Bioanalytical Method Validation of Metformin Hydrochloride in Human Plasma by HPLC-UV for Preliminary Population-Based Pharmacokinetic Modeling Study

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Abstract: *This study aims to validate the method for measuring metformin hydrochloride plasma concentrations using High-Performance Liquid Chromatography (HPLC). This research performed chromatography on a 250 mm 4.6 mm 5 µm purosphere® Star RP-18 column at ambient temperature with a UV detector system at 233 nm. The mobile phase components were 70% phosphate buffer (KH_2PO_4) (10 mM), sodium dodecyl sulfate (0.3 mM), and 30% acetonitrile. It was pumped at an isocratic flow rate of 1.2 mL/min. Metformin HCl and ranitidine HCl (internal standard) were extracted using acetonitrile. The calibration curve was linear (R^2 = 0.9998) in the 0.18–6 µg/mL concentration range. The lower limit of quantification (LLOQ) was 0.18 µg/mL. For intraday accuracy and precision, the percent difference and the coefficient of variation were less than 4 and 7%, and for inter-day were lower than 8 and 6%. The recovery average was 100.96%. The short-term plasma stability test was stable at 24 h at ambient temperature, and the long-term stability test was steady for 30 d at −20 °C. It was also stable after three freeze-thaw cycles. The method meets selectivity, sensitivity, linearity, accuracy, precision, recovery, carryover, and stability requirements and can be applied to population-based pharmacokinetic modeling.*

Keywords: *human plasma; HPLC-UV; metformin HCl; pharmacokinetic; validation*

■ INTRODUCTION

Metformin hydrochloride (metformin HCl) is the recommended starting therapy for diabetes type 2 [1-2] and also for prediabetes and diabetes prevention [3]. It can be used as monotherapy or in combination with insulin or other glucose-lowering treatments [4-5]. In addition, metformin controls gestational diabetes caused by polycystic ovarian syndrome and shows early promise as an anticancer drug [6-7] and anti-aging [7]. Metformin HCl has a chemical name of 1,1-Dimethylbiguanide hydrochloride, a molecular formula of $C_4H_{11}N_5$•HCl, and a molecular weight of 165.62 g/mol [8]. Therefore, monitoring metformin plasma levels are critical for investigating the medication's pharmacokinetics/pharmacodynamics modeling, pharmacogenomics, and therapeutic drug monitoring to achieve a better clinical result for the patient.

Various chromatographic techniques are currently available for metformin HCl analysis in human plasma. A literature review conducted by Kaur et al. states that the High-Performance Liquid Chromatography (HPLC) method is a reasonably good method for analyzing plasma metformin levels compared to other methods such as High-Performance Thin-layer Chromatography (HPTLC), Hydrophilic Interaction Liquid Chromatography HILIC-MS/MS, Liquid Chromatography tandem mass spectrometric (LC-MS-MS), Ultra-High Performance Liquid Chromatography

(UPLC). It is because the HPLC method can separate and quantify metformin levels, has a fast analysis time, minimizes the use of organic solvents, and is affordable for clinical testing of metformin, as mentioned above. The study recommends developing HPLC methods to focus on developing new extraction methods, mobile phases, and adsorbent materials for HPLC separation [9]. LC-MS-MS has better sensitivity than HPLC-UV, but as long as the HPLC-UV method can determine metformin levels according to therapeutic concentrations, this method can still be used according to the objectives of the study [10-11]. However, LC-MS-MS is also not optimal for clinical applications due to the high cost and limited availability of the necessary equipment in clinical laboratories [12].

Several studies have used HPLC-UV to analyze metformin in dosage form and human plasma [12-17]. Because of the need to minimize disruptions while minimizing analyte loss, metformin extraction and purification from human plasma is sometimes the most challenging step in bioanalysis [12-15]. Organic liquid-liquid extraction is an easy, accurate, and effective way to prepare samples for most drugs. However, metformin's high polarity makes the extraction more difficult including its extraction from biological matrices that become more complicated [12-13], and several previous studies reported that this method produces a longer running time [13,18]. Therefore, protein precipitation has been the preferred sample preparation approach to address metformin extraction's difficulty. However, this method's time retention is too short, has poor recovery [19] and is ineffective in removing endogenous interferences [18-19].

