

DEVELOPMENT AND VALIDATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR DETERMINING METFORMIN HYDROCHLORIDE IN DRIED BLOOD SPOTS

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ABSTRACT

Objective: This study aimed to develop an analytical method for determining metformin concentrations in human dried blood spots (DBS) using optimal chromatographic conditions and DBS preparation methods and validate the analytical methods.

Methods: Analysis metformin HCl performed using C-18 columns (250 mm × 4.6 mm) at a column temperature of 40°C with a mobile phase acetonitrile phosphate buffer (40:60% v/v) at pH 7.0 using a flow rate of 0.8 mL/min, a photodiode array detector at a wavelength of 234 nm, and atorvastatin calcium as an internal standard. Samples were prepared by protein precipitation with 60% methanol and then drying using nitrogen gas at 60°C for 15 min before reconstituting in 200 µL of the mobile phase.

Results: Invalidation procedures, the present methods met the acceptance criteria stated in the EMEA Bioanalytical Guideline 2011. The method was also linear at the concentration range of 25.0–5000.0 ng/mL ($r=0.9997$).

Conclusion: The developed method for determination of metformin HCl in DBS sample was valid with a concentration range of 25.0–5000.0 ng/mL and can be applied to *in vivo* studies.

Keywords: Metformin, High-performance liquid chromatography, Optimization, Validation, Dried blood spot.

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INTRODUCTION

In 2012, diabetes caused 1.5 million deaths globally, and high glucose levels contributed to 2.2 million deaths by increasing the risk of cardiovascular diseases and other disorders. Among these 3.7 million deaths, 43% were of people <70 years old and were considered premature. In Indonesia, premature deaths account for 48,300 of 99,400 diabetes-related deaths annually [1].

Metformin is a biguanide medicine that lowers glucose levels by reducing hepatic glucose production and intestinal glucose absorption. Metformin is the most prescribed drug for type 2 diabetes and is the drug of choice for diabetes patients with obesity [2]. Metformin is also present in the List of National Essential Medicines according to the Indonesian Ministry of Health. Hence, metformin is the most needed medicine and must be made available at all health facilities.

Pharmacokinetics studies can be performed using plasma, serum, or whole blood, although most studies are performed with plasma or serum samples because these are easy to prepare. However, the use of these two matrices requires the inconvenient collection of blood samples from patients. In the past few years, a new sampling method has been developed using dried blood spot (DBS) samples on filter paper. Such DBS methods offer improved convenience for patients because they require a small volume of blood [3]. Therefore, DBS methods may facilitate the implementation of pharmacokinetics or bioequivalence (BE) tests for metformin and other medicines because blood sampling is minimal and does not require the imposition of deep wounds with the entry of needles or catheters [4].

Analyzes of metformin in DBS samples will require high selectivity and sensitivity due to minimal amounts of blood collected from peripheral sites. Some high-performance liquid chromatography (HPLC) methods

have been developed for analyzes of DBS samples. HPLC is also commonly available in laboratories and is applicable to BE and other clinical tests. Since HPLC can be combined with various detectors, its uses can be adjusted to suit available tools. Herein, we used photodiode array (PDA) detectors that can perform rapid analyzes at several wavelengths [2].

In drug compound analyses, sample preparation is the most important part. Thus, extraction from DBS samples was conducted and extracts were dissolved in the mobile phase. To optimize the results, protein precipitation was performed, and we used a polar solvent to accommodate the polar properties of metformin, as shown in previous analyzes using HPLC methods [2].

Quantitative analyzes were performed using an internal standard with the similar chemical properties as the analyte, and this was added to samples with as yet unknown concentrations of analyte. In previous analyzes of metformin, diazepam, phenformin HCl, salbutamol, calcium atorvastatin, and ranitidine HCl were used as internal standards. In this study, we used calcium atorvastatin because it has a similar retention time to that of metformin HCl, is readily available, and can be used with a known and validated correction factor to determine analyte concentrations in unknown samples [5].

