 minutes. Centrifugation was performed for 7 minutes. Four mL from the organic layer were transferred into centrifuge tubes and evaporated at 50 ^{o}C then 200 µL mobile phase were used for reconstitution, then 10 µL sample aliquot was injected into the chromatographic system.

2.6. Method validation

Method validation was performed according to the United States Food and Drug Administration (FDA) Guidance for Industry Bioanalytical Method Validation [20].

2.6.1. Selectivity

The contribution of plasma endogenous materials was studied by chromatographing 6 blank plasma samples.

2.6.2. Linearity

Construction of standard curves was done using seven GLM samples (0.1- 1000 ng/mL) and seven MET samples (50 - 2000 ng/mL). Also, a blank sample (without the IS) and a zero sample (plasma with the IS) were also analyzed to be sure that no interferences were present. The calibration curves were plotted using the GLM or MET to IS peak area ratios using least-squares linear regression on different days.

2.6.3. Sensitivity

It was determined by analyzing five plasma samples spiked with the lowest concentration of the standard curve (LLOQ).

2.6.4. Precision

Intra-day precision was estimated through handling of 6 samples representing each QC concentration in the same day. The inter-day precision was assessed by analyzing the same number of samples of each QC concentration during different days.

2.6.5. Matrix interference and recovery

They were clarified by Matuszewski et al. [21]. Efficacy of extraction process was evaluated by comparing their peak areas before and after extraction process. Matrix effect was assessed by analyzing blank plasma samples from six sources extracted and spiked with the studied drugs using QCM concentration. The peak area ratio of GLM or MET after extraction (X) is compared to that of their standard solutions in methanol at equivalent concentrations (Y). The ratio (X/Y) can give an impact on matrix effect. If the ratio is equal to unity, this may lead to absence of matrix effect. A value exceeding unity may suggest ionization enhancement. On the other hand, if a decimal value, this might suggest ionization suppression.

In order to check carryover between the analyte and IS, a test was done. In carryover test, samples should be chromatographed as the following sequence; blank plasma sample followed by upper limit of quantification (ULOQ) sample and finally, blank plasma sample.

2.6.6. Analyte stability

It can be tested as short term stability, post-preparative stability, freeze–thaw stability and long-term stability.

2.6.6.1. Short term storage stability

Three injections of each QC concentration were left at room temperature for four hours on the bench. A standard curve was plotted using all stability samples. These samples were compared to fresh samples with the same concentration.

2.6.6.2. Post preparative stability

Three injections of each QC concentration were prepared and stored in an auto sampler (25 ºC). The prepared samples were analyzed after 12 hours.

2.6.6.3. Freeze and thaw stability

Stability of the studied drugs was evaluated in plasma samples subjected to 3 freeze–thaw cycles in 3 successive days. After the last round, the samples were analysed.

2.6.6.4. Long-term stability

Storage at low temperature (−85 ◦C), for one month, was done for each QC concentration then compared to samples not stored at freeze condition.

3. RESULTS & DISCUSSION

The target of this paper was to adopt a highly sensitive method which can determine GLM and MET in the same time. Also, the method can be applied to quantify the studied drugs in pharmacokinetic studies.

3.1. Optimization of the parameters related to mass spectrometry

Mass spectra of all samples were obtained in positive ion mode by infusing the standard solutions into electrospray ionization source. The $[M + H]^+$ mass spectra of the analytes are shown in Fig. 1. LC-MS/MS in multiple reaction monitoring (MRM) mode was applied to get the required selectivity and sensitivity for analytical methods used for the determination of plasma drug concentrations. The optimized conditions are presented in Table 1.