Another problem that occurred in several previous studies was the use of a relatively narrow range of calibration curves [12,14,18], thus not covering the range of metformin plasma therapeutic levels of 0.4–5 µg/mL [20-21]. Based on these problems, the current study was conducted to modify the extraction method and mobile phase and adjust the calibration curve range to estimate metformin plasma levels in patients with diabetes mellitus so that it is expected to produce better validity parameter results, especially for metformin concentration needs in pharmacokinetic modeling studies. In addition, FDA guidelines state that full validation should be performed on developing new bioanalytical methods or revisions/modifications of existing bioanalytical methods [22].

This study aims to validate a simple and effective metformin plasma extraction technique for plasma concentration measurement by HPLC-UV. The method needs to be validated because there are modifications in the extraction method to get a better recovery test, modifications in the composition of the mobile phase to get a shorter running time, and ranitidine as an internal standard. The study reports on the accuracy, precision, linearity, sensitivity, Lower Limit of Quantification (LLOQ), carryover, selectivity, and stability in stock solutions, human plasma, three freeze-thaw cycles, and results of applying this method in preliminary population-based pharmacokinetic modeling using Monolix 2023R1 software.

■ EXPERIMENTAL SECTION

Materials

Metformin HCl and ranitidine HCl (the internal standard) were secondary reference standards sourced from the National Agency of Drug and Food Control of Indonesia. Acetonitrile and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Merck, Germany. Sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich, and sterile water for injection from Ikapharmindo (Indonesia). In addition, human plasma was taken from healthy volunteers who had signed an informed consent agreement. The Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada approved the study with the reference number: KE/FK/0217/E.C./2021 and conducted it following the Declaration of Helsinki. A preliminary population-based pharmacokinetic modeling study involved 17 patients with type 2 diabetes mellitus receiving 500 mg of metformin every 12 h as a monotherapy.

Instrumentation

Metformin HCl plasma concentration was measured using Shimadzu High-Performance Liquid Chromatography (HPLC) LC-10AD VP manual

injection with a U.V. detector system at 233 nm. The separation was performed on purosphere® Star RP-18 end-capped 250 mm 4.6 mm 5 μm from Merck Germany. The mobile phase was a phosphate buffer (70%) and acetonitrile (30%) mixture. The phosphate buffer consisted of 10 mM potassium dihydrogen phosphate (KH_2PO_4) and 0.3 mM sodium dodecyl sulfate (SDS) at pH 5.2. Analyses were run at a 1.2 mL/min flow rate, and injection volumes were 20 μL at room temperature column. The recorder system used is Shimadzu Class-VP version 6.1 software. A preliminary study of population-based pharmacokinetic modeling was conducted with Monolix 2023R1 software from Lixoft.

Procedure

The metformin assay method used in this study is a modification of several previously published methods developed by Amini et al. and Gabr et al. [12-13] with changes to the extraction method, composition, and procedure of preparation of the mobile phase and the concentration range of the calibration curve and quality control samples considering the therapeutic range of metformin in plasma in diabetes mellitus patients.

Plasma calibration standards and quality control samples

Metformin HCl and ranitidine HCl standard solutions were made by dissolving 10 mg of each reference standard in 10 mL of distilled water to yield a final concentration of 1000 μg/mL and storing them at −20 °C. Metformin HCl working solution was created by diluting aliquots of the standard solutions with distilled water to obtain final levels of 60, 40, 20, 10, 5, 2.5, and 1.8 μg/mL. These solutions were utilized to create plasma calibration standards in the 0.18 to 6.0 μg/mL linear calibration range. The lower limit of quantification (LLOQ), Quality Control Low (QCL), Quality Control Medium (QCM), and Quality Control High (QCH) plasma samples were produced with 0.18, 0.54, 3.0, and 4.5 μg/mL concentrations, respectively.

Extraction procedure

A volume of 500 μL of plasma containing metformin HCl was mixed with 50 μL ranitidine HCl and 1 mL acetonitrile in a test tube. A 2 min vortexing procedure was followed by a 10 min centrifugation step at 10,000 rpm. Then, the supernatant was taken and the second extraction process was performed for the remaining residue by adding 1 mL acetonitrile and conducting the same vortexing and centrifugation procedure. Finally, the supernatant from the first and second extractions were combined and evaporated with nitrogen (N_2). The resulting dry extract was reconstituted with 500 μL of phosphate buffer: acetonitrile (70:30) and vortexed for 3 min. It was then filtered using a 0.45 μm syringe filter, and 20 μL was administered into a previously equilibrated HPLC system.