The methods used herein were modified from the reverse phase HPLC procedures reported by AbuRuz *et al.*, in 2006, and were optimized for determinations of metformin HCl in DBS. After selecting optimal conditions, then validated the method based on the Bioanalytical Guidelines of the European Medicines Agency (EMA; 2011) [6]. Measured parameters included selectivity, linearity, accuracy, precision, stability, recovery, and carry-over. The methods developed herein are expected to be used in pharmacokinetics tests, BE tests, and in other clinical tests of diabetic patients receiving metformin HCl therapy.

MATERIALS AND METHODS

Materials

Metformin HCl and calcium atorvastatin were purchased from British Pharmacopoeia (BP), orthophosphoric acid (Merck), HPLC grade methanol (Merck), sodium dihydrogen phosphate (Merck), acetonitrile (Merck), Aquabidestilata (Ikapharmindo), filter paper (Perkin Elmer), human blood (Indonesian Red Cross Society), Instrumentation HPLC, a C-18 column (Waters, SunFire™ 5 µm; 250 × 4.6 mm), and a PDA detector (Waters 2996).

Standard solution

Metformin HCl was carefully weighed to 10.0 mg and was then dissolved with Aquabidestilata sterilized water in 10.0 mL volumetric flasks to a concentration of 1.0 mg/mL. The internal standard calcium atorvastatin was carefully weighed to 10.0 mg and was then dissolved in methanol in 10.0 mL volumetric flasks to a concentration of 1.0 mg/mL. Dilutions were then performed to generate solutions with a range of concentrations.

Optimization of chromatographic condition

Mixtures containing 10.0-µg/mL HCl metformin and 10.0-µg/mL calcium atorvastatin were injected in 20-µL aliquots into the HPLC instrument and responses were observed. Optimization began with the selection of an optimal wavelength using the PDA detector. Metformin HCl analyzes were performed using the wavelength maxima at 234 nm. Sodium dodecyl sulfate (SDS) was used to provide ion pairs, and SDS concentrations were optimized at 2, 3, and 10 mM. The mobile phase composition and phosphate buffer pH were then optimized, and the best flow rate was established. To optimize the mobile phase, acetonitrile phosphate buffer ratios of 30:70, 40:60, and 35:65 were tested with pH values of 6.0, 6.5, and 7.0. Flow rates were optimized by comparing 0.8, 1.0, and 1.2 mL/min.

System suitability tests

After determining optimum conditions for metformin HCl analysis, mixed solutions of metformin HCl and internal standard were injected 6 times and coefficients of variation (CV) for retention times and areas under the curve for each substance were then calculated as PAR, which should be <2.0%.

Optimization of DBS sample preparation

DBS sample preparation and extraction of metformin HCl were conducted using the protein precipitation method. Protein precipitation was performed by adding 50 µL of 10 µg/mL calcium atorvastatin to DBS samples containing metformin HCl at a concentration of 1 µg/mL. Subsequently, 1.0-mL aliquots of distilled water, 60% methanol, 95% methanol, 100% acetonitrile, and acetonitrile formic acid (90:10) were added to these samples, and selected mixtures were used to optimize volumes of extraction solvent at 0.2, 0.5, 0.7, and 1.0 mL. Sonication times were then optimized by comparing samples after 5-, 10-, and 15-min sonication. Finally, the evaporation step was optimized, and aliquots of up to 100.0 µL were injected into the HPLC.

Validation of metformin HCl methods for DBSs

Full validation of analytical methods for metformin HCl in DBS was conducted, with the parameters selectivity, carry-over, lower limit of quantitation (LLOQ), linearity of calibration curve, accuracy, precision and recovery, dilution integrity, and stability.

