

## EFFECT OF DIFFERENT ANTICOAGULANTS IN DETERMINING METFORMIN HYDROCHLORIDE LEVELS IN HUMAN PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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### ABSTRACT

**Objective:** This study aimed to develop and validate an analytical method and to determine the effect of different anticoagulants for analyzing metformin HCl level in human plasma.

**Methods:** Analysis was performed with high-performance liquid chromatography method using a photodiode array detector set at 234 nm, a C<sub>18</sub> SunFire™ column (5 μm, 250 mm×4.6 mm), a temperature of 40°C, a mobile phase comprising 10 mM sodium dodecyl sulfate and 10 mM phosphate buffer in water-acetonitrile (60:40 v/v) at pH 7.0, with a flow rate of 1.0 mL/min, and atorvastatin calcium as the internal standard. Plasma extraction was performed through protein precipitation using human plasma (300 μL) and acetonitrile (600 μL; 1:2 v/v). The method was linear at a concentration range of 20.0–5000.0 ng/mL, with  $r > 0.9998$ .

**Results:** The stability and recovery of metformin HCl in human plasma were not different between citrate with ethylenediaminetetraacetic acid (EDTA) and heparin with EDTA as anticoagulants. However, a significant difference in the peak area response ratio ( $p < 0.05$ ) was observed between the three anticoagulants.

**Conclusion:** The validation results for the three types of plasma anticoagulants met validation requirements based on the European Medicines Agency bioanalytical guidelines of 2011 for accuracy and precision, selectivity, calibration curve linearity, dilution integrity, carry-over, and stability.

**Keywords:** Anticoagulant, Citrate, Ethylenediaminetetraacetic acid, Heparin, Human plasma, Validation.

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### INTRODUCTION

Metformin HCl is a biguanide drug that lowers blood sugar levels by decreasing the production of hepatic glucose (gluconeogenesis) and increasing the action of insulin (stimulating glycogenesis). Metformin is generally used for patients with Type 2 diabetes and has been proven as an effective antihyperglycemic drug, thus reducing the risk of death [1]. Furthermore, metformin may lower plasma glucose levels by reducing glucose absorption from the intestine [2]. The maximum metformin levels in plasma ( $C_{max}$ ) have been reported to be  $1600 \pm 380$  ng/mL. Metformin is also used for various critical conditions; thus, it requires a definite response and bioequivalence (BE) tests for a copy or generic drugs [3,4]. BE testing is conducted using pharmacokinetic studies involving the determination of plasma drug levels. Therefore, determining metformin concentrations in human plasma are necessary for pharmacokinetic studies [5].

The measurement of drug concentrations in the blood, serum, or plasma is considered the best approach to obtain a pharmacokinetic profile of a given drug [6]. In pharmacokinetic studies, plasma as biological matrix is usually used for analysing analyte and metabolite in plasma. Plasma drug levels are so low that sensitive and selective measurement techniques are required [7]. Plasma is an often-used biological matrix and is obtained through blood sampling using anticoagulants. Thus, one of the most important factors in obtaining plasma is the type of anticoagulant used [8]. Anticoagulants that are frequently used in blood drug analysis include ethylenediaminetetraacetic acid (EDTA), heparin, and citrate [8,9]. The type of anticoagulant commonly used in Indonesia for obtaining plasma is the citrate form as citric phosphate dextrose adenine because it has the ability to extend the plasma storage time [10]. Both citrate and EDTA work by binding calcium ions for the prevention of protein coagulation. In contrast, heparin has mechanisms as an antithrombin activator [8]. These differences between the type

of anticoagulant used during drug analysis may affect small molecules, metabolic profiles, and clinical parameters of the drug to be analyzed [9]. Indeed, the choice of anticoagulant is an important factor because some ions ( $Na^+$  or  $K^+$ ) in the plasma have anticoagulant properties (e.g., Na-citrate) and others such as K-EDTA can cause either the ion suppression or ion enhancement in analyzing analyte or metabolite, which may affect the matrix during analysis [11].

A bioanalysis method should be validated before analysis for the method to be accurate and reliable [7]. Based on the European Medicines Agency (EMA) 2011, full validation is performed for each 1<sup>st</sup>-time analysis method or new compound drugs [12]. If an anticoagulant is used for analysis, then full validation should be performed using the same anticoagulant. For minor change conditions such as anticoagulant changes in an analysis method that was once validated, a partial validation is acceptable.

