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RESEARCH ARTICLE

Effect of Anticoagulant on Acetylsalicylic Acid and Salicylic Acid Analysis in Plasma *in Vitro* with High Performance Liquid Chromatography

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Abstract

Background: Evaluation of the anticoagulant effect and analysis of acetylsalicylic acid (ASA) and salicylic acid (SA) is important. Full validation of *in vitro* methods using citrate anticoagulant in the form of CPD-A has previously been done, whereas in the implementation of *in vivo* studies, EDTA and heparin were used as anticoagulants. Based on EMEA 2011, if there are anticoagulant changes in validated analysis methods then partial validation should be performed. Objective: This study aims to evaluate the effect of different types of anticoagulants on the analysis of ASA and SA in plasma after being preceded by partial validation. Materials and Method: Analysis was performed using high pressure liquid chromatography column C_{18} (Waters, Reliant ™ 5µm; 250 x 4.6 mm); mobile phase acetonitrile - phosphate buffer 20 mM (35:65) with pH 2.5; 1.0 mL/min flow rate; column temperature 35˚C; 14 minutes of time analysis with furosemide as internal standard. Results: Accuracy and precision in plasma citrate, heparin, and EDTA analysis fulfilled linear calibration curves in ASA and SA. Stability and recovery of ASA and SA in plasma with different anticoagulants showed no significant difference, however the peak area response ratio for the three types of anticoagulants between plasma with anticoagulant heparin-citrate and EDTA-citrate showed significant difference and plasma with heparin anticoagulant provides a larger area than plasma with EDTA anticoagulants. Conclusion: From this experiment, the analytical methods obtained were considered to meet the validation requirements for use of citrate, heparin, or EDTA anticoagulants based on EMEA 2011.

Keywords: *Acetylsalicylic acid, Anticoagulant, HPLC, Plasma, Salicylic acid*

Introduction

Acetylsalicylic acid (ASA) is an antiplatelet drug that inhibits platelet aggregation [1]. In low doses, ASA is used as an antithrombotic agent to prevent platelet aggregation through inhibition of the cyclooxygenase enzyme [2]. ASA is absorbed by passive diffusion and is rapidly hydrolyzed to salicylic acid in the liver [3]. The analysis of ASA and salicylic acid (SA) in the body uses plasma as a biological matrix. Plasma is a biological matrix that is often used for the analysis of drugs and their metabolites in the body.

Plasma was obtained from blood sampling using anticoagulants. The purpose obviously is to maintain the sample and the drug or its metabolites of interest in a state that will not degrade from the time of collection to the time of analysis [4]. Common anticoagulants used when collecting blood in a tube are ethylenediamine tetraacetate (EDTA), heparin, and citrate [5]. Selection of anticoagulants has been shown to influence the measurement of small molecules, metabolic profiles, and clinical parameters in the analysis of a drug [6]. In previous studies validation of *in vitro* methods using anticoagulant citrate in the form of CPD-A (Citrate Phosphate Dextrose Adenine) was carried out. However, in the implementation of *in vivo* studies, EDTA and heparin are used as anticoagulants because it is easier to get.

Based on the European Medicines Agency (EMEA) 2011, [7] full validation should be performed for new or published analysis methods. When using anticoagulant, full validation should be performed using citrate anticoagulant.

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If there are anticoagulant changes used in the analysis method that has been validated, partial validation is carried out. The method used for ASA and SA analysis in plasma is rapid and sensitive High Performance Liquid Chromatography [8].The use of ultra-violet (UV) absorption detectors has been widely used for drugs that have chromophore groups that will absorb UV light with appropriate wavelengths [9].

This research is mainly important due to the instability of ASA. Therefore this study conducted the use of commonly used anticoagulant types, namely citrate, heparin, and EDTA on the parameters of ASA and SA analysis in plasma such as stability in plasma, chromatogram form and peak response, and recovery from plasma.

Materials and Methods

Materials

The materials used are acetylsalicylic acid was purchased from Novacyl, France. Salicylic acid was purchased from National Agency of Drug and Food Control, Indonesia. Furosemide was purchased from Ipca Laboratories Limited (Maharashtra, India). Acetonitrile, methanol, perchloric acid, potassium dihydrogen phosphate, ethyl acetate were purchased from Merck Co., Ltd. (Darmstadt, Germany).

