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Research Article

INCURRED SAMPLE STABILITY OF METFORMIN HYDROCHLORIDE IN PLASMA OF SIX HEALTHY SUBJECTS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Objective: This study aimed to measure metformin levels and evaluates the incurred stability in plasma of six healthy subjects after administration 850 mg metformin hydrochloride tablet using high-performance liquid chromatography.

Methods: Samples were collected from six healthy subjects who were administered 850 mg metformin hydrochloride tablet. Blood was collected from the subjects at up to 12-time points throughout a 12 h period. Sample stability was analyzed on days 7, 14, and 30. The analysis was conducted using C-18 columns, a column temperature of 40°C, a mobile phase consisting of acetonitrile-phosphate buffer (pH 7.0; 40:60 v/v), a flow rate of 1 mL/min, the photodiode array detector set at a wavelength of 234 nm, and calcium atorvastatin as the internal standard.

Results: All metformin HCl samples collected on days 7, 14, and 30 from subjects 1 to 6 met stability requirement based on the European Medicines Agency Bioanalytical Guideline (2011), which state that the percentage difference value not >20%. In addition, the developed method for analyzing metformin HCl in plasma was linear in the concentration range of 20.0–5000.0 ng/mL (r=0.9999).

Conclusion: The incurred sample of metformin hydrochloride in plasma of six healthy subjects was stable until 30 days after drug administration.

Keywords: High-performance liquid chromatography, Human plasma, Incurred sample, Metformin hydrochloride, Partial validation.

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INTRODUCTION

Metformin is an oral antidiabetes drug belonging to the biguanide class. Indeed, metformin is the first drug of choice for adults newly diagnosed with type 2 diabetes, particularly for those people who are overweight or obese. The mechanism of action of metformin consists of lowering blood sugar levels by decreasing the production of hepatic sugars and reducing the absorption of glucose in the small intestine. In addition, metformin serves to maintain blood glucose levels in peripheral tissues, particularly after meals and has antilipolytic effects that decrease serum free fatty acid concentrations, thereby reducing substrate availability for glycogenesis [1-4].

Metformin is considered a critical-use drug, which includes drugs for serious conditions that require a definite response. The study of the pharmacokinetics of metformin has included a series of tests to determine certain pharmacokinetics parameters [5,6]. This pharmacokinetic test data can then be used to assess the rate of change or the degree of availability between formulations (i.e., bioequivalence [BE]). However, before performing pharmacokinetic studies, it is necessary to validate the bioanalysis method for the drug. In this process, one of the parameters of interest is the incurred sample reanalysis (ISR) [7,8].

The assessment of recurrent levels of study samples as part of the ISR has the purpose of evaluating the reproducibility of the results of bioanalysis tests. As such, ISR is considered routine practice in bioanalysis, which is a requirement based on regulations and guidelines concerning the validation of bioanalytical methods, including those stipulated by the European Medicines Agency and the Food and Drug Administration. Specifically, the incurred sample stability is a reanalysis of some incurred samples over a period of time to determine whether the analyte is stable and present in reproducible analytical concentrations. In general, analyte stability can be assessed by measuring *in vitro* QC samples known to be concentrated at a given time. However, in some cases, the QC sample is not capable of representing analytical stability. For example, component formations due to other metabolic processes in *in vivo* generated samples may significantly affect the quantity of the analyte [9].

Several methods for analyzing metformin in plasma using high-performance liquid chromatography (HPLC) have been reported. In a previous study, the development and validation of the determination of metformin by HPLC were only determined in spiked plasma, and no studies have evaluated ISS. Therefore, in this study, we sought to measure metformin levels and evaluate incurred sample stability in plasma samples from healthy subjects. HPLC was selected as the analytical method because it includes tools that are generally present in the laboratory; thus, methods can be applied for BE testing as well as other clinical trials. In this study, we used a photodiode array detector, which allowed for a quick and accurate analysis [10].