RESULTS AND DISCUSSION

Optimization of chromatography conditions

Initially, we optimized detection wavelengths for metformin HCl and then adjusted the SDS concentration to 10 mM for optimal provision of an ion pair that acts perfectly with metformin HCl. The mobile phase was optimized to maximize peak responses of the combination of acetonitrile phosphate buffer (40:60; pH 7.0). Mixing of the mobile phase was also optimized because it can affect the pH of the phosphate buffer and influence reactions between metformin HCl and SDS ion pair [7-11]. The preferred mixing method involved manual mixing by the analyst. Finally, the flow rate was optimized to 0.8 mL/min.

Metformin HCl was detected using a PDA detector at 234 nm after elution from a C-18 column (Waters, SunFire™ 5 µm; 250 mm × 4.6 mm) under optimal conditions. These included a mobile phase of acetonitrile phosphate buffer (pH 7.0; 40:60) at a flow rate of 0.8 mL/min and column temperature of 40°C using calcium atorvastatin as an internal standard. These analytical conditions produced the best separation parameters, including the largest area values, relatively quick retention times, a tailing factor close to 1, a resolution (R) of >2, the largest theoretical (N) plate, and an HETP value that was close to zero.

System suitability test

After identifying optimal conditions, system suitability tests were conducted with six injections and retention time, area under the curve, and PAR were obtained with a CV of <2.0%. Retention time of metformin is 6.872 min and retention time of atorvastatin is 7.869 min (Fig. 1).

Optimization of DBS sample preparation

Preparation or extraction of metformin HCl was conducted using the protein precipitation method. The protein precipitation solvents used were the polar solvents methanol and acetonitrile and 60% methanol gave the highest peak analyte and internal standard responses. This was due to the highly polar nature of metformin HCl, which led to greater solubility in a polar solvent. Further studies with the selected solvent were performed to optimize the amount of solvent volume added, the sonication time, and the evaporation step. The optimum sonication time was 10 min, and longer agitation times caused degradation of the analyte. The evaporation step was then optimized to produce a sharper peak (Fig. 2).

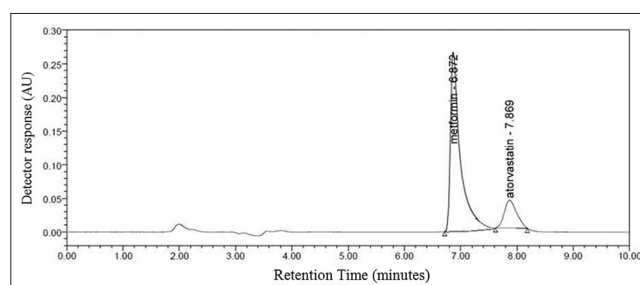


Fig. 1: Chromatogram in system suitability test

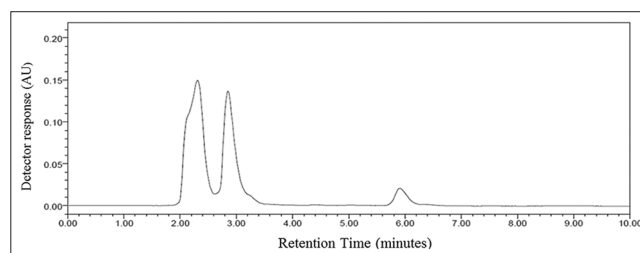


Fig. 2: Chromatogram of dried blood spots extracted blanks

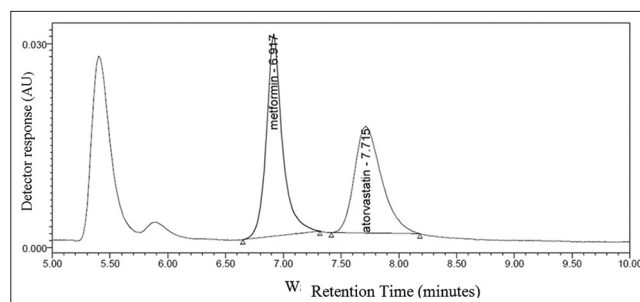


Fig. 3: Chromatogram of metformin HCl at the upper limit of quantitation concentration of 5000 ng/ml

Validation of metformin HCl methods in DBSs

Selectivity

Analyzes of selectivity were performed on blank and LLOQ concentrations using blood from six different sources. The results showed no interference with internal standard or with impurities at the retention time of analyte.

