

PHARMACOKINETIC PROFILE OF METFORMIN HYDROCHLORIDE IN DRIED BLOOD SPOT OF HEALTHY SUBJECTS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-PHOTODIODE ARRAY

YAHDIANA HARAHAP*, CITRA DARA MALIA, SUNARSIH

Bioavailability/Bioequivalence Laboratory, Faculty of Pharmacy, Universitas Indonesia, Depok 16424, Indonesia.

Email: yahdiana03@yahoo.com

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ABSTRACT

Objective: This study aimed to analyze the metformin hydrochloride in dried blood spot (DBS) sample and evaluate a pharmacokinetic profile in six healthy subjects who administered 850-mg metformin hydrochloride in tablet using high-performance liquid chromatography-photodiode array (HPLC-PDA).

Methods: Metformin analysis was performed on data of six healthy subjects who administered 850 mg of metformin hydrochloride as tablets. Blood samples were taken at 12-time points at intervals of up to 12 h, and data were analyzed using HPLC-PDA.

Results: A linear calibration curve was obtained in the range of 25–5000 ng/mL, with $r=0.9990$. Pharmacokinetic profiles of metformin were obtained using DBS samples from six healthy participants, with a C_{max} of 347.3–416.22 ng/mL. The average t_{max} and $t_{1/2}$ were obtained at 3 and 2 h, respectively, and the area under the curve (AUC)_{0-t}/AUC_{0-∞} ratio was >80% for all subjects.

Conclusion: The DBS biosampling technique can be developed for application in pharmacokinetic study of metformin HCl.

Keywords: Dried blood spot, High-performance liquid chromatography, Metformin hydrochloride, Pharmacokinetic profile, Validation.

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INTRODUCTION

Diabetes mellitus (DM) is a disorder of carbohydrate, protein, and fat metabolism due to decreased insulin secretion, insulin receptor sensitivity, or both and can be identified by hyperglycemia in the patient [1]. In 2015, the International Diabetes Federation rated Indonesia as the country with the seventh greatest number of people with diabetes aged 20–79 years, with 10 million people with the disease. By 2040, the number of people with diabetes in Indonesia is projected rise to 16.3 million. Furthermore, 90–95% of all cases of diabetes are type 2 DM [2].

Metformin is the first-line medication for patients with type 2 DM and is the drug of choice for type 2 DM patients with obesity [3]. Metformin is included in the National List of Essential Medicines, the list of drugs that are assessed using a bioequivalence (BE) test, and was one of the most commonly prescribed drugs worldwide in 2014 [4–8].

Pharmacokinetic studies comprise a series of tests to determine the pharmacokinetic parameters of a drug [4]. They generally use plasma samples, and biosampling techniques are performed by taking a volume of approximately 5–15 mL of blood through venipuncture, often causing discomfort for the subject. However, over the past 5–7 years, a dried blood spot (DBS) technique has been intensively developed and evaluated for the quantification of drug exposure in non-clinical as well as clinical studies at various phases for drug research and development. DBS is an innovative biosampling technique in which small quantities (10–80 µL) of wet blood samples are collected on DBS paper or other paper materials then dried for 2–3 h at room temperature or under nitrogen gas flow with controlled air humidity [9]. This method is preferable as it is minimally invasive for the patient (using a sterile lancet), the volume of blood required is low, it is simple and does not cost much to implement, and the analytes in the dry blood sample are relatively stable in the long term. Metformin is reported to be partitioned into erythrocytes; therefore,

the use of DBS could be considered instead of plasma samples to measure metformin concentrations [10,11].

Metformin hydrochloride analysis using DBS samples requires a device that has high selectivity and sensitivity due to the low blood volume and the fact it is derived from the periphery [12]. High-performance liquid chromatography (HPLC) is the most widely used technique for drug analysis in biological matrices. It is a commonly used instrument that is available in the laboratory and analytical methods are more applicable [13]. Photodiode array (PDA) detectors are commonly used and are advantageous in sensitive and selective HPLC systems for the analysis of drugs and their metabolites in biological matrices [14].

To date, there have been no reports of pharmacokinetic tests of metformin hydrochloride using the DBS biosampling technique. Further, analysis is required to determine whether metformin hydrochloride bioanalysis using the DBS biosampling technique can be applied *in vivo* as well as more widely.

In the present study, six healthy subjects administered 850 mg of metformin hydrochloride in tablet formulation. Blood samples were taken at 12-time points at various intervals up to 12 h. The study was approved Ethical Clearance from the Medical Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia (no. 63/UN2.FI/ETIK/2017).