Assay validation

Assessment for the validation of the analytical method refers to the guidelines from the US Food and Drug Administration (FDA) and European Medicines Agencies (EMA) [22-23]. The specificity was determined by comparing metformin and internal standard-containing samples' chromatograms to those of blank samples. In addition to the calibration standards curve, further tests were conducted to establish intra-day and inter-day assay precision and accuracy, selectivity, sensitivity, recovery, carryover, and stability. Besides the LLOQ concentration, which should be at most 20% of the coefficient of variation (CV) and percentage of difference (% diff), the acceptance criterion for the CV and % diff should be at most 15% [22-23].

Preliminary population-based pharmacokinetic modeling study

Seventeen patients with type 2 diabetes mellitus who received Metformin HCl 500 mg twice daily in 12 h intervals as monotherapy were subjected to periodic blood sampling. Metformin blood levels were determined at two sampling points for each patient. The first sampling of all subjects was carried out at pre-dose/just before taking metformin. The second collection was carried out randomly at one of the times chosen between the time ranges of 0.5, 1, 2, 3, 4, 6, 8, and 10 h post-dose [20,24]. Modeling population-based pharmacokinetic parameters was performed using the monolix 2023R1 software [25]. A volume of 500 μL of plasma samples were taken from patients, and extracted in the same methods described above.

■ RESULTS AND DISCUSSION

Method Development

The significant modification from the previous study [12-15,18-19,26] concerns the extraction method, composition, and preparation procedure of the mobile phase, as well as the concentration range of the calibration curve and quality control samples, taking into account the therapeutic range of metformin in the plasma of the patients with diabetes mellitus. Optimization of several alternative extraction methods was carried out in the preliminary study, including protein precipitation with trichloroacetic acid and acetonitrile. However, this method was less effective in removing the peaks of endogenous plasma compounds causing them to coincide with the peaks of metformin. Furthermore, liquid-liquid extraction was carried out with acetonitrile added with 100 µL NaOH 8 M to alkalinize the pH. However, the metformin peak results obtained were less symmetric. Finally, the extraction was conducted in a more straightforward method and by only using acetonitrile as the solvent without alkalizing the pH with NaOH and without adding acetic acid. In addition, we also performed evaporation with nitrogen gas to produce dry extracts to concentrate the concentration of analytes, because in bioanalytic studies, it is challenging to quantify small amounts of the drug in plasma with the tailing factor level still according to the recommendations.

A preliminary study was also conducted to optimize the mobile phase's performance. Initially, a combination of acetonitrile and sterile water for injection was used with various compositions. As a result, metformin appeared in a short retention time because it is a highly polar compound. However, in the early minutes, a peak of endogenous compounds will also appear in the plasma and can interfere with metformin peaks [19]. In modifying the mobile phase composition, phosphate buffer was used with SDS admixture. SDS is an anionic surfactant that can be a quasi-stationary phase that can delay the appearance of metformin peaks to a more optimal retention time [27]. SDS will form micelles above the critical micelle concentration, resulting in a pseudo-stationary phase that can partition components/analytes according to their partition coefficient [28]. The final mobile phase components were 70% phosphate buffer (KH_2PO_4) 10 mM, sodium dodecyl sulfate (0.3 mM), and 30% acetonitrile. Pure acetonitrile and its water (buffering) mixtures have unique chromatographic extraction characteristics and significant applications [29]. Fig. 2 shows an excellent peak symmetry, and both the analyte and internal standard were effectively separated at 4.8 and 6.7 min retention times with running time at 8 min. This result is better than previous references [12-15,19], which show a longer running time, thus less efficient. The resulting retention time was optimal, not too fast, so it did not coincide with endogenous plasma compounds. It was under 8 min, so it was efficient and suitable for laboratory and clinical pharmacokinetics services.