Carry-over

Carry-over tests showed no carry-over effects in blanks after the highest concentration (upper limit of quantitation) of metformin HCl was injected (Fig. 3). The carry-over percentage met the requirement (<20%) for analytes, as shown in Table 1.

Calibration curve and LLOQ

The calibration curve was linear and had a correlation coefficient (r) of >0.999 in the concentration range of 25.0–5000.0 ng/mL. The concentration of the LLOQ of metformin HCl was 25.0 ng/mL, with a CV of 10.72% and a percentage difference of - 14.36%–14.10% (Fig. 4).

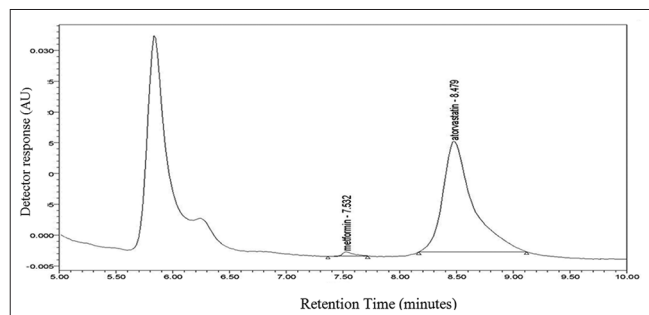


Fig. 4: Chromatogram of metformin HCl at the lower limit of quantitation concentration of 25.0 ng/mL

Accuracy, precision, and recovery

Estimates of accuracy are based on the proximity of measurements with actual concentrations (bias), whereas those of precision are based on repeatability of determinations (CV). Accuracy and precision tests were conducted within and between runs using concentrations that corresponded with the LLOQ, quality control low (QCL), quality control mid, and quality control high (QCH). Accuracy and precision data from within and between days are shown in Table 2.

The recovery test was performed by comparing analytical peak responses in DBS with those of standard solution at the same concentrations (Table 3).

Dilution integrity

Dilution integrity tests were conducted to assess accuracy, precision, and reliability of dilution processes during bioanalysis. If *in vivo* measurements of metformin HCl levels in biological matrices exceed the upper limit of 5000 ng/mL, then dilution to within the range of the calibration curve is required. The results of dilution integrity tests are shown in Table 4.

Stability

The stability of metformin HCl was tested to determine whether degradation occurred during storage. Stability tests were performed by analyzing QCL and QCH samples with three replicates for each sample. In short-term stability tests, samples were stored at room temperature and were stable after 0, 6, and 24 h. The results shown in Table 5 indicate that metformin HCl samples are stable during storage at room temperature for at least 24 h.

In long-term stability tests, samples were stored at room temperature with desiccants for 7–45 days. These tests showed that metformin

Table 1: Data from carry-over measurements

Blanks sample	Carry-over metformin HCl (%)	Carry-over IS (%)
Blank-1	8.7795	0.0740
Blank-2	8.6564	0.0871
Blank-3	8.1641	0.0868
Blank-4	9.2923	0.0780
Blank-5	8.8410	0.0901
Average	8.7467	0.0832

Table 2: Accuracy and precision within-run and between-run

Actual concentration (ng/mL)	Within-run			Between-run		
	Measured concentration (Average±SD; ng/mL)	CV (%)	Bias (%)	Measured concentration (average±SD; ng/mL)	CV (%)	Bias (%)
25.0	25.68±1.88	7.31	2.73	26.32±2.52	9.58	5.26
75.0	76.49±0.95	1.24	1.99	78.92±3.46	4.38	5.22
2500	2520.61±72.19	2.86	0.83	2473.21±78.59	3.18	-1.07
3750	3885.96±89.01	2.29	3.62	3909.65±119.14	3.05	4.26