Several methods are available for analyzing metformin HCl level in the plasma, including high-performance liquid chromatography (HPLC), which is the most widely used technique for the analysis of drugs in biological matrices [13,14]. In general, the use of a photodiode array (PDA) detector provides advantages for sensitive and selective HPLC systems during the analysis of drugs and their metabolites in biological matrices [15,16]. Using these principles, various analytical methods for determining plasma metformin levels have been widely developed and accepted [5,17-20].

Research into the effects of different anticoagulants on the determination of metformin HCl levels *in vitro* using HPLC has been limited; however, this information is important to better understand the pharmacokinetics of metformin in human plasma and its analysis. Therefore, in this study, we developed an *in vitro* metformin HCl analytical method for human plasma, with a focus on evaluating the

influence of commonly used anticoagulants, such as EDTA, citrate, and heparin, on specific analysis parameters, such as stability, recovery, chromatogram interference, and peak analytical response.

## METHODS

### Equipment

A set of HPLC devices (Waters 2965; Separation Module) consisting of pumps, an autosampler, C18 columns (Waters, SunFire™ 5 µm; 250 mm×4.6 mm), PDA detectors (Waters 2996) set at 234 nm wavelength, with data processing on a computer were used. In addition, other equipment used in this study included a pH meter (Eutech pH 510), vacuum tubes and Vacutainers with K3EDTA anticoagulant (Vacuette®), vacuum tubes with Li-Heparin anticoagulant (Vacuette®), blood collection tubes (BD Vacutainer® Beckton, Dickinson and Company), analytical scales (Acculab), eluent filter (Whatman), busting/degasser (Elmasonic S60H), microcentrifuge (Spectrafuge 16M), a -80°C freezer (Biomedical Labtech Deep Freezer), a -20°C freezer (Biomedical Labtech Deep Freezer), refrigerator set to 4°C-8°C, vortex (Maxi Mix II), Eppendorf micropipettes (Socorex), centrifuge tubes, sample cups, blue tips, yellow tips, filter paper (Perkin Elmer), and various glassware.

### Materials

The reagents used in this study were of analytical grade and included metformin HCl (United States Pharmacopeia [USP]), calcium atorvastatin (USP), methanol pro HPLC (Merck), orthophosphoric acid (Merck), acetonitrile pro HPLC (Merck), sodium dodecyl sulfate (SDS) (Merck), sodium dihydrogen phosphate (Merck), sodium hydroxide (Merck), aquabidestilata, and human plasma (Indonesian Red Cross). Blood samples were obtained directly from healthy subjects.

### Stock solution

Standards of metformin HCl and atorvastatin calcium were each weighed to obtain 10 mg. The compounds were then placed into a 10-mL measuring flask. From this solution, 1 mL was then further diluted into 10 mL. Water was then added to the metformin HCl solution, while methanol was added to the calcium atorvastatin solution to prepare test concentrations of the stock solution.

### Optimization of chromatographic conditions

The analysis was conducted using HPLC equipped with detector PDA at wavelength (λ) 234 nm, C18 column (Waters, SunFire™ 5 µm; 250 mm×4.6 mm). 10 mM SDS and 10 mM phosphate buffer in water-acetonitrile (60:40) were used as the mobile phase, and was adjusted to pH 7.0 with 98% orthophosphoric acid. The column temperature was set to 40°C. The flow rate was adjusted from 0.8 mL/min to 1.0 mL/min over a 10-min analysis time.

### System suitability test

A mixture of 10 mg µg/mL of metformin HCl and 10 µg/mL of calcium atorvastatin was injected as 50 µL into a HPLC equipped with PDA detector instrument at a wavelength of 234 nm. The injection results were recorded and counted for the coefficient of variation (% CV) of the 5 times injection. The recorded parameters included the area and peak retention time.