Method Validation

System suitability test

Table 1 demonstrates that the HPLC-UV system satisfies all suitability test parameters. Resolution and relative retention parameters indicate that plasma matrices containing metformin and ranitidine (internal standard) are well separated. The tailing factor (TR) level also meets the requirements to guarantee accuracy in quantifying the area of the metformin peak [30]. The column used also has good efficiency based on the value of the Plate Number (N) and HETP parameters [30].

Table 1. System suitability test

No.	Parameters	Acceptability criteria [30]	Metformin	Ranitidine [IS]
1.	Capacity factor (k')	> 2	3.94	5.83
2.	Resolution (Rs)	> 2	3.8	5.93
3.	Tailing factor (TF)	≤ 2	1.39	1.38
4.	Relative retention (α)	> 1	2	1.39
5	Plate number (N)	> 2000	2526.4	2125
6	Height Equivalent Theoretical Plate (HETP)	0.01–1	0.098	0.117

Selectivity

Six individual plasma samples were analyzed by chromatography to examine the possibility of endogenous compounds in plasma that could interfere with the appearance of metformin hydrochloride peaks and internal standards. As illustrated in Fig. 2(a), no endogenous plasma peak interfered with the elution of metformin or ranitidine. It is generally considered that there are no interfering components when the analyte's response is less than 20% of LLOQ and the internal standard is less than 5% [22]. The result shows no significant interfering components at the retention time of the analyte and internal standards of the blank matrix.

Lower limit of quantification [LLOQ]

LLOQ was the least amount at which repeatability was within 20% of CV, and the measured concentration was within 20% of the actual attention [22-23]. Determining the LLOQ value was conducted using the response method's standard deviation and the calibration curve's slope [31]. In this study, we decided on the LLOQ value at 0.18 µg/mL with the CV values being 6.542% and % diff of 0.905%, which is better than the previous study reported by Ningrum et al. [19]. The LLOQ value set at 0.18 µg/mL indicates that the method can meet the sensitivity requirements in the analysis of metformin because the range of metformin levels in plasma is 0.4–5 µg/mL [20,24].

Linearity

The standard calibration curve was drawn using seven spiking metformin plasma levels (0.18, 0.25, 0.5, 1, 2, 4, 6 µg/mL), including the LLOQ. The standard curve is based on the metformin level series (x) versus the chromatogram area ratio of the metformin to ranitidine (y). The correlation coefficient was 0.9998 with the linear regression equation y = 0.1595x + 0.0043. Fig. 1 displays the calibration curve from metformin spiked in human plasma. Several previous studies employed a calibration curve with a relatively limited range that did not correspond well with plasma metformin levels, particularly in the upper range (above 2 µg/mL) [12,14,18]. In addition, some studies had linearity with an R^2 value under 0.999 [14,18].

Accuracy and precision

Accuracy refers to the proximity of the obtained concentration value to the actual concentration of the analyte, expressed as a percentage difference (% diff). Precision refers to the similarity of repeated individual analyte measurements, denoted by the coefficient of variation (CV). The acceptance limits for % diff, and CV are 20% for LLOQ and 15% for QCL, QCM, and QCH [22-23]. Table 2 shows intra- and inter-day accuracy and precision data for measuring metformin in human plasma. For intraday accuracy and precision, the percent difference was less than 4%, and the coefficient of variation was less than 7%. The results were below 8 and 6% on day-to-day accuracy and precision. Fig. 2 shows

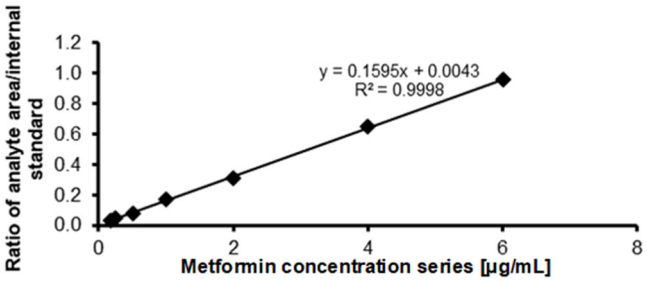

Fig 1. Spiked plasma metformin calibration curve

Table 2. The intra- and inter-day accuracy and precision plasma spiking metformin