Table 3: Recovery of metformin HCl

Actual concentration (ng/mL)	Amount of replication	Recovery (average±SD; %)	CV (%)
75.0	3	77.48±0.83	1.07
2500	3	77.22±0.12	0.16
3750	3	77.88±1.21	1.55

Table 4: Dilution integrity test data within and between runs

Actual concentrations (ng/mL)	Dilution factor	Within-run			Between-run		
		Measured concentrations (average±SD; ng/mL)	CV (%)	Bias (%)	Measured concentrations (average±SD; ng/mL)	CV (%)	Bias (%)
3750	½	3844.45±68.81	1.79	2.52	3838.72±67.76	1.77	2.36
1875	¼	1939.23±76.26	3.93	3.43	1940.78±50.95	2.63	3.51

Table 5: Short-term stability of metformin HCl in DBS at room temperature

Hour	QCL 75.0 ng/mL			QCH 3750 ng/mL		
	Measured concentrations (average±SD; ng/mL)	CV (%)	Bias (%)	Measured concentrations (average±SD; ng/mL)	CV (%)	Bias (%)
0	73.82±1.51	2.05	-1.58	3869.06±44.50	1.15	3.17
6	71.37±2.00	2.80	-4.84	3829.97±17.64	0.46	2.13
24	74.99±0.74	0.98	-0.02	3827.45±4.53	0.12	2.06

Table 6: Long-term stability of metformin HCl in DBS at room temperature

Day	QCL 75.0 ng/mL			QCH 3750 ng/mL		
	Measured concentrations (average±SD; ng/mL)	CV (%)	Bias (%)	Measured concentrations (average±SD; ng/mL)	CV (%)	Bias (%)
0	75.40±1.48	1.96	0.53	3787.54±43.52	1.15	1.00
7	73.46±0.34	0.47	-2.05	3776.92±27.36	0.72	0.72
45	71.81±1.05	1.46	-4.25	3823.65±33.46	0.88	1.96

Table 7: Post-preparation stability (autosampler) of metformin HCl in DBS

Hour	QCL 75.0 ng/mL			QCH 3750 ng/mL		
	Measured concentrations (average±SD; ng/mL)	CV (%)	Bias (%)	Measured concentrations (average±SD; ng/mL)	CV (%)	Bias (%)
0	75.40±1.48	1.96	1.60	3787.54±43.52	1.15	1.00
24	72.82±0.27	0.37	-2.91	3672.88±17.57	0.48	2.05

HCl samples are stable at room temperature for at least 45 days in the presence of desiccants (Table 6).

To determine the stability of metformin HCl in the autosampler after preparation of DBS samples, post-preparation stability tests were necessary for 24 h at the autosampler temperature. As shown in Table 7, HCl metformin preparations were stable for at least 24 h in the autosampler.

Short-term stability tests of standard metformin HCl and internal standard solution were conducted at room temperature for 24 h, and long-term stability tests of standard solution were performed after storage at -80°C for 7–45 days. The resulting percentage difference for the stability of metformin HCl stock solution was -1.95%–0.27% and that for calcium atorvastatin stock solution was -1.76%–0.73%. These data suggest that stock solutions of metformin HCl and calcium atorvastatin are stable at room temperature for at least 24 h and at -80 °C for 45 days.

CONCLUSION

The developed method for determination of metformin HCl in DBS sample was valid in the range of concentration of 25.0–5000.0 ng/mL and can be applied to *in vivo* studies. Moreover, metformin HCl samples are stable during storage at room temperature for at least 24 h and stock solutions of metformin HCl and calcium atorvastatin are stable at room temperature for at least 24 h and at -80 °C for 45 days.

CONFLICTS OF INTEREST

None declared.

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