### Sample preparation

Sample preparation of metformin HCl in plasma was performed by protein precipitation. A 300-µL aliquot of plasma containing a concentration of metformin HCl was added to the sample cup, and 50.0 µL of standard calcium atorvastatin 10 µg/mL was added. 600 µL acetonitrile was then added as a protein precipitator. The mixture was vortexed for 3 min to extract the metformin HCl from the plasma. The mixture was then centrifuged to precipitate proteins at 13,000 rpm for 10 min. Aliquots of 1000 µL were centrifuged and transferred into vials. Aliquot (50.0 µL) was then injected into the HPLC system.

### Validation of bioanalysis methods

In this study, the validation of the metformin HCl method in plasma was performed with the use of different types of anticoagulants:

Citrate, EDTA, and heparin. Full validation was performed using plasma with the citrate anticoagulant and monitored for selectivity, carry-over parameters, lowest quantity limit measurement (lower limit of quantification [LLOQ]), linearity of the calibration curve, accuracy, precision and recovery, dilution integrity, and stability. Partial validation was performed using EDTA and heparin for the plasma, with minimum parameters of accuracy and precision. However, in this study, we also performed recovery and stability tests on plasma EDTA and heparin to determine the influence of the type of anticoagulant used. Validation of the analytical methods was evaluated according to the EMEA guidelines 2011 for the validation of bioanalysis methods.

## RESULTS AND DISCUSSION

### Optimization of conditions for the analysis method

The analysis method we used in this study was based on an HPLC method. The analysis conditions used C18 column (Waters, SunFire™ 5 µm; 250 mm×4.6 mm). In addition, 10 mM SDS and 10 mM 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, with a flow rate of 1.0 mL/min over a 10-min analysis time. Calcium atorvastatin was selected as an internal standard due to its similar physicochemical properties, including pKa value. The analytical conditions resulted in the good separation of the targeted compound. The retention time of metformin hydrochloride was 5.4081 min and that of calcium atorvastatin was 7.021 min (Fig. 1). The runtime was quite short at 10 min, which was considered as an optimum condition.

### System suitability test

CV requirements should be <2.0%. The results obtained from each system suitability test consistently yielded CV values <2.0%. In addition to CV, tailing factor was close to 1 (1.698), N (theoretical plates) was high and height equivalent to the theoretical plate (HETP) values were close to zero. Our results indicated that the N (theoretical plate) was 2698.88 and the HETP value was 0.0092. The analysis time for each injection was 10 min. The system suitability test was performed every time to perform the analysis on a daily basis and to confirm the parameters met the requirements of the validation process.

Based on the results of the optimization of the centrifugation time, we obtained the highest area under curve of metformin HCl with a centrifugation time of 10 min at a speed of 13,000 rpm with an area of 115,432. Therefore, the extraction conditions were considered optimal for extracting the analytes from plasma for the types of anticoagulants used (plasma citrate, plasma heparin, and EDTA plasma).

### Validation of bioanalysis methods

#### LLOQ (citrate plasma)

In this study, the measurement of the LLOQ value was performed by creating an analytical calibration curve with range concentration of 20.0–5000 ng/mL. In this process, the concentration of 20.0 ng/mL becomes the temporary limit of LLOQ, and the replication is carried out for 5 times. The values of percentage difference and CV at a

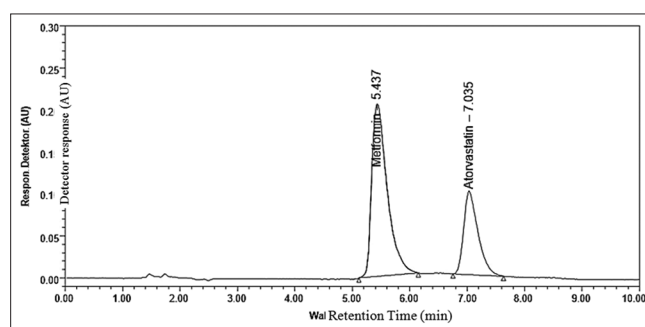


Fig. 1: Chromatogram of the system suitability test. Optimization of sample preparation

concentration of 20.0 ng/mL still met the requirement of not more than 20%; thus, the concentration met the criteria of accuracy and precision.

#### Selectivity test (citrate plasma)

In this study, we get the percentage difference values of LLOQ is 3.11–19.77%, which still fulfilled the requirement ( $<\pm 20\%$ ) and yielded a coefficient of 4.05%. In addition, at the time of analysis, the retention and standard plasma chromatogram for the blank showed some interference, but this is not significant. Interference for metformin HCl was 2.36–2.61% and for calcium atorvastatin it was 0.02–0.04%, indicating the method used in this study met the selective criteria.

