

Determination of metformin hydrochloride in human plasma by UPLC/MS/MS: Application in bioequivalence study

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ABSTRACT

Metformin hydrochloride was quantified in human plasma by Ultra Performance Liquid Chromatography with Tandem Mass spectrometric determination (UPLC-MS/MS). The mobile phase used was (Water + 0.1% Formic acid) and (Acetonitrile + 0.1% Formic acid) in the ratio of 80:20 (v:v). An Acquity UPLC HSS T3 1.8 μ m 2.1 \times 50 mm column was used. The detection was performed on a mass spectrometer (ESI+) using chlorpheniramine as an internal standard. The method was fully validated and it was applied to bioequivalence study of metformin hydrochloride in two brands of the drug with relative bioequivalence of 94.7%.

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1. Introduction

Metformin hydrochloride is *N,N*-dimethyldiguanide hydrochloride (Figure 1). It is an inexpensive biguanide oral anti hyperglycemic agent [1], it exerts its glucose-lowering effects primarily through increased hepatic insulin sensitivity and the resultant suppression of hepatic glucose output. Metformin hydrochloride may also modestly enhance glucose uptake in peripheral tissues and increase glucose metabolism in the splanchnic bed [2]. It is also used for diabetes prevention, particularly in overweight obese people and those with unimpaired renal function [3].

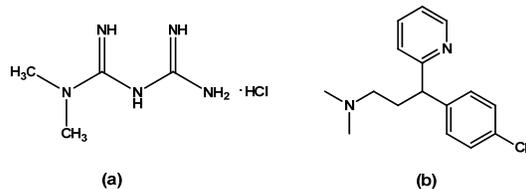


Figure 1. Structure of metformin hydrochloride (a) and chlorpheniramine (b).

Our goal is to develop and validate a fast bio analytical method for the analysis of metformin hydrochloride to support bioequivalence studies. Direct injection of the extracted samples is preferred. Analysis of metformin hydrochloride is a challenge owing to its high polarity and small molecular size, which lead to poor retention of metformin hydrochloride on reversed-phase liquid chromatographic columns. There have been some published applications, which used reversed-phase columns such as C₁₈ and C₈. Highly aqueous mobile phases were used for the analyte retention. High organic extracts are not conducive for direct injection onto reversed phase columns with highly aqueous mobile phases, which may lead to poor chromatograms with peak broadening and distortion [4-6]. Therefore, many of these applications did not support direct injection of extracted sample solution containing high organic concentration, thus requiring additional time-consuming evaporation and reconstitution procedures during sample preparation. These additional steps could also lead to additional analyte loss [7-11]. Using of solid phase extraction increases the cost of the method to be applicable for analysis large number of samples in bioequivalence studies [12]. Only one method was published for direct injection using

hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-MS/MS) [13]. The present paper describes a fast and highly sensitive approach that enables the determination of metformin hydrochloride at 0.20 min with a total analysis time of 0.5 min. Using Acquity UPLC HSS T3 column, which is suitable for analysis of highly organic samples with direct injection. This method was fully validated and it was applied to bioequivalence study of two brands of the drug.

2. Experimental

2.1. Materials

Metformin hydrochloride and chlorpheniramine (Internal standard, IS) were purchased from Sigma Aldrich, USA. The chemical structures of metformin hydrochloride and IS are shown in Figure 1. Acetonitrile of HPLC grade was purchased from E. Merck, Darmstadt, Germany. Formic acid was purchased from Sigma Aldrich, USA. Purified water was processed using an ELGA Pure Lab Classic water polisher and deionizing unit (Marlow, UK). Blank human plasma was purchased from VACSERA, Giza, Egypt and was stored frozen at -70 °C. All mobile phase solvents were HPLC grade and were filtered through 0.22 µm filters (HNWP membrane filters, Millipore).