The aim of the present study was to analyze metformin hydrochloride in DBS sample and evaluate a pharmacokinetic profile in six healthy subjects who administered 850-mg metformin hydrochloride in tablet using HPLC-PDA.

METHODS

Instruments

HPLC (Waters 2695 Separation Mode) consisting of pumps, an autosampler, C18 column (Waters, Sunfire™ 5 µm; 250×4.6 mm),

PDA (Waters 2996), and a computer for data processing. Other equipment used included a pH meter (Eutech pH 510), evaporator (Turbovap Caliper), analytical scales (Acculab), eluent filters and samples (Whatman), degasser (Elmasonic S60H), vortex (Maxi Mix II), Eppendorf micropipettes (Socorex), microtubes, sample cups, pipette tips, and glassware.

Materials

Test materials

DBS samples were obtained from blood samples collected on DBS paper from six healthy subjects who administered 850 mg of metformin hydrochloride in tablet.

Chemical materials

The materials used included metformin hydrochloride and calcium atorvastatin were purchased from British Pharmacopoeia, HPLC grade acetonitrile (Merck), HPLC grade methanol (Merck), sodium dihydrogen phosphate monohydrate (Merck), sodium dodecyl sulfate (SDS) (Merck), orthophosphoric acid (Merck), sodium hydroxide (NaOH) (Merck), aquabidest (Ikapharmindo), DBS paper (Perkin Elmer), human blood (Indonesian Red Cross), and 850-mg immediate release metformin hydrochloride tablets (Merck).

Sampling materials

Sampling materials included sterile pens and lancets (sterile needle; Medilance), Eppendorf micropipettes (Socorex), alcohol swabs (OneMed), and 0.5-mL vacutainers (GM Micro Tube).

Stock solution of metformin HCl and atorvastatin

10 milligrams of standard metformin hydrochloride were carefully weighed and added into a 10-mL measuring flask. The substance was dissolved in aquabidest, to a final volume of 10 mL, giving a standard compound solution of 1000 µg/mL. The stock solution was diluted to obtain solutions of various concentrations for a calibration curve. 10 mg of standard atorvastatin compound were also carefully weighed and dissolved with methanol to a final volume of 10 mL. The resulting stock solution of 1000 µg/mL was diluted to obtain solutions of various concentrations.

Chromatography conditions

HPLC-PDA was performed using a detector with a wavelength of 234 nm. The column used was a C18 (Waters, Sunfire™ 5 µm; 250×4.6 mm). The mobile phase was 10-mM SDS and 10-mM phosphate buffer in water-acetonitrile (60:40) and then was adjusted to pH 7.0 with orthophosphoric acid and NaOH; the column temperature was 40°C; flow rate was 0.8 mL/min, with an analysis time of 10 min.

Sample preparation

A volume of 20 µL of blood containing analytes was pipetted and spotted on DBS paper. The samples were then dried at room temperature (20–25°C) for 3 h. After drying, the paper was cut according to the marking on the paper, then inserted into a microtube, and 50 µL of atorvastatin (10 µg/mL) as an internal standard and 0.5 mL of methanol (60%) as the extracting solvent were added. Sonication was performed for 10 min. The process was continued by mixing the solution using a vortex for 90 s. The solution was then transferred into the microtubes and evaporated to dryness at 60°C for 15 min under nitrogen gas. The residue was reconstituted by adding 200 µL of mobile phase followed by sonication for 3 min. The solution was then transferred to an autosampler vial and centrifuged for 10 min (room temperature, 3000 rpm, 10 min). A 100-µL sample volume was injected into HPLC system.

Partial validation of bioanalysis methods

Calibration curve and linearity test

Blank samples (whole blood without analyte), zero samples (whole blood with internal standard), and metformin hydrochloride solution in whole blood at concentrations of 25, 50, 75, 100, 500, 1000, 2500, 3750,

and 5000 ng/mL were collected. A calibration curve was obtained by spiking each blood sample to obtain the desired concentration. Then, 20 µL of the spiked blood was collected on DBS paper and dried for around 3 h. The paper was cut according to the marking on the paper and inserted into microtubes. Next, 50 µL of atorvastatin (10 µg/mL) was added as an internal standard and extracted using the same extraction method as used for the sample preparation. A 100-µL sample volume was injected into the HPLC system.

