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The Effect of Using Different Anticoagulant Types for Determination of Esomeprazole Levels in Human Plasma by High-Performance Liquid Chromatography

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ABSTRACT

Esomeprazole is a Proton Pump Inhibitor (PPI) drug formulated in delayed-release tablets, that are included in the mandatory bioequivalence test. In vitro method validation used human plasma from the Indonesian Red Cross that used citrate as an anticoagulant. In the implementation of in vivo study, usually using human plasma used EDTA or heparin as an anticoagulant. This study aims to evaluate the effect of using anticoagulant types that may affect the analysis of esomeprazole in human plasma. Optimum chromatographic conditions used column C18 SunfireTM (5 µm, 250 mm x 4.6 mm); column temperature 40°C; mobile phase acetonitrile - phosphate buffer (40:60% v/v) pH 7.6; 1.0 mL/min flow rate with lansoprazole as an internal standard and wavelength 300 nm (PDA). The extraction was carried out by liquid-liquid extraction method using 500 µl plasma and 5 ml dichloromethane as extraction solvent. The result showed that the concentration range of calibration curve linearity in 5 – 1500 ng/mL. Recovery and broad peak response data of esomeprazole in plasma have significant differences between heparin-EDTA and citrate-EDTA anticoagulants ($p<0.05$), but there is no significant difference for the stability test. In conclusion, heparin is better than EDTA as an anticoagulant for esomeprazole bioanalysis

INTRODUCTION

Esomeprazole is the S-isomer of omeprazole which is a weak acid character and a class of proton pump inhibitors (PPIs) that is made in delayed-release tablets because it is easily degraded in an acidic condition (European Medicines Agency, 2013; Food and Drug Administration, 2014a). Esomeprazole is more effective for the treatment of gastroesophageal reflux disease (GERD) symptoms and provides better acid control than omeprazole (Satyadev et al, 2013).

Quantitative measurements of drug compounds in the biological matrix such as blood, serum, or plasma were very important aspects in the development of new drug products or variations in generic drugs such as pharmacokinetic studies, toxicokinetic, bioavailability, bioequivalence, and monitoring of drug therapy (Finkel et al, 2015). Plasma as a biological matrix was needed by the anticoagulants to separate them from other blood components. Anticoagulant was used to prevent coagulation in blood samples, but anticoagulant and different counter ions could affect plasma pH (Bergeron et al, 2009; Hansen & Pedersen, 2015).

The type of anticoagulant commonly used in Indonesia (Indonesian Red Cross) for plasma was citrate, especially in the solution of CPD-A (Citric Phosphate Dextrose Adenine) form. Anticoagulants could affect the stability of plasma storage. Esomeprazole was an unstable drug in an acidic environment that needs proper anticoagulant selection to maintain its stability during storage (Kulkarni et al, 2016). Therefore, it was necessary to evaluate the effect of any anticoagulant with the drug or analytes to be analyzed.

A bioanalytical method must be validated before analyzing the subject sample and has undergone the development of previous analytical methods because there is no compendial analysis method that can be used directly to analyze drugs in the biological matrix until now (Harahap, 2010). Full validation should be performed if new methods and literature were used. This validation is partial to the validation that has been done in previous research (Harahap et al, 2017). The partial validation needs to be done when using different types of anticoagulants must be partially validated to the previous analysis method by determining the accuracy and precision value of the samples analyzed as minimum parameters until the validation is almost full (European Medicines Agency, 2011). In the bioanalysis method validation, plasma samples from the Indonesian Red Cross were used with citrate anticoagulants, whereas in the bioanalysis of drug samples in plasma, heparin and EDTA anticoagulants were used (Harahap, 2010).

This study involved 6 healthy subjects who were taken their blood to get a plasma that contained heparin and EDTA anticoagulant. The study of the effect of anticoagulant types on the determination of esomeprazole levels in vitro using a high-performance liquid chromatography system has never been done before so it is necessary to conduct a study on the analysis of the different anticoagulant types to can be used for pharmacokinetics studies of drugs in human plasma and useful in the future. Therefore, this study observed the effect of commonly used anticoagulant types such as EDTA, citrate, and heparin on the analysis of esomeprazole in human plasma. The parameters analyzed were accuracy and precision, stability, recovery, the shape of the chromatogram, and peak area response of analytes from each plasma using different anticoagulants.

METHODS

The calibration curve is made using plasma consisting of blank samples, zero, and 8 samples containing esomeprazole with different concentrations in 5 ng/mL –1500 ng/mL concentration range. The correlation coefficient (r) of the linear regression equation is calculated to see the linearity of the curve. Also calculated the diff% value for each calibration curve manufacture used with the requirements should not exceed ± 15% except for LLOQ should not exceed ± 20% (EMA, 2011).