2.2. Instrumentations and chromatographic conditions

2.2.1. Liquid chromatography

Chromatographic separation was performed on Waters UPLC system (Milford, Massachusetts, USA) with an auto sampler and a column oven that enabled temperature control of the analytical column. Acquity UPLC HSS T3 1.8 µm 2.1×50 mm column was employed and maintained at 40 °C. The mobile phase consisted of (Water + 0.1% Formic acid):(Acetonitrile + 0.1% Formic acid) in the ratio of 80:20 (v:v) at an isocratic flow rate of 0.65 mL/min. The injection volume was 10 µL.

2.2.2. Mass spectrometry

Waters UPLC Xevo TQD system (Milford, Massachusetts, USA) equipped with a Turbo Ion spray interface. Mass spectrometer settings in positive-ion mode (ESI+) with ion spray voltage at 5000 V, temperature at 400 °C, collision gas (N₂) at 15 psi, curtain gas at 20 psi, declustering potential (DP) and collision energy (CE) were set at 40 and 60. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of (precursor to product) monitored were *m/z* 129.93/59.99 for metformin hydrochloride and *m/z* 275.14/229.93 for chlorpheniramine. Data acquisition and processing were performed with the Analyst software.

2.3. Calibration standards and quality control samples

Standards and quality control samples (QCs) of metformin hydrochloride were prepared from stock solutions (5 µg/mL in water). Working calibration standards at concentrations of 5.00, 25.00, 50.00, 100.00, 500.00, 1000.00, 2000.00, 3000.00 and 4000.00 ng/mL were prepared in blank plasma. Chlorpheniramine was prepared in water with concentration of 50.00 µg/mL. Three levels of QC samples at 10.00, 2000 and 3000.00 ng/mL, respectively, were prepared in plasma for the determination of inter-assay accuracy and precision. Aliquots of the standards and QC samples were stored frozen at -70 °C.

2.4. Sample preparation

50 µL of Chlorpheniramine IS (50 µg/mL) was added to 200 µL metformin hydrochloride working plasma solutions, Vortex (30 s). The plasma was deprotonated with acetonitrile

in a ratio of plasma:acetonitrile (2:3, v:v). After mixing (30 s) and centrifugation (10 minutes at 3500 rpm), 10 µL of the clear supernatant was injected into the liquid chromatographic system after filtration through Whatman Filter paper No. 22.

2.5. Validation of UPLC/MS/MS method

The method was validated for accuracy, precision, sensitivity, recovery, matrix effect, specificity, linearity and reproducibility according to the FDA guidance for bio-analytical method validation [14] over a concentration range of 5.00-4000,000 ng/mL using six calibration standards and six replicates of QC samples at each concentration level in three separate batch runs. Analyte stability was tested using QC samples for multiple freeze-thaw cycles, on the bench at room temperature (Short-term stability), or frozen at -70 °C (Long-term storage). Processed sample stability and stock solution stability were also determined. The extraction recovery of metformin hydrochloride was calculated by comparing the peak areas of extracted plasma standards with the peak areas of metformin hydrochloride in the solvent at the corresponding concentrations. The method specificity was evaluated by screening six lots of blank human plasma.

2.6. Study design

This was a randomized, open-label, 2 ways, crossover trial in 24 healthy volunteers aged 18 to 40 years, with body mass indices between 18 and 27 kg/m². Subjects were given single oral doses of the following two treatments after an overnight fast: Mepaphage 500 mg Film Coated Tablets (MEPACO, Elsharkya, Egypt) and Glucophage 500 mg Film Coated Tablets (Merck Sante S.A.S., France) with 250 mL of water. Treatments were separated by 1 week washout periods between consecutive doses. Each subject was randomized to receive the two treatments. Key exclusion criteria included use of prescription or nonprescription drugs, vitamins, or dietary supplements within 7 days or 5 half-lives (whichever is longer) before the first dose of study medication, except for acetaminophen at doses of ≤1 g/day; any clinically significant disease or drug allergies; febrile illness within the 5 days before first administration of study medication; sensitivity to heparin or heparin-induced thrombocytopenia; a positive test result for a drug of abuse; regular consumption of alcohol and excessive tobacco or nicotine use (equivalent to 5 cigars per day). Blood samples were drawn from subjects before dosing and at 0.30, 0.60, 1.00, 1.30, 1.60, 2.00, 2.50, 3.00, 3.50, 4.00, 5.00, 6.00, 8.00, 10.00, 12.00, 24.00 and 30.00 h post dose in each treatment period. Samples were collected in 6 mL Vacutainer tubes (BD Diagnostics, Franklin Lakes, NJ) using heparin as anticoagulant, then centrifuged at 4500 rpm for 5 minutes to separate the plasma. Plasma samples were transferred to two 2 mL polypropylene cryogenic tubes (Labeled retention sample and analyte, respectively) and immediately stored at -70 °C.