The regression value of the peak area (y) to the concentration of analyte in plasma (x) was calculated for each concentration, and a calibration curve was prepared. A linearity test was performed using data from the calibration curve, then analyzed using peak area regression to the metformin concentration in the DBS sample, and a correlation coefficient (r) was obtained, demonstrating its linearity. In addition, calculation of the percentage difference (% diff) with the limit value of % diff did not exceed 20% for the lower limit of quantification (LLOQ) and 15% for all other concentrations.

Within-run accuracy and precision

Metformin hydrochloride solutions in whole blood were made with LLOQ concentration and metformin quality control (QC) solutions with low, medium, and high (QC low [QCL], QC medium [QCM], and QC high [QCH], respectively) concentrations. A volume of 20 µL of blood was spotted on DBS paper, dried for around 3 h, and the paper was cut according to the marking on the paper. The paper was inserted into microtubes, then 50 µL of atorvastatin (1 µg/mL) was added as an internal standard and extracted using the same method as the sample preparation. Next, a 100-µL sample volume was injected into HPLC system. Accuracy and precision tests were performed using five replicas at each concentration in one run (intrabatch/within-run accuracy). The % diff of data obtained was calculated to determine the accuracy. The precision was measured using the calculation of the coefficient of variation (% CV) data obtained. The requirement % diff and % CV values were <20% for LLOQ and <15% for all other concentrations.

Treatment of healthy subjects

The scope of the study was explained to the six healthy subjects, and all gave signed informed consent and passed the screening. The day before the sampling, the subjects quarantined at the Bioavailability and Bioequivalence Laboratory of the Faculty of Pharmacy, Universitas Indonesia. After an 8-h fast, each subject received an oral dose of 850-mg metformin hydrochloride tablets, with 240 mL of water. To prevent hypoglycemia, subjects were given 100 mL of a 10% glucose solution every hour for 3 h. Subsequently, at 4, 8, and 12 h after administration of the 850-mg metformin hydrochloride tablet, subjects were fed standard foods. Standardization of body position and physical activity throughout the study day was ensured due to their effects on motility and blood flow of the gastrointestinal tract.

Sampling and preparation of DBS samples from healthy subjects

Blood samples were taken at 0 (predose), 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, and 12 h after administration of 850-mg metformin hydrochloride tablets in six healthy subjects (12-point collection). Blood collection was performed through finger prick using a sterile lancet needle. The amount of blood used for this study was approximately 40 µL. Blood was collected in 0.5-mL vacutainers, and 20 µL was spotted on DBS paper and dried for approximately 3 h. The paper was cut out according to the marking on the paper, inserted into microtubes.

Metformin hydrochloride analysis in the sample

Analysis was performed using HPLC-PDA (100-µL sample injection volume) with an optimized mobile phase to determine the concentration of metformin hydrochloride in healthy subjects who administered 850-mg metformin hydrochloride in tablet to evaluate the pharmacokinetic profile. From the peak area obtained, the concentration of metformin hydrochloride was calculated using a standard calibration curve.

Calculation of pharmacokinetic parameters

Metformin hydrochloride blood concentration data were plotted to form a pharmacokinetic profile curve (concentration vs. time). From these data, the pharmacokinetic parameters of the maximum concentration in blood (C_{max}) and the maximum time (t_{max}) were calculated. Other pharmacokinetic parameters determined included half-life ($t_{1/2}$), area under the curve (AUC_{0-t}) and $AUC_{0-\infty}$.

RESULTS AND DISCUSSION

Calibration curves

The results indicated that the range of the calibration curve for metformin was 25–5000 ng/mL. The calibration curve consisted of blanks (whole blood without analyte and internal standard), zero samples (whole blood with internal standard), and non-zero blood (whole blood with analyte and internal standard) using nine different concentrations: 25, 50, 75, 100, 500, 1000, 2500, 3750, and 5000 ng/mL. The calibration curve yielded a linear regression equation of $y=0.0003x+0.0114$ with $r=0.9990$, where x represents the concentration of metformin (ng/mL) and y represents the peak area ratio between metformin and atorvastatin. These data show that the calibration curve obtained met the requirements of $\leq 20\%$ at concentrations of LLOQ and $\leq 15\%$ at other concentrations.

Within-run accuracy and precision

In the present study, only partial validation was performed, including precision and intraday accuracy. According to the 2011 guideline of Bioanalytical Method Validation from the EMEA, partial validation can be performed, starting with intraday accuracy and precision parameters until almost all validation parameters are full. Full validation was not performed because the analytical methods were fully validated in other studies using the same research conditions including location, instruments, methods of extraction, and bioanalysis methods. An accuracy test was performed to assess the similarity between the measured and actual concentrations by determining its bias value (% diff). A precision test was conducted to determine the proximity of the repetition of the measurement analytes by analyzing the value of % CV. The accuracy and precision tests were performed using five replicas of four different concentrations of metformin hydrochloride in whole blood, namely LLOQ, QCL, QCM, and QCH. It was essential that both accuracy and precision met the requirement stated in the guidelines by the EMEA, that is, $\leq 20\%$ at concentrations of LLOQ and $\leq 15\%$ at other concentrations.