After validation of analysis method, we considered it necessary to further evaluate the stability of metformin HCl in the plasma of healthy subjects after administration of an 850-mg metformin tablet. We performed an ISS on six subjects by reanalyzing some of the samples that were around the C_{max} and in the elimination phase. Blood sampling in the subjects was performed at up to 12 time points during a 12 h period.

METHODS

This research passed the ethical review of the Medical Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia, with number 63/UN2.FI/ETIK/2017.

Solution preparation

Preparation of the metformin HCl solution for the calibration curve

A standard metformin HCl stock solution was prepared by dissolving 10.0 mg into 10 mL of methanol. From the solution, 1 mL was further

diluted to 100 mL. From this dilute solution, methanol was added to obtain different concentrations of the calibration curve.

Preparation of the metformin HCl solution for quality control

A standard metformin HCl stock solution was prepared by dissolving 10.0 mg into 10 mL of methanol. From this solution, 1 mL was further diluted to 100 mL. From this dilute solution, aquadest was added to obtain several concentrations of the QC sample.

Preparation of the atorvastatin solution as an internal standard

The stock solution was prepared by dissolving 10 mg in 100 mL of methanol followed by dilution at 1:100.

Experimental steps

Verification of the chromatographic conditions for metformin HCl analysis

The analytical method used HPLC method in conjunction with a photodiode array detector set at a wavelength of 234 nm. The separation used a C18 column (Waters, SunFire™ 5 µm; 250×4.6 mm). The mobile phase consists of 10 mM sodium dodecyl sulfate and 10 Mm of phosphate buffer in water-acetonitrile (60:40). The mobile phase was adjusted to pH 7.0 with 98% orthophosphoric acid; the column temperature was set to 40°C, and the flow rate was 1 mL/min over a 10-min analysis period.

Sample preparation from healthy subjects

Sampling procedure

Blood samples were taken at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, and 12 h. As much as 5 mL was taken at each time, after the healthy subjects were administered a tablet containing 850 mg metformin HCl. Blood was collected into a 5-mL vacuum tube containing a citrate anticoagulant.

Sample preparation

Sample preparation of metformin HCl in plasma was performed by protein precipitation. A 300 µL plasma aliquot containing a concentration of metformin HCl was added to the sample cup followed by the addition of atorvastatin (50.0 µL; 100 µg/mL) and 600 µL acetonitrile as a protein precipitator. The solution was then shaken and vortexed for 30 s to extract the metformin HCl from the plasma. The mixture was then centrifuged to precipitate proteins at 13,000 rpm for 10 min. The supernatant was transferred into a vial. Then, as much as 50.0 µL was injected into the HPLC system.

Partial validation of analytical method

The lower limit of quantitation (LLOQ)

The calibration curve of metformin HCl solution was prepared using concentrations of 12.5, 50, 100, 500, 1000, 2500, and 5000 ng/mL, and 50.0 µL of atorvastatin (100 µg/mL). Each concentration was extracted as described above and aliquots (as much as 50 µL) were injected into the HPLC system.

Calibration curve

The calibration curve solution was prepared by diluting the standard metformin HCl 10 µg/mL solution with plasma to produce concentrations of 20, 50, 100, 250, 500, 1000, 2500, 3750, and 5000 ng/mL. At each concentration, 50.0 µL of atorvastatin (100 µg/mL) was added and the solution was extracted according to the sample preparation method. Aliquots generated from each sample were then injected (as much as 50.0 µL) into the HPLC system under the selected chromatographic conditions.

Accuracy and precision

The plasma was prepared with metformin HCl at concentrations of the LLOQ and QC. Standard solution of metformin HCl 10 µg/mL was diluted with plasma to give the following concentrations:

a. QCH = 75%×ULOQ (5000 ng/mL)=3750 ng/mL
b. QCM = 50% the concentration range of the calibration curve= 2500 ng/mL
c. QCL = 3×LLOQ concentration (12.5 ng/mL)=37.5 ng/mL

At each concentration, 50.0 µL atorvastatin (100 µg/mL) was added and the solution was extracted according to the sample preparation method. A total of 50.0 µL aliquots of each solution were then injected into the HPLC system.