2.7. Statistical analyses

Non compartmental pharmacokinetic analysis was performed using Kinetica Version 5.0 software and the following pharmacokinetic parameters were calculated: Maximum plasma concentration C_{max}, Time to reach C_{max} following drug administration T_{max}, AUC_{0-t}: Area under the plasma concentration-time curve, AUC_{0-inf}: Area under the plasma concentration-time curve from time 0 (administration) extrapolated to infinity, K_e: Terminal elimination rate constant, t_{1/2}: Elimination half-life.

Table 1. Accuracy of calibration standards, n = 5.

Statistical parameters	Concentration of metformin hydrochloride (ng/mL)								
	5	25	50	100	500	1000	2000	3000	4000
Found mean plasma conc.	5.15	24.8	50.4	97.0	491.0	960.5	1910.3	2890.6	4078.0
SD ^a	0.10	0.34	0.96	1.5	2.5	11.2	14.9	23.5	44.6
CV ^b	1.9	1.4	1.9	1.5	0.5	1.2	0.8	0.8	1.1
Accuracy percent	102.70	99.20	100.80	97.00	98.20	96.05	95.50	96.35	101.00

^a SD, standard deviation.^b CV, coefficient of variation.

3. Results and discussion

3.1. Chromatography and mass spectrometry

Metformin hydrochloride is a small molecule with high polarity. The retention of metformin hydrochloride on reversed-phase columns was very poor. The use of column suitable for separation of high polar compounds as HSS T3 permits the separation of polar compound as metformin hydrochloride. In order to minimize the run time of the assay, an ultra-performance column was used. The extracted samples by protein precipitation, which typically contain a high concentration of organic solvent is not compatible with the separation using reversed column, which needs high aqueous mobile phase to be separated, while the use of ultra-performance column HSS T3 permits the use of aqueous mobile phase with good separation. Therefore a mixture of water with 0.1% formic acid: acetonitrile in ratio of 80:20 (v:v) was finally adopted as the mobile phase thus making direct injection of extracted samples possible. The total run time was 0.5 min per sample. The shortest analysis time in the literature was 2 min. The shorter analysis time would better meet the requirements for high sample throughput in bioequivalence study.

The low limit of detection (LLOD) for metformin hydrochloride was 5.00 ng/mL. Due to the lower injection volume of 10 μ L, the on column sensitivity in our study (The quantity of drug injected on the column per injection) was 50.00 pg which is lower than the published values. UPLC-MS-MS operation parameters were carefully optimized for the determination of metformin hydrochloride. The mass spectrometer was in positive ionization modes for metformin hydrochloride. In the precursor ion full-scan spectra, the most abundant ions were protonated molecules (M+H)⁺ m/z 129.93, and 275.14 for metformin hydrochloride and I.S., respectively. The product ion scan spectra showed high abundance fragment ions at m/z 59.99 and 229.93 for metformin hydrochloride and I.S., respectively. Retention time of metformin hydrochloride was 0.20 min and that for chlorpheniramine was 0.21 min, Figure 2.

3.2. Sample preparation

Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are techniques often used in the preparation of biological samples as they often improve the sensitivity and robustness of the assay. However, metformin hydrochloride had a very high polarity, so it was impossible to extract it from biological fluids using liquid-liquid extraction method. On the other hand, SPE would not be cost effective in a high throughput analysis involving many samples. Therefore, in the present experiment, a simple protein precipitation procedure was developed to reduce sample preparation time. No further concentration procedure was needed and the sample preparation procedure was simplified. To test extraction efficiency, three different protein precipitation agents (acetonitrile, methanol, and acetone) were investigated. Acetonitrile had a higher efficiency of precipitation of protein with minimal loss of extracted drug sample.