The results of the within-run accuracy testing on metformin met the requirements; the % diff value at LLOQ concentration was 12.35%–17.43%, and the QC concentration was 1.48–13.60%. The within-run precision test also met the requirements for the analyte with a % CV value for metformin at 2.79% for LLOQ, 2.75% for QCL, 1.56% for QCM, and 2.37% for QCH. Although the obtained % CV and % diff were different from previous studies, the accuracy and precision test results in the analytes remained within the required range, and still qualified, demonstrating that the method of analysis was accurate and precise.

Analysis of samples from healthy subjects

Sampling process

Blood samples were taken under the supervision of a doctor. The subjects then received an oral dose of 850-mg metformin hydrochloride in tablet (Glucophage) with 240 mL of water. To prevent hypoglycemia, subjects were given 100 mL of a 10% glucose solution every hour for 3 h. Subsequently, at 4, 8, and 12 h after administration of metformin hydrochloride, the subjects were fed standard meals. Body position and physical activity were standardized throughout the study day due to their effects on motility and blood flow of the gastrointestinal tract.

Up to 40 μ L of blood was collected at 12-time points: 0 (predose), 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, and 12 h after administration of 850-mg metformin hydrochloride tablets. Blood collection was performed

through finger prick using a sterile lancet needle. The collected blood was then transferred to 0.5-mL vacutainers. Side effects were monitored during the study based on direct questions, spontaneous reports, and clinical parameters measured during the sampling process. Adverse event during the sampling process or complaints from the subject were monitored and studied by the doctor.

After blood was collected, 20 μ L was directly spotted on DBS paper and dried in a horizontal position for 3 h in an open space at room temperature (20–25°C). Samples were stored in an airtight plastic (sealable plastic) with a silica pack to control the moisture during storage. Humidity was well controlled to reduce the risk of analytical degradation due to bacterial growth that may have affected the results of the analysis. The dried blood samples were stored at room temperature in dry and cool (20–25°C) conditions before analysis. The DBS sample was then prepared for partial validation methods.

Sample analysis results

Analysis was performed on one batch to generate three calibration curves (CC1, CC2, and CC3). Sample QC 1 consisted of QCL, QCM, and QCH of each of two replicas, and 12 sample points of subject 1; sample QC 2 consisted of QCL, QCM, and QCH of each of two replicas, 12 sample points of participant 2; and sample QC 3 consisted of QCM and QCH of each of the two replicas. The calibration curve yielded a linear regression equation of $y=0.0003x+0.0116$, with $r=0.9980$ for CC1; $y=0.0003x+0.0119$, with $r=0.9990$ for CC2; and $y=0.0003x+0.0112$, with $r=0.9992$ for CC3. Sample analysis was completed within 1 month after sampling. The analysis time still met the requirements as the long-term stability validation was 45 days.

Fig. 1 shows the metformin hydrochloride concentrations in blood plotted on a pharmacokinetic profile curve (concentration vs. time). The data show the pharmacokinetic parameters, that is, AUC, the maximum concentration in blood (C_{max}), maximum time (t_{max}), and halftime ($t_{1/2}$). The calculated pharmacokinetic parameters for each participant are shown in Table 1.

The results showed that the C_{max} obtained from six healthy subjects ranged from 347.3 to 416.22 ng/mL. The mean C_{max} was 367 ng/mL with a standard deviation of 26.14 ng/mL. The lowest C_{max} concentration was 347.3 ng/mL, observed in subject CF1, and the highest concentration was 416.22 ng/mL, observed in subject CF4. There was a slight difference in the maximum concentration measured in each participant. This was due to variations in the body's response to metformin and metabolic processes occurring in each participant, resulting in variations in concentrations. The t_{max} in the six test subjects was observed at the sixth and seventh blood sampling time points, that is, at 2.5 h after drug administration for subjects CF1, CF2, CF4, and CF5 and 3 h after drug administration for subjects CF3 and CF6. The mean t_{max} for the six subjects was 3 h. This was in accordance with the literature showing that t_{max} was obtained around 2–3 h after drug