Stability test

Stability of stock solutions of metformin HCl and atorvastatin

The metformin HCl stock solution in aquabidestilata was prepared at a concentration of 5000 ng/mL and the standard stock solution of atorvastatin was prepared at a concentration of 10 µg/mL. Stock solutions of metformin HCl and the atorvastatin were stored at room temperature for 0, 6, and 24 h as a short-term stability test. A portion of the solution was again stored at 4 °C (refrigerator) over a range of 0, 7, 14, and 30 days as a long-term stability test. Each stock solution was then injected (as much as 50.0 µL) into the HPLC system.

Freeze and thaw stability

Plasma samples were subjected to three freeze-thaw cycles. Samples were then stored and frozen at a temperature −20°C in the freezer for 24 h, then discharged and melted at room temperature. Samples were then refrozen for 12 h. The analysis was performed on the 1st and 3rd cycles. Samples were thawed first at room temperature and then added with 50.0 µL standard atorvastatin solution (100 µg/mL), then extracted as described for sample preparation. Aliquots (50.0 µL) were then injected as the supernatant for each concentration into the HPLC system.

Short-term stability of metformin HCl in plasma

Plasma samples were stored at room temperature for 0, 6, and 24 h. Thereafter, each concentration was added with 50.0 µL of standard atorvastatin solution (100 µg/mL), then extracted as in the sample preparation. Aliquots of each concentration were then injected (as much as 50.0 µL) into the HPLC system.

Long-term stability of metformin HCl in plasma

Plasma samples were stored at −20°C for a defined time period. On days 0, 7, and 14 at each sample concentration, 50.0 µL atorvastatin (100 µg/mL) was added, and the sample was extracted as in the sample preparation. Aliquots for each concentration were injected (as much as 50 µL) into the HPLC system.

Post-preparation stability (autosampler)

Plasma samples were added with 50.0 µL atorvastatin (100 µg/mL), then extracted as in the sample preparation. Aliquots were stored on the autosampler at room temperature for 24 h and then analyzed at the 0 and 24 h.

Pharmacokinetics parameter analysis of six healthy subjects in plasma

In this study, pharmacokinetic parameters were analyzed for six healthy subjects in plasma on days 0, 7, 14, and 30. For each subject, the average of AUC_{0-t}, C_{max}, t_{max}, and $t_{1/2}$ was calculated.

ISS of metformin HCl in plasma

The ISS of metformin HCl in plasma was performed over a period of time to determine the stability of the analyte. The ISS was performed with plasma samples stored at −20°C for a defined time period. On days 0, 7, 14, and 30, 50.0 µL atorvastatin (100 µg/mL) was added, and the sample was extracted as in the sample preparation. The supernatants from each concentration were then injected (as much as 50 µL) into the HPLC system. ISS was then analyzed in the C_{max} and elimination phase for each healthy subject.

RESULTS AND DISCUSSION

Validation of metformin HCl methods in plasma

LLOQ measurement

In this study, the measurement of the LLOQ value was done by making an analytical calibration curve with a concentration of 20 ng/mL, up to 5000 ng/mL. The concentration of 20 ng/mL becomes LLOQ, and the repetition of these concentrations was carried out for five replicas. The values of percentage difference and coefficient of variation (CV) at concentrations of 20 ng/mL still met the accuracy and precision criteria of ≤20%.

Results of the calibration curve

The calibration curve consisted of zero samples (plasma with internal standard) and non-zero plasma samples (plasma with analyte and internal standard) of 9 concentrations, namely, 20, 50, 100, 250, 500, 1000, 2500, 3750, and 5000 ng/mL (Fig. 1).

Accuracy and precision

From the within-run accuracy measurements, we obtained a percentage difference value for the LLOQ concentrations ranging from −7.64% to +9.03%, while that for the QC concentrations ranged from −3.39% to +2.95%. For the measurement of within-run precision, we obtained a CV value of <6.68% for the LLOQ and QC concentrations. For the between-run measurement, we obtained an accuracy for the LLOQ concentration with the percentage difference range from −15.67% to +19.05%, while that for the concentration of QC had a percentage difference range from −5.56% to +13.43%. For the measurement of between-run precision, we obtained a CV value <7.76%. Both the within-run and between-run percentage difference and CV values met the precision and precision requirements.