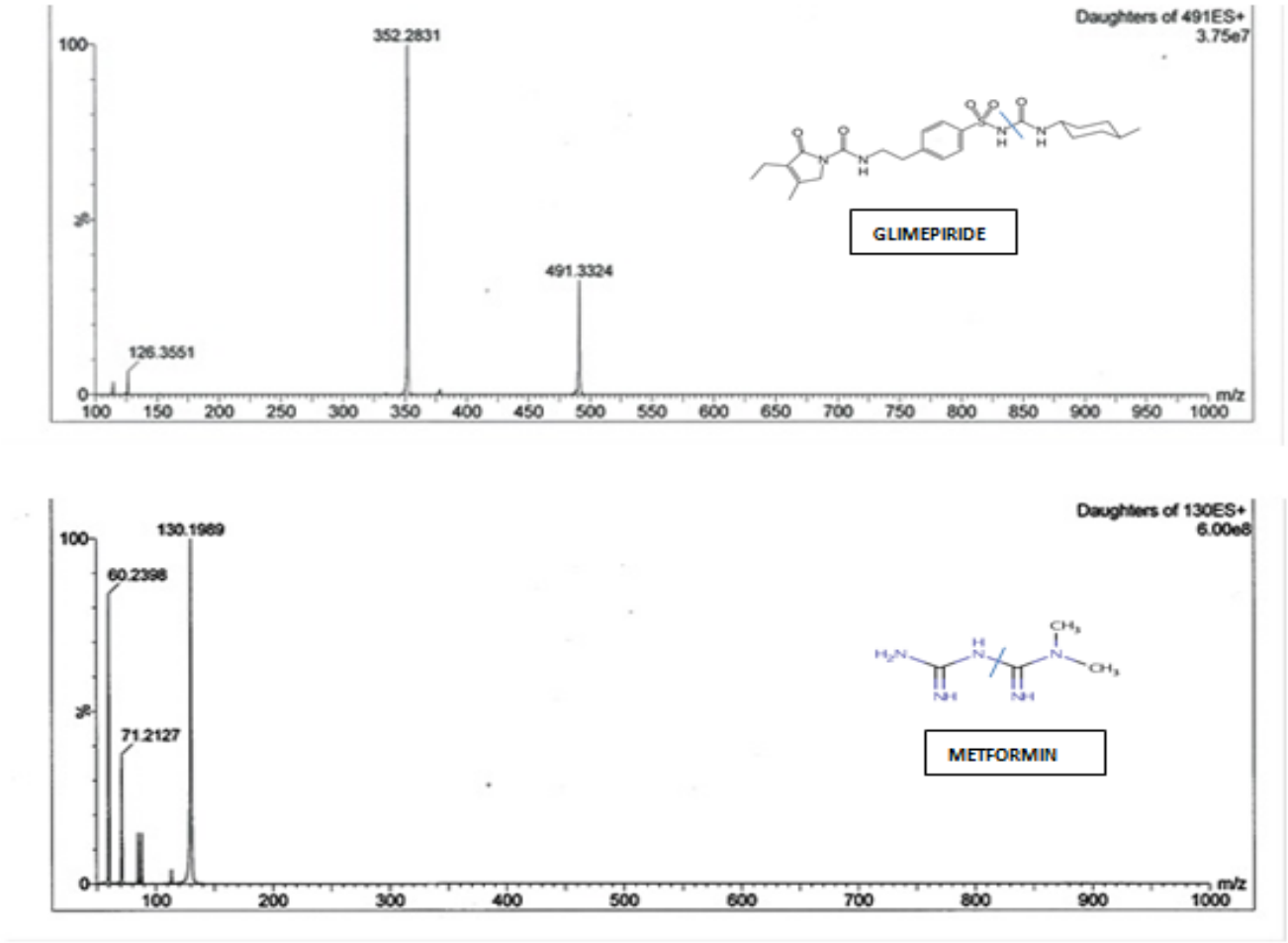

Fig. 1. Mass spectra of the fragmentation products for glimepiride and metformin

Table 1. The optimized conditions of the tandem mass spectrometer

Parameter	Value		
	Glimepiride	**Metformin**	**IS**
Source temperature (ºC)	120 ºC	120 ºC	120 ºC
Dwell time per transition (second)	0.061	0.061	0.145
Capillary (kV)	3 kV	3 kV	3 kV
Desolvation temperature (ºC)	400 ºC	400 ºC	400 ºC
Desolvation gas flow (L/Hr)	800 L/hr	800 L/hr	800 L/hr
Cone (V)	25	20	35
Collision energy (V)	15	15	20
Mode of analysis	Positive	Positive	Positive
Ion transition (Da) m/z	491.22/ 352.28	129.90/ 60.24	559.39/ 440.27

3.2. Sample preparation and chromatographic condition optimization

One of the most important steps in the development of a bioanalytical method is the optimization of the extraction protocol to get the highest sensitivity. Several extracting solvents were tried to adopt maximum sensitivity. Good extraction for all samples was achieved using n-heptane: propanol (3:2, v/v).