Accuracy and precision were performed by the determination of LLOQ and QC samples (QCL, QCM, and QCH) from plasma samples that contained anticoagulant citrate, heparin, and EDTA

with five replicates in one day(within-run). The acceptance criteria for precision was % CV not more than ±15%, and the accuracy was % diff not more than ±15% while for LLOQ was not more than ±20% [10]. The absolute recovery esomeprazole and IS were determined by comparing the peak areas between the extracted plasma standards and the unextracted plasma (post-extraction plasma blanks spiked) (FDA, 2013).

The stability of analytes and internal standard in plasma containing each anticoagulant was made in two QC samples concentrations (QCL and QCH), in triplicate was then analyzed to determine short-term stability, long-term stability, freeze and thaw stability, and autosampler stability. The results obtained were compared with the new QC samples and the % diff and % CV value were calculated (EMA, 2011).

Comparison of Citrate Anticoagulant, EDTA, and Heparin as Anticoagulant for Esomeprazole Analysis

Comparative analysis of the effect of different anticoagulants type was carried out using statistical analysis by observing the shape of a chromatogram from each plasma, recovery, peak area ratio and stability of esomeprazole in each plasma analyzed statistically using ANOVA (LSD Post Hoc), Mann Withney, and Kruskal-Wallis to compare between the three anticoagulants (citrate, heparin, and EDTA).

RESULTS AND DISCUSSION

System Suitability Test

Tailing factor (Tf) which is less than 2, the result obtained is equal to 1.6019. Besides that, the value of N (theoretical plate) obtained must be large (> 2000) and the small HETP value is close to 0. The results of the analysis obtained show the value of N (theoretical plate) of 11465.28 and the value of HETP can be obtained for 0.00218, the value obtained is following the requirements under the Food and Drug Administration (Food and Drug Administration, 2014b). The time used for analysis in every injection is 10 minutes. The retention time of esomeprazole appeared about 5.2 minutes while lansoprazole was about 7.9 minutes. The chromatogram of system suitability can be shown in Figure 1.

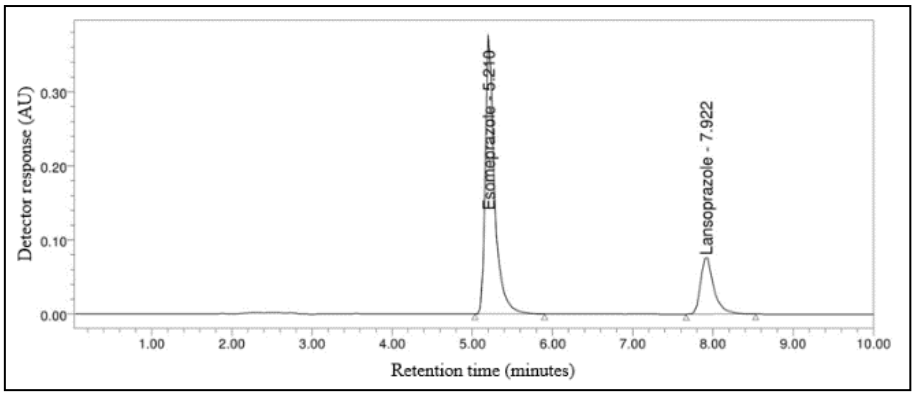

Figure 1. Chromatogram of the System Suitability Test

In this study, the system suitability test has fulfilled the requirements because of each parameter analyzed namely retention time, area, tailing factor, theoretical plate (N), HETP, and the resolution value has a coefficient of variation ≤ 2.0% (United States Pharmacopeia, 2017). It is mean that the analysis is done in the appropriate chromatographic condition.

Partial Validation Analysis Methods of Esomeprazole in Citrate, Heparin, and EDTA

Full validation has been carried out in previous studies (Harahap et al, 2017). Partial validation of the esomeprazole analysis method was carried out using citrate which aims to prove that the method used is valid and reliable (EMA, 2013). Parameters performed in partial validation of the method include measurement of linearity of calibration curves, accuracy and precision, and stability.

Calibration Curve

The calibration curves from citrate, heparin, and EDTA showed good linearity in the concentration range 5 – 1500 ng/mL with a correlation coefficient (r of ≥ 0.995) with CV and differential not more than ± 15% and LLOQ for esomeprazole was 5 ng/mL with CV and differential, not more than ± 20%.

Accuracy, Precision, and Recovery Test

The result of accuracy using citrate, heparin, and EDTA that is carried out within-run (one day) was shown in Table 1 with ranged from -11.25% to 0.87% for citrate, -9.22% to -4.41% for heparin, and -6.48% to -0.84% for EDTA. The precision value (%CV) was less than 3.83% for citrate, 3.42% for heparin, and 3.15% for EDTA

The result of esomeprazole recovery in citrate, heparin, and EDTA respectively was obtained with ranged from 86.92% to 103.65% with % CV was 3.55% for citrate, 81.12% to 98.97% with %CV was 2.64% for heparin and 85.53% to 100.30% with %CV was 1.51% for EDTA.