Stability of stock solutions of metformin HCl and atorvastatin

The stability test of the metformin HCl atorvastatin stock solution met the requirement that the percentage difference value should not exceed ±2%.

Stability of freeze-thaw metformin HCl in plasma

The results we obtained for the percentage difference values for QCL ranged from −0.72% to +4.13%, while that for QCH ranged from −0.72% to −0.77%. The freeze-thaw stability test met the requirements that the percentage difference and CV values should not exceed ±15%.

Short-term stability of metformin HCl in plasma

Based on these data, the percentage difference after 24 h for the QCL concentrations ranged from −0.72% to +3.14%, while that for the QCH concentrations ranged from −0.30% to −0.78%. This indicates that plasma samples of metformin HCl can be stored at room temperature for at least 24 h as it met the stability requirements.

Long-term stability of metformin HCl in plasma

From the results obtained at −20°C, the percentage difference values on the 14th day of the QCL concentration ranged from 1.03% to 3.24%, while that at QCH concentrations ranged from−0.31% to −0.76%. For the results obtained at −80°C, the percentage difference value on the 14th day of the QCL concentration ranged from 0.71% to 3.52%, while that at QCH concentrations ranged from −0.27% to −0.83%. This indicates that plasma samples of metformin HCl are still stable and can be stored at −20°C or−80°C for at least 14 days as it met the stability requirements.

Post-preparation stability (autosampler) of metformin HCl in plasma

Based on the data, the value of percentage difference after 24 h for QCL ranged from 2.08% to 4.41%, while that for QCH ranged from −0.29% to +0.09%. Thus, the extracted plasma can be stored in the autosampler for at least 24 h before because it met the requirements that the percentage difference value ≤15%.

Pharmacokinetic parameter analysis of six healthy subjects in plasma

The analysis of the pharmacokinetics parameters in plasma from six healthy subjects who administered 850 mg metformin hydrochloride tablets obtained median values as follows (Figs. 2-5):