High sensitivity and optimum peak shape for all samples were acquired on using Waters Acquity™ ultra performance C_{18} column with 15 cm length and 2.1 mm internal diameter. The packing material was 1.7 µm particle size. The mobile phase was water containing 0.1% formic acid: methanol (3: 7, v/v). The flow rate was 0.2 mL/min.

3.3. Method validation

3.3.1. Selectivity

It was checked by analyzing five blank human plasma extract (Fig.2). Endogenous substances offer no significant interference at used parameters.

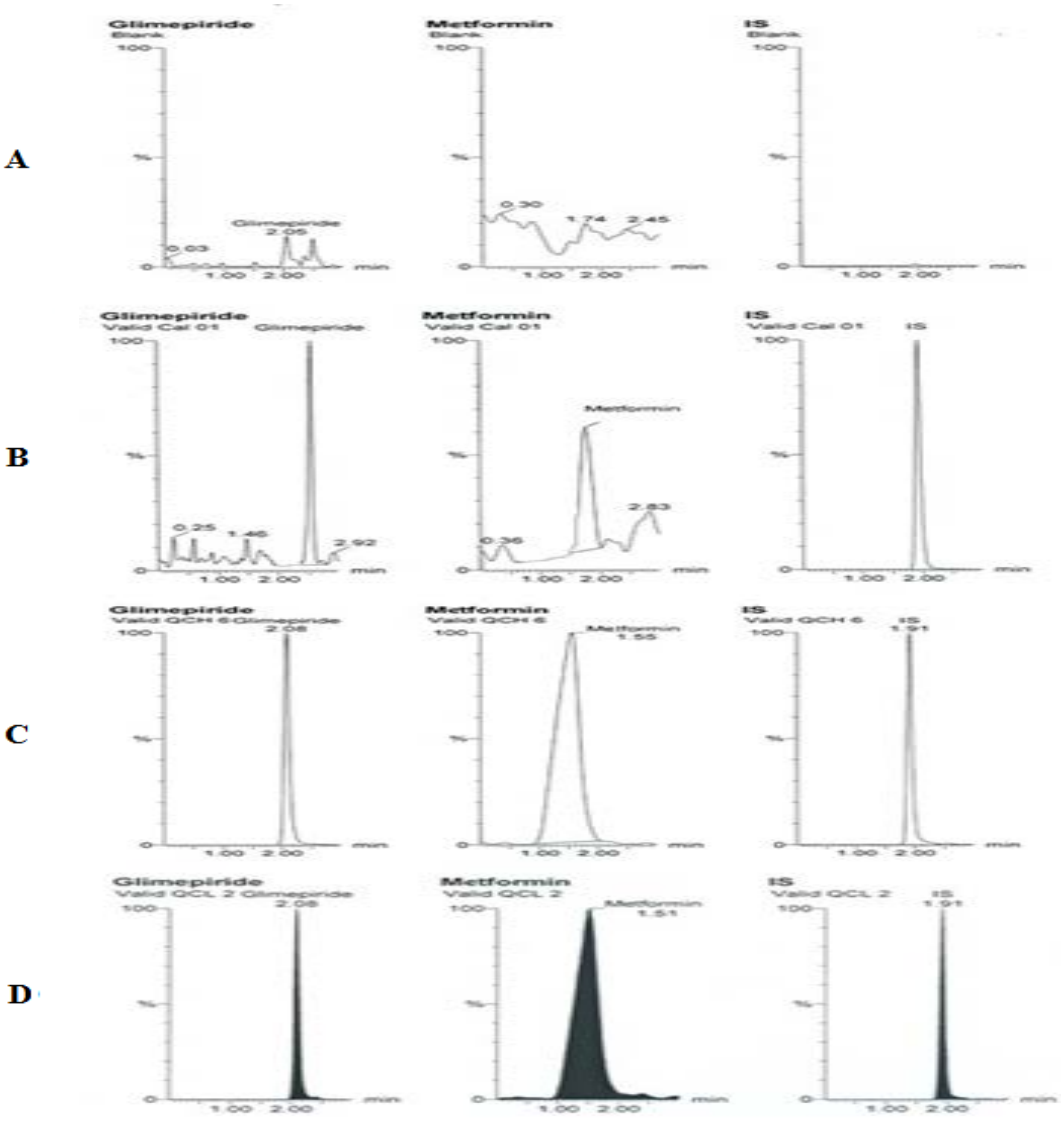

Fig. 2. Representative MRM chromatogram of (A) blank plasma samples, (B) spiked plasma samples at LLOQ with the IS, (C) spiked plasma samples at QCH with the IS and (D) spiked plasma samples at QCL with the IS

3.3.2. Linearity and lower quantification limit (LLOQ)

Standard curves were linear over the concentration range 0.1 - 1000 ng/mL GLM and 50- 2000 ng/mL MET. Linearity was checked by calculating the correlation coefficient and by evaluating the back calculated concentrations of the calibration standards. Table 2 gives the standard curve results. LLOQ is the smallest concentration in the linearity range with the highest accuracy and precision [21]. Figure 2 shows the LLOQ chromatograms.

Table 2 Regression parameters for the standard curves of studied drugs

	Glimepiride	**Metformin**
Correlation coefficient	0.9921	0.9911
Slope	0.0038	0.0008
Intercept	0.0551	0.0162

3.3.3. Accuracy and precision measurement

Table 3 gives the intra and inter day accuracy and precision. Their values were below 15%, which is acceptable.

Table 3 Intra and inter day recoveries for the determination of glimepiride and metformin

	Recovery Mean recovery ± RSD%*			
	Intra-day		**Inter-day**	
	Glimepiride	Metformin	Glimepiride	Metformin
QCL	94.21 ±6.41	97.54±.5.32	106.71±9.21	98.12±10.51
QCM	96.98±3.21	95.82±3.99	104.15±8.51	98.93±4.81
QCH	102.51±4.21	97.51±3.11	95.66±2.43	101.78±1.98

* Mean percentage recovery and RSD% were calculated using six determinations