Aquabidestilata was purchased from Ikapharmindo Putramas Pharmaceutical Laboratories (Jakarta, Indonesia). Human plasma with citrate anticoagulant was purchased from The Indonesian Red Cross (Jakarta, Indonesia). Blood samples with EDTA and heparin anticoagulants were taken directly from healthy subjects.

Ethical Consideration and Consent to Participate

This research had obtained ethical approval from Ethics Committee of Faculty of Medicine, Universitas Indonesia. The written informed consent form had been signed by all participants before conducting the study.

Preparation of Stock Solutions

Stock solutions of ASA, SA, and furosemide were prepared by weighing each of 10 mg of ASA, 10 mg SA, and 10 mg of furosemide. Each of the substances was put into three different 10 mL volumetric flasks, added acetonitrile for ASA and SA to the limit and methanol for furosemide to the limit then shaken until dissolved.

Chromatographic Conditions

The analytical method used is a high performance liquid chromatography (HPLC) UV-Vis detector at wavelength (λ) 230 nm. Column C_{18} (Waters, Reliant TM 5µm; 250 x 4, 6 mm).The analysis conditions used acetonitrile with 20 mM phosphate buffer (35:65) at pH 2.5; flow rate of 1.0 mL/minute; 35˚C column temperature.

The sample injection volume was 20 µL with the auto sampler maintained at ambient temperature. The run time for each sample was typically 14 min.

Preparation of Standard Solutions and Samples

20 µL internal standards (10 µg/mL) were added into 250 µL plasma containing ASA and SA. 20 µL perchloric acid 15% was vortex mixed for 10 seconds, 500 µL ethyl acetate was added and vortex mixed for 3 min then centrifuged at 12000 rpm for 3 min. The organic layer was separated and evaporated at 50°C for 20 minute. The residue obtained was reconstituted with acetonitrile - 20 mM phosphate buffer (35:65).

Method Validation

Linearity was assessed by analyzing ASA and SA standard samples over the concentration range of 0.05–1.50 µg/mL for ASA and 0.20-5.00 µg/mL for SA. The intra-assay recovery was evaluated using five replicates of standard samples at the concentrations of 0.050, 0.150, 0.725, and 1.125 µg/mL for ASA and 0.200, 0.600, 2.400, and 3.750 µg/mL for SA.

ASA and SA stability were evaluated at the concentrations of 0.150 and 1.125 µg/mL for ASA and 0.600 and 3.750 µg/mL for SA. Stability tests in plasma include freeze and thaw stability, short-term stability, long-term stability, auto sampler stability, and stability of stock solution.

Comparison of ASA and SA in Three Types of Plasma

Comparison analysis of three types of anticoagulants can be seen by observing the chromatogram formation for each plasma as well as assessing the recovery, the peak analytical response to the internal standard,

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and the stability of the analyte in the three plasma.

Results

Chromatography

Under the chosen chromatographic conditions, ASA showed a retention time of 5.0 min, SA time of 8.0 min and the internal standard time of 11.4 min.

Linearity

The mean correlation coefficients (r) of the respective calibration curves generated during the validation were 0.998 for ASA and 0.999 for SA in citrate plasma, 0.999 for ASA and 0.999 for SA in heparin plasma, and 0.998 for ASA and 0.998 for SA in EDTA plasma.

Accuracy, Precision and Recovery

Accuracy, precision and recovery results in three different plasma are all well within accepted values for clinical analysis.

Stability

ASA and SA in three different plasma were stable for at least 24 h in the autosampler, stable for at least 3 h (stored at ±25˚C) and 25 days (stored at -80˚C). After 25 days, stock solutions (stored at +4˚C and protected from light) were stable.

Three Different Plasma Comparisons

Chromatogram and stability of the three plasma showed no significant difference. The peak area ratio (PAR) at QCL, QCM and QCH concentrations in each plasma obtained $p < 0.05$ (Kruskal Wallis) for ASA and SA which showed significant differences between PAR values generated from the three types of plasma anticoagulants, the data showed there is a difference between EDTA, citrate and heparin anticoagulant both in plasma ASA and SA.