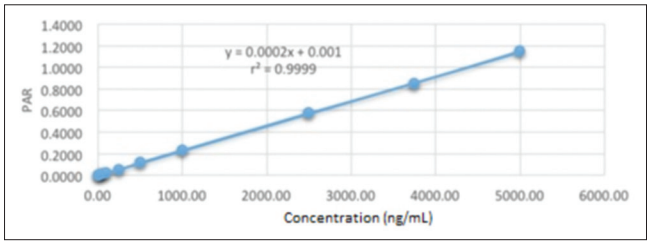

Fig. 1: Metformin HCl calibration curve

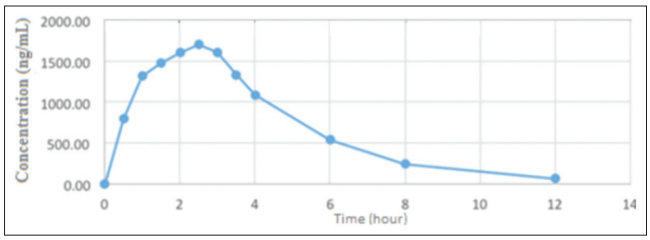

Fig. 2: Pharmacokinetic profiles of six healthy subjects in plasma day 0

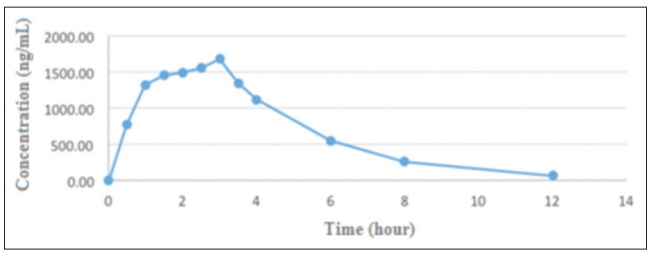

Fig. 3: Pharmacokinetic profile of six healthy subjects in 7th-day plasma

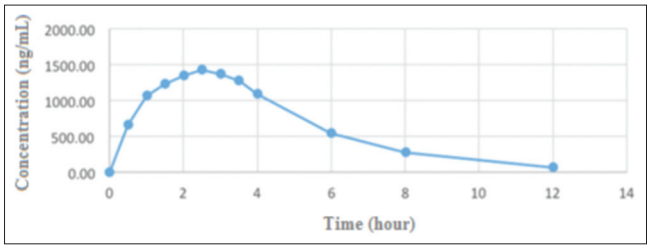

Fig. 4: Pharmacokinetic profile of six healthy subjects in the 14th-day plasma

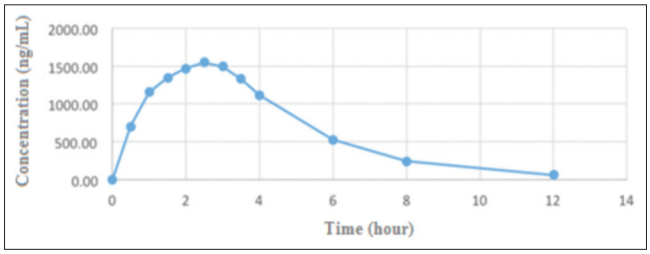

Fig. 5: Pharmacokinetic profile of six healthy subjects in the 30th-day plasma

Table 1: Data of incurred sample stability of subjects

Subject	Period	Days		
		7	14	30
Subject 1	5	1.9699	8.1338	17.6180
	6	4.8268	7.7860	17.2180
	9	3.1591	7.8418	9.55000
Subject 2	5	4.5311	13.8201	19.2554
	6	5.3822	9.2911	16.9648
	9	5.3812	7.5837	18.4434
Subject 3	5	0.1345	8.8426	15.4791
	6	4.5965	10.1975	16.4481
	9	3.4614	3.4769	11.4038
Subject 4	5	4.6369	6.7828	12.8174
	6	5.1418	8.6638	16.2470
	9	3.5954	8.6945	15.8748
Subject 5	5	0.8373	7.3097	16.1559
	6	4.9249	10.6615	14.1513
	9	2.0786	10.0936	16.5275
Subject 6	6	4.2576	8.8480	16.3667
	7	1.5022	7.7817	14.2778
	9	4.5763	7.3055	9.6193

Day 0 C_{max}=1724 ng/mL; t_{max}=3 h; $t_{1/2}$=2 h; AUC_{0-t}=8248 ng h/mL; $AUC_{0-\infty}$=8457 ng h/mL; day 7 C_{max}=1649 ng/mL; t_{max}=3 h; $t_{1/2}$=2 h; AUC_{0-t}=7987 ng h/mL; $AUC_{0-\infty}$=8188 ng h/mL; day 14 C_{max}=1568 ng/mL; t_{max}=3 h; $t_{1/2}$=2 h; AUC_{0-t}=7810 ng h/mL; $AUC_{0-\infty}$=8007 ng h/mL; and day 30 C_{max}=1450 ng/mL; t_{max}=3 h; $t_{1/2}$=2 h; AUC_{0-t}=7584 ng h/mL; $AUC_{0-\infty}$=7796 ng h/mL.

The ISS of metformin HCl in plasma

The ISS of metformin HCl in plasma was performed over a period of time to determine the stability of the analyte. The ISS was done by storing the plasma at −20°C for a defined time period. On days 0, 7, 14, and 30, at each sample concentration, 50.0 µL atorvastatin (100 µg/mL) was added. The sample was then extracted as in the sample preparation. ISS data obtained can be seen in Table 1. Based on the calculation of ISS analysis, all subjects met the requirements that the percentage difference value should not be >20% of the mean in at least 67% or two-thirds of the total sample conducted as stated in the EMEA Bioanalytical Guidelines (2011).

CONCLUSION

The incurred sample of metformin hydrochloride in plasma od six healthy subjects was stable until 30 days after administration of 850 mg HCl tablets.

CONFLICTS OF INTEREST

All authors have none to declare.

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