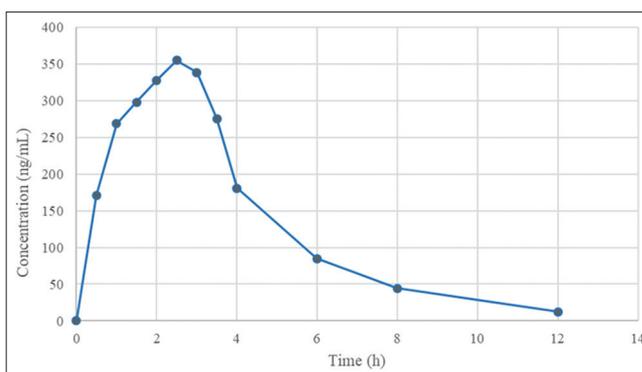


Fig. 1: Mean pharmacokinetic profile versus time of metformin HCL on six healthy subjects in dried blood spot samples

Table 1: Results of pharmacokinetic parameters

Subject	C _{max} (ng/mL)	t _{max} (h)	t _{1/2} (h)	AUC _{0-t} (ng.h/mL)	AUC _{0-∞} (ng.h/mL)	AUC _{0-t} /AUC _{0-∞} (%)
CF1	347.3	2.5	1.67	1309.36	1309.36	100
CF2	348.4	2.5	1.67	1349.87	1349.87	100
CF3	362.94	3	2.80	1729.3	1833.51	94.32
CF4	416.22	2.5	2.94	1861.79	1968.31	94.59
CF5	352.64	2.5	1.65	1404.53	1404.53	100
CF6	373.02	3	2.48	1772.04	1861.68	95.18
Mean±SD	367±26.14	3±0.26	2±0.61	1571±242.94	1621±297.05	97.35±2.92
% CV	7.13	9.68	27.63	15.46	18.32	3.00

% CV: Coefficient of variation, SD: Standard deviation, AUC: Area under the curve

administration [15]. Our results showed that the AUC_{0-t}/AUC_{0-∞} met the requirements of >80% for all test subjects.

The C_{max} values obtained from the six subjects showed that the achieved LLOQ was in the range of 6.00–7.10% of the maximum concentration of the analyte in the body (C_{max}), while the EMEA requirement is 5%. As there were no previous studies on the pharmacokinetic profile of metformin hydrochloride with biosampling of DBS, the concentration of metformin in plasma was used as a reference for C_{max}. However, we demonstrated that the C_{max} for metformin using DBS was lower than the C_{max} for metformin in plasma; therefore, a more sensitive LLOQ was required in the range of 17.4–20.91 ng/mL. This could have been caused by unextracted analytes in the DBS paper, making the LLOQ measurements less sensitive. The DBS paper used was polar and made of cellulose, so there was a high probability of metformin having polar properties, strongly bound to the DBS paper.

The metformin concentrations in DBS were lower than those in plasma. Following administration of 850-mg metformin hydrochloride tablets, the C_{max} of metformin in plasma was 1000–1500 ng/mL (Porta *et al.*, 2008). A major contributor to the lower concentration of metformin in DBS samples compared with plasma is due to the blood samples being taken from the periphery and in small volumes. Whole blood from the periphery comprises blood cells, plasma, and interstitial fluid, as well as more intrusive components. In addition, there was a difference in analyte concentration in the periphery (capillary vessels) with the veins [12,16]. The distribution coefficient (cP/cBC) for metformin was 4.65±0.73. This suggests that the plasma concentration of metformin is 4 times higher than that of the blood cell concentration [11]. This may have contributed to the lower concentration of metformin observed with DBS biosampling compared with venous biosampling. Therefore, a multiplier factor is required to convert the concentration from the DBS analyzer to the plasma analyzer concentration.

The DBS method has more advantages than vein biosampling method. It is a more comfortable method of biosampling as it is minimally invasive, especially during the pharmacokinetic and BE tests, where blood were collected for several points (12-18 times) over a period of time. The technique is also simpler and does not cost much to implement, and the analytes in the sample tend to be more stable compared with other biosampling techniques [11]. Metformin is partitioned in erythrocytes; therefore, DBS biosampling for metformin analysis is possible and can be used for pharmacokinetic, BE, or other metformin clinical trials. This method has been demonstrated to meet the validation criteria and can be applied *in vivo*. However, future studies are required to develop further methods of extraction to improve the recovery so to achieve LLOQ analysis.

CONCLUSION

The DBS biosampling technique can be developed for application in pharmacokinetic study of metformin HCl.

CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest.

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