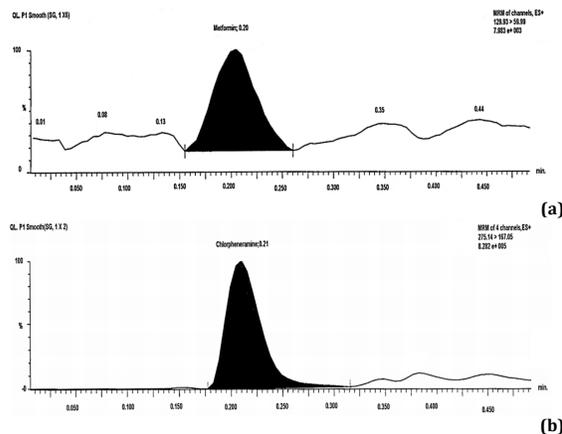


Figure 2. Typical chromatogram of metformin hydrochloride (R_t : 2.00 min) (a) and chlorpheniramine (R_t : 2.10 min) (b).

3.3. Method validation

3.3.1. Selectivity

Selectivity is defined as the ability of a chromatographic method to measure a response of the analyte without interference from the biological matrix. This was assessed by evaluating six individual lots of human plasma with the corresponding spiked plasma. There were no endogenous peaks that interfered with the quantitation of metformin hydrochloride or internal standard.

3.3.2. Linearity and LLOQ

The standard calibration curves for metformin hydrochloride was linear over the concentration range of 5.00-4000.00 ng/mL ($r^2 > 0.999$). The typical equations for the calibration curves for metformin hydrochloride was $y = 0.0005-0.0074x$ & $r^2 = 0.9997$. The lower limit of quantification for metformin hydrochloride was 10.000 ng/mL. A typical chromatogram is shown in Figure 2.

3.3.3. Precision, accuracy and dilution integrity

Table 1 shows the validation data on accuracy of each standard concentration. The CVs for the back-calculated calibration standards at 5.00 and 4000.0 ng/mL were 1.9 and 1.1%, respectively. The precision and accuracy data for QCs are summarized in Table 2. For QCs at 10.00, 2000.00 and 4000.00 ng/mL, inter-assay CV values were 1.5, 0.4 and 2.0%, respectively, and the percentage of nominal ranged from 99.0 to 101.8%. Intra-assay CV values for QCs at 10, 2000 and 4000 ng/mL were 2.1, 0.9 and 1.3 %, and the percentage of nominal ranged from 98.9 to 105.5%. These CV and percentage of nominal values indicate reproducibility and reliability of the UPLC-MS/MS method.

Table 2. Precision for the determination of metformin hydrochloride and in human plasma.

Added concentration of metformin hydrochloride (ng/mL)	CV (%) ^a	
	Intra-day	Inter-day
10.00	2.1	1.5
2000.00	0.9	0.4
4000.00	1.3	2.0

^a CV, coefficient of variation.**Table 3.** Stability of metformin hydrochloride in human plasma, n = 6.

Storage stability conditions	Statistical parameters	Metformin, ng/mL		
		10.000	2000.00	4000.00
Bench top stability (8 h)	Found mean plasma conc.	9.93	2049.00	4071.20
	SD ^a	0.35	24.79	85.50
	CV ^b	3.52	1.21	2.10
	Percentage of nominal	99.31	102.45	101.78
Three freeze-thaw cycles	Found mean plasma conc.	10.15	1989.20	4084.80
	SD ^a	0.152	39.78	52.00
	CV ^b	1.50	2.00	1.30
	Percentage of nominal	101.52	99.46	102.12
Long-term stability (30 days)	Found mean plasma conc.	11.02	2110.60	4338.80
	SD ^a	0.69	45.00	160.97
	CV ^b	6.25	2.13	3.71
	Percentage of nominal	110.21	105.53	108.47

^a SD, standard deviation.^b CV, coefficient of variation.**Table 4.** Pharmacokinetics parameters of metformin hydrochloride after administration of single oral dose of Mepaphage 500 mg Tablet, the test product (MEPACO, Egypt.) and Glucophage 500 mg Tablet, the reference product (Merck Sante, S.A.S., France).