Table 1. The Result of the Accuracy and Precision of Citrate, Heparin, and EDTA

Conc. (ng/mL)	Type of Anticoagulant	Measured Conc. (Mean; ng/mL±SD)	%CV	%*diff*
5.00	Citrate	4.44±0.17	3.83	-11.25
	Heparin	4.78±0.16	3.42	-4.41
	EDTA	4.83±0.07	1.37	-3.38
15.00	Citrate	14.10±0.37	2.61	-5.97
	Heparin	13.62±0.34	2.49	-9.22
	EDTA	14.87±0.40	2.71	-0.84
750.00	Citrate	739.27±7.66	1.04	-1.43
	Heparin	705.08±16.32	2.32	-5.99
	EDTA	701.38±22.06	3.15	-6.48
1125.00	Citrate	1180.74±16.74	1.42	0.87
	Heparin	1070.22±2.02	0.19	-4.87
	EDTA	1079.95±16.20	1.50	-4.00

Esomeprazole Stability Test

The stability of esomeprazole was evaluated to determine the storage condition during the analysis to ensure that the analyte was stable from degradation in long-term storage. Short-term stability was determined by analyzing QC samples (low and high) at room temperature for 0, 6, and 24 hours. The result indicates esomeprazole was still stable for 24 hours at room temperature. The long-term stability was also tested for long-term storage conditions using QC samples at -20 °C and -80 °C for 0, 7, 14, and 30 days. The result indicated that esomeprazole was still stable for 30 days in long-term storage conditions. The analyte was also evaluated for freeze-thaw stability. The ability of analytes in plasma to be analyzed which have been freeze-thaw for several cycles could be seen using this test. The QC samples were stored in the freezer for at least 12 hours then thawed and re-freeze for three cycles. The result indicated that esomeprazole was still stable for 3 cycles after freeze-thaw. Besides that, the autosampler stability was also evaluated. The autosampler stability test was carried out using QCL and QCH concentration as analytes to be stored in the autosampler for 24 hours to see the stability of

the analyte after being prepared. The result showed that esomeprazole was still stable in the autosampler for 24 hours.

Based on the results obtained indicate that the parameters tested in plasma with anticoagulant citrate, heparin, and EDTA fulfill the requirements with good that has a value of % CV and % diff ≤ ± 15% except for LLOQ ± 20%.

Comparison of Citrate Anticoagulant, EDTA, and Heparin as Anticoagulant for Esomeprazole Analysis

This research was performed using 3 types of anticoagulants such as citrate, heparin, and EDTA to isolate human plasma from whole blood to analyze esomeprazole using HPLC. Lansoprazole was used as an internal standard when esomeprazole was extracted from plasma with dichloromethane by liquid-liquid extraction. Dichloromethane was used as an extraction solution because it could have a good result in separation and extraction for the analyte.

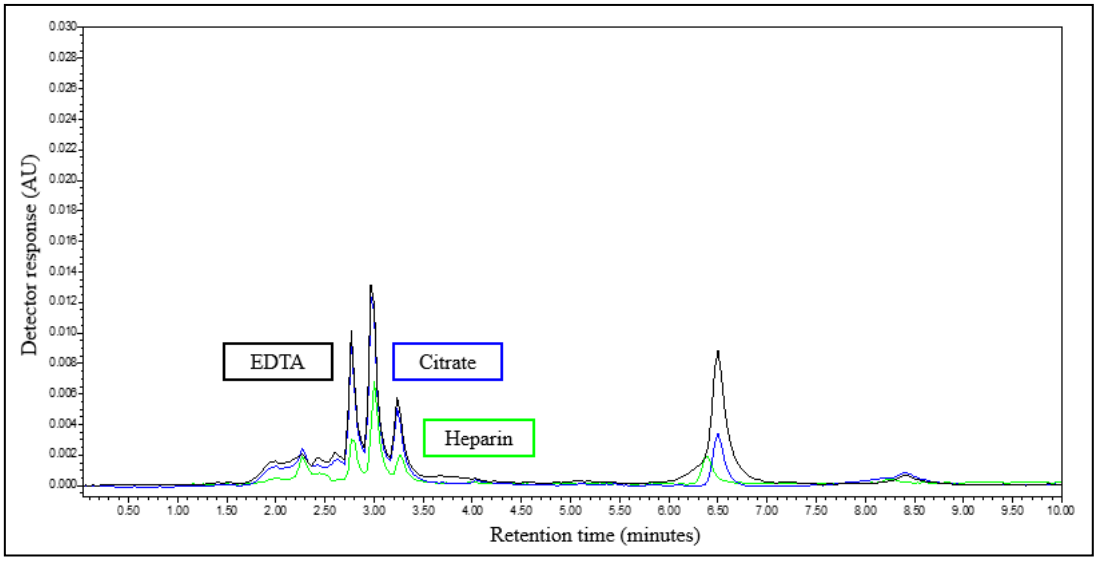

Figure 2. Comparison of Blank Chromatograms Plasma Extract with Citrate, Heparin, and EDTA as an Anticoagulant