	Intra-day (n = 5)		Inter-day (n = 5)			
			Day 2		Day 3	
Conc. (µg/mL)	Accuracy (% diff)	Precision (% CV)	Accuracy (% diff)	Precision (% CV)	Accuracy (% diff)	Precision (% CV)
0.18	0.905	6.542	2.663	1.301	-3.893	5.305
0.54	3.059	4.509	5.879	1.125	7.723	3.095
3	-3.295	3.295	-3.877	2.014	-0.397	1.782
4.5	0.415	2.008	-5.931	2.996	-0.622	2.264

The acceptance limits for % difference [% diff] and CV are below 20% for LLOQ and < 15% for QCL, QCM, and QCH [22-23]

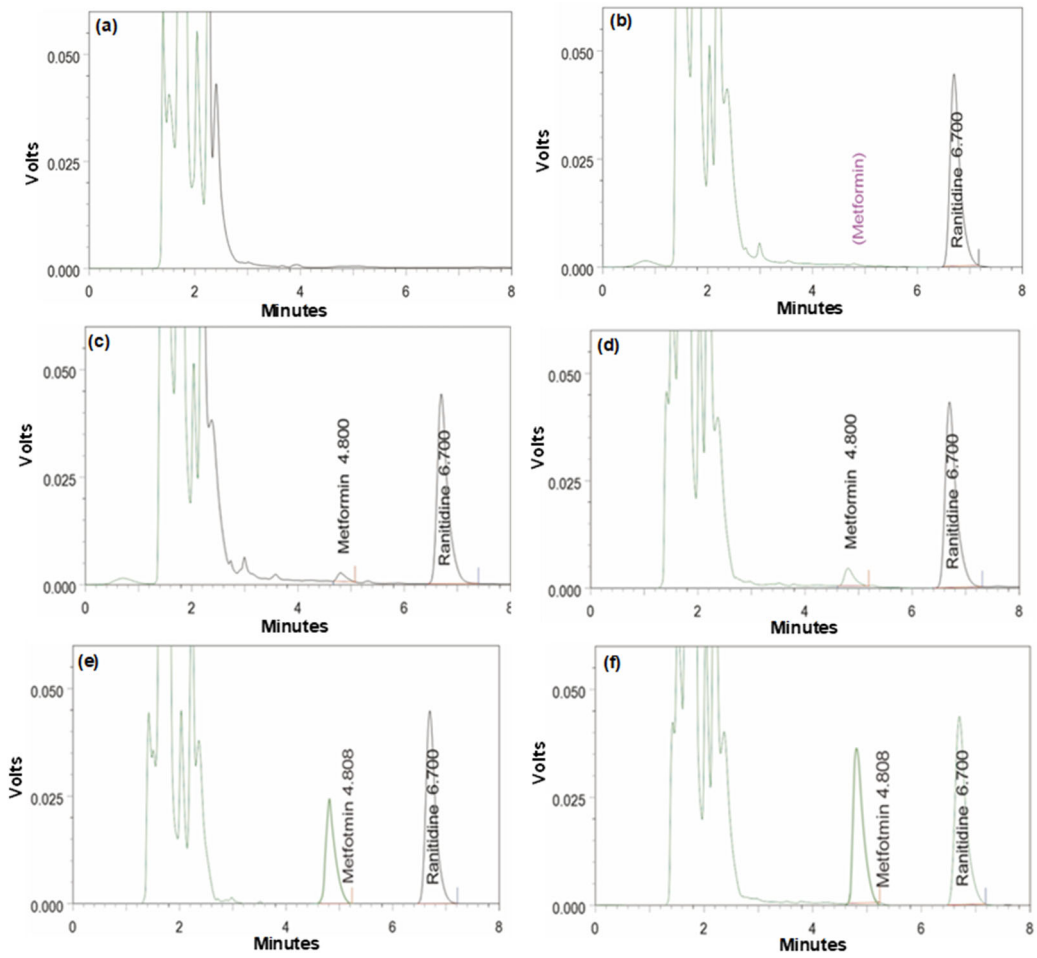

Fig 2. Chromatograms for a blank plasma sample (a), a zero sample (blank+internal standard 10 µg/mL) (b), spiking plasma metformin LLOQ sample (c), spiking plasma metformin QCL sample (d), spiking plasma metformin QCM sample (e) and spiking plasma metformin QCH sample (f). Zero, LLOQ, QCL, QCM, and QCH samples were spiked with the ranitidine 10 µg/mL as the internal standard

an example of a chromatogram peak from LLOQ, QCL, QCM, and QCH samples.