Pharmacokinetic parameter	Mepaphage (Test drug) (Mean±SD)	Glucophage (Reference drug) (Mean±SD)
AUC _{0-t} (ng.h/mL)	4595±2070	4837±2794
AUC _{0-∞} (ng.h/mL)	4808±2115	5079±2840
C _{max} (ng/mL)	711±429	747±557
T _{max} (h) (Median)	2.5	2.5
K _{el} (h ⁻¹)	0.115±0.045	0.12±0.051
T _{1/2} (h)	7.12±3.3	7.27±3.95

3.3.4. Recovery

The extraction recovery was determined by comparing the peak areas of extracted plasma QC samples at 10, 2000 and 4000 ng/mL levels to the peak areas of metformin hydrochloride in solvent at the corresponding concentrations. The average recovery for metformin hydrochloride was 97.0%.

3.3.5. Stability of the analyte

Stock solution of analyte and IS were stable at room temperature for 22 h and at 2-8 °C for 30 days. The analyte in control human plasma at room temperature was stable at least for 8 h (bench top stability), stable for minimum of three freeze and thaw cycles. Spiked plasma samples, stored at -70 °C for long term stability experiment, were stable for minimum of 30 days. Different stability experiments in plasma with values for precision and percent change are shown in Table 3.

3.4. Bioequivalence study

The present method was successfully applied to a bioequivalence study of metformin hydrochloride after oral administration of two brands of metformin hydrochloride (Mepaphage 500 mg tablet MEPACO, Elsharkya, Egypt and Glucophage 500 mg film coated tablet MERCK SANTE S.A.S., France) in 24 healthy volunteers. Mean plasma concentration-time curves of metformin hydrochloride in a single dose study are shown in Figure 3. Bioequivalence criteria were met for the Mepaphage 500 mg tablet formulation relative to the Glucophage 500 mg film coated tablet formulation as the corresponding 94.7%. The pharmacokinetic parameters are shown in Table 4.

4. Conclusion

A relatively simple, accurate, precise and rapid method suitable for the determination of metformin hydrochloride in

plasma for bioequivalence studies was developed and validated. This method has several advantages compared to previously reported methods such as using of UPLC column, reducing flow rate and consequently decreased the volume of organic solvent used in the mobile phase and time of analysis (about 0.5 min). In addition, the method was comparatively simple, due to the use of direct solvent extraction and provided accuracy and precision, which was better than most previously reported methods. The current relatively very short run time of 0.5 min. has the advantage of facilitating and enhancing the efficiency of processing large numbers of plasma samples obtained from bioequivalence studies in healthy human subjects.

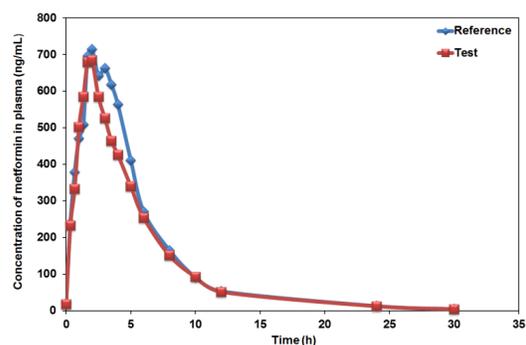


Figure 3. Mean plasma concentration of metformin after administration of single oral dose of Mepaphage (Metformin hydrochloride) 500 mg Film Coated Tablet, the test product (MEPACO, Elsharkya, Egypt) and Glucophage (Metformin hydrochloride) 500 mg Film Coated Tablet, the reference product (Merck Sante, S.A.S., France) in 24 healthy volunteers.

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