#### Calibration curve (citrate plasma, heparin, and EDTA)

Calibration curve analysis was performed regarding the linearity parameter ( $r > 0.9990$ ), accuracy (% difference)  $> \pm 15\%$ , with the exception of that the LLOQ concentration should  $< \pm 20\%$ . The calibration curve for plasma citrate yielded a linear regression equation of  $y = 0.0016 + 0.0002x$ , with  $r = 0.9999$ . The heparin plasma calibration curve yielded a linear regression equation of  $y = 0.0041 + 0.0002x$ , with  $r = 0.9996$ . The EDTA calibration curve yielded a linear regression equation of  $y = 0.0022 + 0.0002x$ , with  $r = 0.9995$ , where  $x$  is the concentration of metformin HCl ( $\mu\text{g/mL}$ ) and  $y$  is peak area ratio (PAR) between the response of the analyte area and the inner standard.

#### Test accuracy, precision (citrate plasma, heparin, and EDTA)

For the measurement of accuracy using plasma citrate, we obtained a percentage difference value with the range of +5.85–+18.93% for the LLOQ concentration and a range of -14.67–14.31% for the QC concentration. For the measurement of intraday precision using plasma citrate, we obtained a CV value  $< 4.65\%$  for the LLOQ concentration and  $< 8.56\%$  for QC samples. For the precision interday values, we obtained a CV value of  $< 7.97\%$  for both the LLOQ and QC. Both intraday and interday accuracy and precision values still met the requirements for the LLOQ  $< \pm 20\%$  and QC samples  $< \pm 15\%$ . In measuring the accuracy of heparin plasma, the values of the percentage difference ranged from -15.26% to +10.50% for the LLOQ concentration, and ranged from -14.64% to +13.88% for plasma QC. The measurement of the intraday precision for heparin plasma obtained a CV value  $< 9.50\%$  for the LLOQ and QC concentrations. For the precision interday evaluation in plasma heparin, we obtained a CV value  $< 8.46\%$  for both LLOQ and QC concentrations. For the measurement of accuracy in plasma EDTA, we obtained a value of percentage difference of -15.59–+14.21% for the LLOQ concentration, with a range of -10.30–+14.61% for QC samples. For the intraday precision measurement in plasma EDTA, we obtained CV values  $< 11.24\%$  for LLOQ concentrations and QC samples. For the precision interday values, we obtained CV values  $< 11.37\%$  for both LLOQ concentrations and QC samples.

#### Dilution integrity test (plasma citrate)

To perform the dilution integrity test, we made an analyte solution in plasma with the concentration exceeding the LLOQ, which was equal to 7500.0 ng/L. We then diluted this solution by a of  $\frac{1}{2}$  and  $\frac{1}{4}$ . The results of the dilution were tested for accuracy and precision, both for intraday and interday parameters. The values of the % diff were -4.34–7.63% for dilution  $\frac{1}{2}$  times and percentage difference of -5.86–11.55% for dilution  $\frac{1}{4}$  times. As for the value of the intraday variation coefficient (KV), we obtained a value of  $< 7.53\%$  with the interday KV value  $< 3.08\%$  for both the dilution  $\frac{1}{2}$  and  $\frac{1}{4}$  times. The accuracy and precision values of the dilution still fulfilled the precise criteria; thus, the dilution described in this study was considered validated.

#### Carry-over (plasma citrat)

In this study, the percentage of followings for the analyte was 8.10%, while for the standard it was 0.03%. The percentage of the follow-up still met the requirements for analytes at  $< 20\%$ , while for standards it is  $< 5\%$ . This indicates that, during the analysis process, there was no meaningful carry-over between injections.

#### Stability test of metformin HCl and calcium atorvastatin stock solution

The results of the stability test in this study indicated a value of percentage difference for metformin stock solution HCl and calcium atorvastatin for 24 h at room temperature of -0.75–-0.16%, with a value of percentage difference of -1.78–0.20% after 3 days storage. Therefore, the standard analytical and raw stock solutions used for 24 h and stored at room temperature were stable for at least 31 days in storage at 4°C.