The effect of using different three types of anticoagulants can be observed by comparing the shape of chromatograms of each plasma, peak area ratio, recovery, and stability from citrate, heparin, and EDTA plasma. The results obtained do not show a significant difference between blank chromatograms plasma each other. Interference appeared at a retention time of less than 4 minutes for citrate, heparin, and EDTA and showed unknown peaks between the sixth and seventh minutes, but did not interfere with the analysis of esomeprazole compounds and lansoprazole for the internal standard. Then there is a small peak at the time of retention of lansoprazole, but this does not interfere with the results of the analysis because the value is less than 5% of the area of lansoprazole. The blank chromatograms of esomeprazole using different anticoagulants were shown in Figure 2.

The peak area ratio was analyzed using the Kruskal-Wallis test and obtained a value of $p < 0.05$ at each measured concentration. This indicates that there are significant differences in PAR values obtained on plasma citrate, plasma heparin, and plasma EDTA. After that, further analysis was carried out using the Mann-Withney method to determine the differences between the two anticoagulant groups. The results showed that for anticoagulant citrate-EDTA and heparin-EDTA had $p<0.05$ which indicated that there was a significant difference between peak area response between the two anticoagulants while for anticoagulant citrate-heparin had $p>0.05$ which indicates that there is no

significant result of the response of peak area between the two.

Recovery values of plasma citrate, plasma heparin, and plasma EDTA were compared to determine differences in the efficiency of analyte extraction using statistical tests (ANOVA, LSD Post Hoc). The result shows that QCL, QCM, and QCH get p-value <0.05 indicating that there is a significant difference that is the difference of result of recovery value get from each of the three plasmas. This indicates that despite the recovery value > 80%, there are still significant differences in results between the three types of plasma. The result of recovery were shown in Table 2.

Table 2. The Result Comparison of Citrate Anticoagulant, EDTA, and Heparin as an Anticoagulant for Esomeprazole Analysis Parameters

Conc. added (ng/mL)	Type of Anticoagulant	Number of samples	Absolute recovery (Mean ± SD; %)	%CV
15.00	Citrate	3	90.01±3.20	3.56
	Heparin	3	94.99±3.55	3.74
	EDTA	3	86.76±1.21	1.40
750.00	Citrate	3	102.97±0.69	0.67
	Heparin	3	96.36±1.97	2.04
	EDTA	3	99.31±1.14	1.15
1125.00	Citrate	3	94.07±6.05	6.44
	Heparin	3	82.90±1.78	2.15
	EDTA	3	90.42±1.81	2.00

The stability of esomeprazole in plasma citrate, heparin, and EDTA was compared with long-term stability, short-term stability, freeze and thaw stability, and autosampler stability. Esomeprazole is stable in plasma citrate, plasma heparin, and plasma EDTA within 24 hours at room temperature and autosampler. In addition, the three plasma types were also stable for 30 days in a freezer with temperatures of -20 °C and -80 °C and three cycles of freeze and thaw during use. Therefore, based on the data obtained during the study showed that there was no significant difference in the stability of esomeprazole in plasma citrate, plasma heparin, and EDTA plasma.

Based on the results obtained, for in vivo studies it is recommended the anticoagulant that can be used to obtain plasma samples from the whole blood of healthy subjects are heparin. Heparin has a comparison of the peak area response which is not significantly different from the citrate used in the validation of the analysis method.

CONCLUSION

Based on the comparison of the results of esomeprazole analysis in several parameters using different anticoagulants, there were significant differences between plasma using citrate, heparin, and EDTA anticoagulant in recovery and peak response of esomeprazole in plasma between anticoagulant heparin-EDTA and citrate-EDTA (p <0.05), but for plasma stability citrate, plasma heparin, and plasma EDTA were not significantly different. Esomeprazole is stable within 24 hours at room temperature or 30 days during storage in the freezer at -20 ° C and -80 ° C. In plasma blank chromatograms with anticoagulant citrate, heparin and EDTA there is plasma interference at a retention time of between 6 to 7 minutes which can be quite disturbing if column efficiency is low.

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