Statistical analysis was performed using Mann-Whitney method in two types of plasma. Based on the data, the comparison of heparin-EDTA plasma has $p > 0.05$, meaning there is no significant difference between heparin and EDTA anticoagulant both in plasma ASA and SA, which means there is no difference between using heparin or EDTA anticoagulant. However, the comparison of plasma citrate-EDTA and citrate-heparin has a value of $p < 0.05$, meaning that there is a significant difference in citrate anticoagulant both in plasma ASA and SA. Results are shown in Table 1 and 2.

Tabel 1: Comparison of analysis results of plasma citrate, heparin, EDTA on several parameters of acetylsalicylic acid analysis

Analysis Parameter		Plasma Types			P Value	Explanation
		Citrate	Heparin	EDTA		
Peak Area Ratio (±SD)						
1	QCL	0.0766 ± 0.01	0.0843 ± 0.00	0.0599 ± 0.01	P < 0.05	Significant difference
2	QCM	0.3377 ± 0.04	0.2783 ± 0.06	0.2534 ± 0.04	P < 0.05	Significant difference
3	QCH	0.5209 ± 0.01	0.3596 ± 0.11	0.3364 ± 0.03	P < 0.05	Significant difference
Recovery (% ± SD)						
1	QCL	87.76 ± 5.47	93.89 ± 3.19	88.91 ± 5.44	P > 0.05	No significant difference
2	QCH	106.92 ± 8.47	109.60 ± 6.64	89.93 ± 8.19	P > 0.05	No significant difference
Short Term Stability (±25˚C)						
1	QCL	Minimum 3 hours	Minimum 3 hours	Minimum 3 hours	-	-
2	QCH	Minimum 3 hours	Minimum 3 hours	Minimum 3 hours	-	-
Auto sampler Stability						
1	QCL	Minimum 24 hours	Minimum 24 hours	Minimum 24 hours	-	-
2	QCH	Minimum 24 hours	Minimum 24 hours	Minimum 24 hours	-	-
Freeze and Thaw Stability						
1	QCL	Minimum 3 cycle	Minimum 3 cycle	Minimum 3 cycle	-	-
2	QCH	Minimum 3 cycle	Minimum 3 cycle	Minimum 3 cycle	-	-
Long Term Stability (-80˚C)						
1	QCL	Minimum 25 days	Minimum 25 days	Minimum 25 days	-	-
2	QCH	Minimum 25 days	Minimum 25 days	Minimum 25 days	-	-

Analytical condition: Waters Reliant™ C_{18} column (5.0 µm; 250 mm x 4.6 mm); Mobile phases acetonitrile – phosphate buffer 20 mM pH 2.5 (35:65); Flow rate 1.0 mL/min; Column temperature 35ºC; UV-Vis Detector; Injection volume 20.0 µL; ASA concentration 10.0 µg/mL; SA concentration 10.0 µg/mL; Furosemide concentration 10.0 µg/mL; Analysis time 14 min.

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Table 2: Comparison of analysis results of plasma citrate, heparin, EDTA on several parameters of salicylic acid analysis

Analysis Parameter		Plasma Types			P Value	Explanation
		Citrate	Heparin	EDTA		
Peak Area Ratio (±SD)						
1	QCL	0.5333 ± 0.02	0.5650 ± 0.02	0.6560 ± 0.06	P < 0.05	Significant difference
2	QCM	2.0740 ± 0.03	2.0890 ± 0.08	2.1590 ± 0.11	P < 0.05	Significant difference
3	QCH	3.6760 ± 0.20	3.2270 ± 0.02	3.2450 ± 0.32	P < 0.05	Significant difference
Recovery (% ± SD)						
1	QCL	100.88 ± 5.22	111.68 ± 1.27	103.62 ± 13.64	P > 0.05	No significant difference
2	QCH	87.55 ± 4.17	91.38 ± 18.72	89.49 ± 10.83	P > 0.05	No significant difference
Short Term Stability (±25˚C)						
1	QCL	Minimum 3 hours	Minimum 3 hours	Minimum 3 hours	-	-
2	QCH	Minimum 3 hours	Minimum 3 hours	Minimum 3 hours	-	-
Auto sampler Stability						
1	QCL	Minimum 24 hours	Minimum 24 hours	Minimum 24 hours	-	-
2	QCH	Minimum 24 hours	Minimum 24 hours	Minimum 24 hours	-	-
Freeze and Thaw Stability						
1	QCL	Minimum 3 cycle	Minimum 3 cycle	Minimum 3 cycle	-	-
2	QCH	Minimum 3 cycle	Minimum 3 cycle	Minimum 3 cycle	-	-
Long Term Stability (-80˚C)						
1	QCL	Minimum 25 days	Minimum 25 days	Minimum 25 days	-	-
2	QCH	Minimum 25 days	Minimum 25 days	Minimum 25 days	-	-