#### Comparison of chromatograms of plasma citrate, heparin, and EDTA

There were no significant differences between the three chromatogram forms. We did observe some interference with plasma citrate at the 4-min retention time, but this did not interfere with the analytical process (Fig. 2).

#### Comparison of metformin HCl stability in plasma citrate, heparin, and EDTA

To compare the stability of metformin HCl using different anticoagulants, including citrate, heparin, and EDTA, we determined if each stability test fulfilled the requirement that at both low and high concentration, quality control dictates percentage difference values of  $\pm 15\%$ . Therefore, during the bioanalysis process, the storage of metformin HCl in plasma citrate, heparin, and EDTA in the analysis room was still stable for 31 days and can be used for the analysis process at  $-20^\circ\text{C}$ – $-80^\circ\text{C}$ .

#### Comparison of the peak analytical response of plasma citrate, heparin, and EDTA

Given the different characteristics of anticoagulants, including differing physicochemical properties, plasma pH, and ionic strength, it is necessary to observe the chromatogram form and peak area response generated by the analyte and the standard with each anticoagulant type. In this study, we compared the PAR at the concentrations of Quality Control Low (QCL), Quality Control Medium (QCM) and Quality Control High (QCH) in each plasma sample. The analysis was done statistically to observe any differences. Based on the statistical analysis of PAR for each plasma sample, we obtained a  $p < 0.05$  (Kruskal-Wallis), which was good for the concentrations of QCL, QCM, and QCH. Therefore, the hypothesis that there is no significant difference was rejected because the  $p$  value we obtained was  $< 0.05$ , indicating a significant difference between the PAR values generated from plasma citrate, heparin, and EDTA.

#### Comparison of the absolute recovery value and the modified recovery with plasma citrate, heparin, and EDTA

In the comparative analysis of modified recovery values using the plasma matrix as a comparison (not the standard solution, which is used for the absolute recovery), we obtained statistical results for QCL

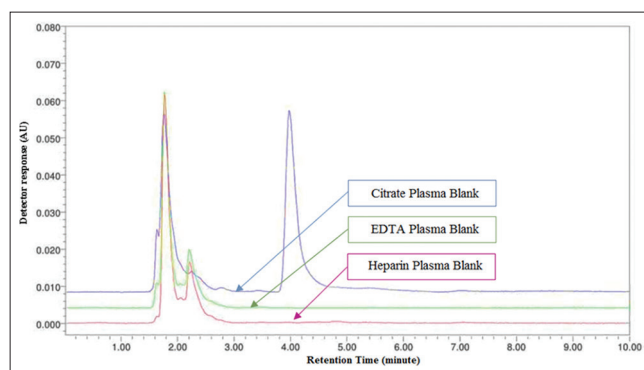


Fig. 2: Overlay chromatogram of plasma citrate blanks, heparin, and ethylenediaminetetraacetic acid

and QCH concentrations at  $p=0.602$  and  $p=0.129$ , respectively. Since  $p>0.05$  (ANOVA, least significant difference *post hoc*), the hypothesis was accepted, indicating no significant difference in recovery between plasma citrate, heparin, and EDTA.

## CONCLUSION

The method developed in this study to analyze plasma concentrations of metformin HCl using HPLC obtained an LLOQ of 20 ng/mL. The validation results for the three types of plasma anticoagulants met validation requirements based on the EMEA bioanalytical guidelines of 2011 for accuracy and precision, selectivity, calibration curve linearity, dilution integrity, carry-over, and stability.

Based on the comparison of several analytical parameters, there were no significant differences for plasma with citrate, heparin, or EDTA anticoagulants regarding stability and recovery of metformin HCl in plasma ( $p>0.05$ , ANOVA). However, for the peak plasma response ratio of metformin HCl with the plasma anticoagulants citrate, heparin, and EDTA, a significant difference ( $p<0.05$ ) was observed between plasma with the anticoagulant citrate-EDTA and plasma with the heparin-EDTA anticoagulant for low, moderate, and high concentrations. In the plasma blast chromatogram with citrate anticoagulant, there was considerable interference of plasma at a retention time  $<5$  min, whereas in plasma with heparin and EDTA anticoagulants, no interference was observed.

## CONFLICTS OF INTEREST

None declared.

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