Recovery

Recovery is a term that relates to an analytical process's extraction efficiency, expressed as the ability to recover a predetermined amount of analyte from a sample after sample extraction and processing, and is measured in terms of recovery percentage. The acceptance limit for % recovery is 80–120% for LLOQ and 85–115% for QCL, QCM, and QCH [31]. Table 3 shows the average recovery of LLOQ, QCL, QCM, and QCH, which ranged from 96.159–104.905%. The relative recovery ranged between 92.749–99.352% (LLOQ), 102.327–109.778% (QCL), 95.357–102.524% (QCM), and 101.477–106.714% (QCH).

Table 3. Metformin plasma recovery

Conc. (µg/mL)	Mean recovery (n = 3)	SD	% CV
0.18	96.159	3.307	3.439
0.54	104.905	4.223	4.025
3	98.693	3.609	3.657
4.5	104.078	2.619	2.516

The acceptance limits for % difference [% diff] and CV are below 20% for LLOQ and < 15% for QCL, QCM, and QCH [22-23]

The result indicated that the extraction method produced a better recovery test than the previous study by Gabr et al., with complex liquid-liquid extraction (recovery ranging from 93.7–88.5%) [13], Ningrum et al., that used a simple protein precipitation method (recovery ranged

from 59.98–93%) [19], and Nielsen et al., that used a solid-phase extraction (recovery ranged 80–88%) [14].

Carryover

After injecting the high concentration standard, carryover should not exceed 20% of the LLOQ and should not exceed 5% for the internal standard [22-23]. Results show that the average metformin level after injecting blank samples after a high concentration (6 μg/mL) was 0.038 μg/mL (not greater than the LLOQ value of 0.18 μg/mL) and 0.051% for the internal standard (lower than 5%).

Stability

Every step in the sample preparation and analysis and the storage conditions employed should be examined to verify that the analyte concentration does not change. The stability tests that were carried out were stock solution storage stability (Table 4) and plasma storage stability (Table 5).

Stability in stock solution (short and long term). The stock solution was tested for stability for 24 h at ambient temperature and 30 d under freezing (−20 °C). Testing was conducted using QCL and QCH samples. Table 4 shows that the stock solution stability test was excellent, as demonstrated by the accuracy value (% diff) and precision (CV) being less than 15%. In conclusion, the metformin stock solution was steady at ambient temperature for 24 h and after 30 d under freezing (−20 °C).

Stability in human plasma

(i) **Short term stability.** Stability testing with QCL and QCH samples was conducted at 25 °C for baseline and after 24 h (Table 5). As a result, the concentration of metformin HCl in plasma remained constant for 24 h at room temperature because both CV and % diff was less than 15%.

(ii) **Long term stability.** Two QCL and QCH plasma samples were subjected to stability testing at −20 °C storage conditions for 0, 7, and 30 d. Table 5 informs that the accuracy value (% diff) was −11.324% (QCL) and −8.753% (QCH), and the precision (CV) was 0.853% (QCL) and 1.925% (QCH) after 30 d at −20 °C storage. Therefore, metformin plasma spiking was steady for 30 d at −20 °C.

(iii) **Freeze and thaw stability.** The effect of three freeze/thaw cycles on the plasma metformin concentrations of the QCL and QCH samples in duplicate was evaluated. Table 6 shows that after three freeze/thaw cycles, the accuracy value (% diff) was 0.535% (QCL) and 4.75% (QCH), respectively. The precision parameter (CV) was 1.89% (QCL) and 1% (QCH). The results show a good test for three freeze/thaw cycles from accuracy and precision parameters.

The stability test results on stock solutions and human plasma showed results that met the stability criteria, both under short-term and long-term (30 d) storage conditions. The stability criteria following FDA and EMEA guidelines were % diff and CV below 15% [22-23]. For the short-term stability test, storage at room temperature for 24 h was carried out to ensure that the metformin HCl stock solution and metformin HCl in human plasma were stable during the analysis. Meanwhile, the results of long-term stability testing both in stock solutions and human plasma showed long-lasting results in storage at −20 °C for 30 d. The method also offers sample stability after three freeze-thaw cycles.