Analytical condition: Waters Reliant^{TM} C_{18} column (5.0 µm; 250 mm x 4.6 mm); Mobile phases acetonitrile – phosphate buffer 20 mM pH 2.5 (35:65); Flow rate 1.0 mL/min; Column temperature 35ºC; UV-Vis Detector; Injection volume 20.0 µL; ASA concentration 10.0 µg/mL; SA concentration 10.0 µg/mL; Furosemide concentration 10.0 µg/mL; Analysis time 14 min.

Discussion

Looking at the interference (impurity) found in each plasma with different types of anticoagulants has no significant differences. Based on data analysis of PAR using Mann-Whitney statistical analysis method in two types of plasma, the ratio of citrate-EDTA and citrate-heparin plasma has a value of $p < 0.05$, meaning there is a significant difference for both ASA and SA. Significant differences in plasma citrate are due to plasma citrate obtained from Indonesia Red Cross, while plasma EDTA and heparin are obtained from blood taken directly from healthy subjects.

The anticoagulants EDTA and heparin are present in solid form in blood collection tubes. Citrate however, is added as a premeasured liquid to attain a 0.38 % (%v/v) final blood citrate concentration [6]. Use of EDTA tubes prevented ex vivo oxidation of oxidative stress biomarkers ascorbate and dehydroascorbic acid when compared to 5 other anticoagulants. A study by Wiese et al. (1997) recommended the use of EDTA or heparin tubes over citrate tubes as they led to lower lactate concentration measurement in critically ill[4]. EDTA may interfere with some immunoassay detection systems, EDTA tubes are generally recommended for proteomic analyses [5]. EDTA is the anticoagulant of choice if delayed blood processing is anticipated [12]. There was a significantly reduced level of TC, and LDL-c by when K_2EDTA anticoagulant tube was used [13]. It is important to store evacuated blood collection tubes under the condition recommended by the manufacturer to assure an accurate draw volume and clinical results [10]. Heparin was generally recommended as the most suitable anticoagulant for plasma biochemical measurements [11].In the comparison of the recovery values, both in plasma citrate, heparin, and EDTA there were no significant differences ($p > 0.05$; ANOVA). Based on these statistics, the extraction efficiency shown by recovery does not differ between plasma with citrate, heparin, and EDTA anticoagulants. The next study about analysis of ASA and SA in plasma *in vivo* with High Performance Liquid Chromatography should use heparin anticoagulant, because in this study heparin gave a best result than other anticoagulant.

Conclusion

Based on the comparison of several parameters of analysis, there were no significant differences for plasma with citrate, heparin, or EDTA anticoagulants on the stability and recovery of ASA and SA in plasma ($p > 0.05$; ANOVA), but for the peak area response ratio ($p < 0.05$) for the three types of anticoagulants between plasma with anticoagulant heparin-citrate and EDTA-citrate showed significant difference and plasma with heparin anticoagulant provides a larger area than plasma with EDTA anticoagulants therefore heparin anticoagulant showed the best result on ASA and SA analysis.

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Significant Statement

This study discovered the best anticoagulant to use on ASA and SA analysis in plasma *in vitro* with High Performance Liquid Chromatography that can be beneficial for researchers and this study will help the researchers to uncover critical areas of ASA and SA stability in plasma with different anticoagulant that many researchers were not be able to explore.

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