3.3.4. Matrix effect

The matrix effect value was fraction for GLM and MET (0.89 and 0.98, respectively), so ionization suppression may be suggested.

3.3.5. Carryover test

There is no carryover between any of the samples and the following samples.

3.3.6. Stability assessment

The analyte stability at room temperature, for four hours, (the maximum time for sample preparation) was assured. No significant degradation of the studied drugs and IS was found when the handled samples were kept in the auto-sampler at 25 ^{o}C for 12 hours. With regard to freeze thaw stability, no difference was observed in QC sample concentrations in comparison to the freshly prepared one. Stability under long term storage was clarified for at least 30 days at −85^{o}C temperature. These findings were introduced in Table 4.

Table 4. Glimepiride and metformin stability data

Item	% Deviation *from fresh sample concentration	
	Glimepiride	Metformin
Short term stability (4h at room temp)		
QCH	-6.52	0.42
QCM	8.41	11.24
QCH	-2.54	-2.10
Post preparative stability (12 h at 25ºC)		
QCH	7.41	3.51
QCM	1.42	5.27
QCH	4.57	1.12
Long term stability (-20ºC for 30 days)		
QCH	-7.95	0.14
QCM	2.14	4.95
QCH	-8.54	-3.51
Freeze- thaw cycles (three cycles)		
QCH	-7.11	0.28
QCM	2.13	7.84
QCH	-2.91	-2.42

*% deviation = 100 x (Stability sample – Fresh sample/ Fresh sample)

4. CONCLUSION

This work offers a tandem mass spectrometric method, which is characterized with higher sensitivity and selectivity if compared with the previously adopted methods for the simultaneous determination of the studied drugs in human plasma. Also, the very small values of LLOQ favours the applicability of this method for monitoring of the studied drugs in plasma during the pharmacokinetic studies.

ACKNOWLEDGEMENTS

This project was supported by the Deanship of Scientific Research at Prince Sattam bin Abdulaziz University under the research group number (2016/03/6600).

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