Table 4. Short and long-term stock solution stability

Conc. (μg/mL)	After 24 h at room temperature (n = 3)		After 30 d at −20 °C (n = 3)	
	% diff	% CV	% diff	% CV
0.54	-1.486	4.118	-4.086	9.391
4.5	-4.246	0.672	-1.163	2.712

Note: acceptance criteria limit % diff and CV are below 15% [22-23]

Table 5. Stability of metformin in human plasma

Conc. (μg/mL)	Room temperature				In −20 °C					
	Baseline		After 24 h		After 24 h		After 7 d		After 30 d	
	% diff	% CV	% diff	% CV	% diff	% CV	% diff	% CV	% diff	% CV
0.54	0.616	2.201	-4.946	6.505	-5.883	0.760	-8.416	0.971	-11.324	0.853
4.5	0.24	0.415	-1.482	0.606	1.222	0.600	1.020	0.312	-8.753	1.925

Note: acceptance criteria limit % diff and CV are below 15% [22-23]

Table 6. Freeze/thaw stability test

Conc. (μg/mL)	After three freeze/thaw cycles (n = 3)	
	% diff	% CV
0.54	-0.79	1.896
4.5	5.53	1

Note: acceptance criteria limit % diff and CV are below 15% [22-23]

Application in Preliminary Population-Based Pharmacokinetic Modeling Study

Metformin concentrations in human plasma samples were collected from 17 type two diabetes mellitus patients who received metformin HCl 500 mg twice daily in 12 h intervals as monotherapy. Fig. 3 shows the chromatogram profile of metformin in the patient's plasma, which looks similar to the chromatogram profile during the method validation test. Preliminary population-based pharmacokinetic modeling studies were done using Monolix 2023R1 software. Fig. 4 shows the profile of the level-versus-time curve of 17 patients based on a population. Plasma sampling was conducted

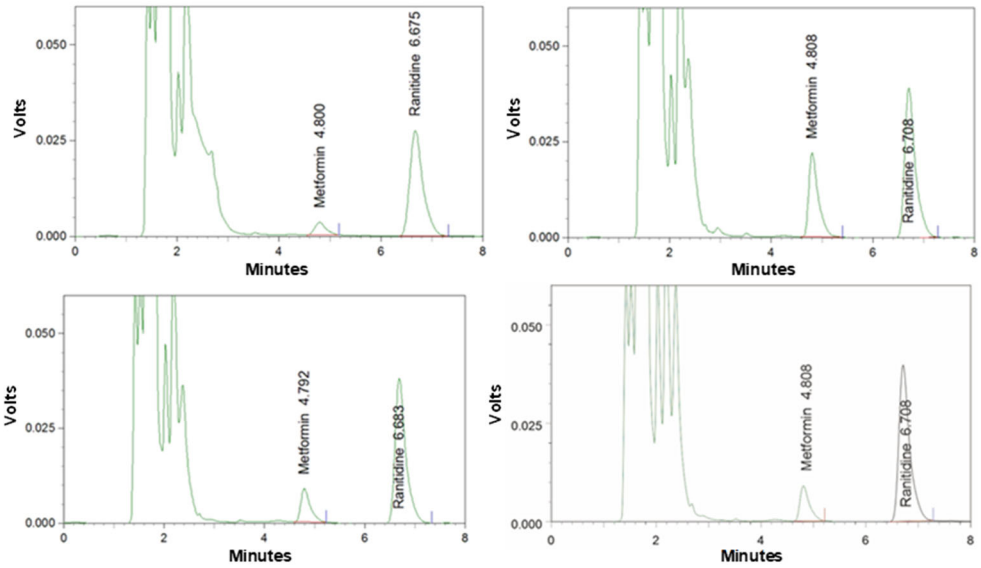

Fig 3. Representative chromatograms from diabetes mellitus patients receiving metformin 500 mg twice daily with spiking internal standard 10 μg/mL

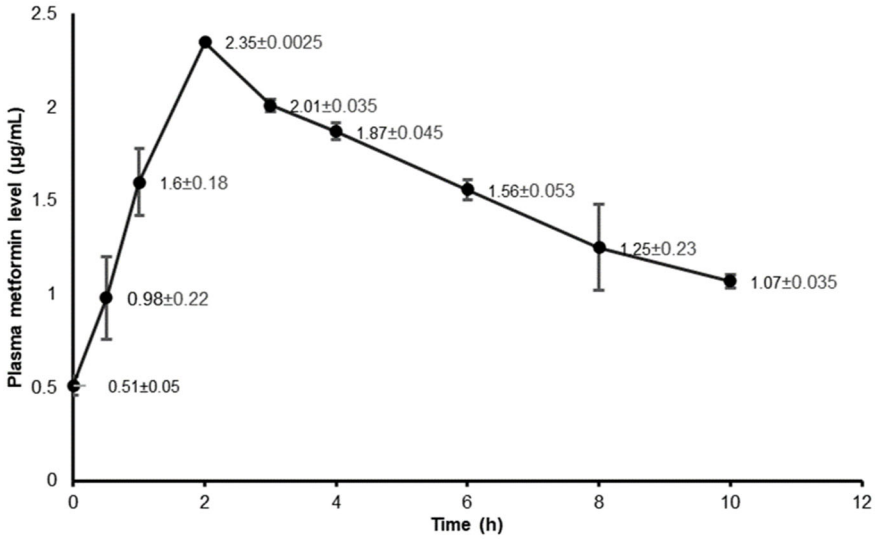

Fig 4. Population-based plasma metformin levels (± error standard) vs. time from 17 diabetes mellitus patients receiving metformin 500 mg twice daily

Table 7. Population-based pharmacokinetics parameters from 17 types two diabetes mellitus 500 mg twice daily

Parameters	Value	SE	RSE (%)
ka_pop (h^{-1})	0.751	0.147	19.648
V_pop (L)	209.567	14.049	6.704
k_pop (h^{-1})	0.165	0.0138	8.387
C_{max}_pop* (µg/mL)	2.35	0.0025	0.106
C_{min}_pop* (µg/mL)	0.51	0.05	9.8

SE: Standard error
RSE: relative standard error
ka_pop: absorption rate constant from the population
V_pop: apparent volume of drug distribution in the body from the population
k_pop: elimination rate constant from the population
C_{max}: maximum plasma drug concentration from the population
C_{min}: minimum plasma drug concentration from the population
*: values based on population-based metformin plasma level curve profiles versus time created by Monolix 2023R1 (Fig. 4)

twice as blood sampling from each patient will be combined in 1 observation data curve. The curves of plasma metformin level versus time indicate ideal pharmacokinetic phases, namely the absorption, peak, and elimination phases. Table 7 informs the value of population pharmacokinetic parameters from the Monolix 2023R1 software processing results using the pharmacokinetics structural model. The pharmacokinetic structural model equation used is as follows: Cc =Cc0 + (ka*amtDose/(V*(ka-k))*(exp(-k*t)-exp(-ka*t))) with the PK model definition; Cc = pkmodel(ka, V, k).

Curves of metformin plasma level versus time and population parameter values from an initial population-based pharmacokinetic study exhibited encouraging results. In addition, it demonstrates that the method can be utilized in a population-based pharmacokinetic study.

■ CONCLUSION

The method performed in the current study meets the requirements for selectivity, sensitivity, linearity, accuracy, precision, recovery, carryover, and stability requirements based on EMA and FDA guidelines. The modifications in the extraction method obtained a better recovery test and the composition of the mobile phase obtained a shorter running time. A preliminary population-based pharmacokinetic study showed promising results from the curve of metformin plasma levels versus time and population parameter values. Furthermore, it shows that this method can be applied in a population-based pharmacokinetic study in diabetes mellitus patients who receive metformin 500 mg every 12 h as monotherapy.

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■ AUTHOR CONTRIBUTIONS

Dimas Adhi Pradana: Methodology, Investigation, Resources, Data Curation, Writing (original draft). Erna Kristin: Conceptualization, Methodology, Writing (review and editing). Akhmad Kharis Nugroho: Conceptualization, Methodology, Writing (review and editing). Dwi Aris Agung Nugrahaningsih: writing (review and editing). Mustofa: Conceptualization, Methodology, Writing (review and editing). Ari Wibowo: Methodology, Writing (review and editing).

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