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Research Article

INCURRED SAMPLE STABILITY OF AMLODIPINE BESYLATE AND VALSARTAN IN HEALTHY HUMAN PLASMA BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Yahdiana Harahap [1]*, Meidi Rani Sitompul [1], Sunarsih [2]
[1] Faculty of Pharmacy, Universitas Indonesia, Depok 16424, West Java, Indonesia
[2] Dea Medica Clinic, Bogor, 16961, Indonesia
*Corresponding Author Email: yahdiana03@yahoo.com

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ABSTRACT

This study aimed to evaluate incurred sample stability of amlodipine besylate and valsartan in plasma after administration of fixed dose combination tablet of amlodipine besylate and valsartan using Liquid Chromatography – Tandem Mass Spectrometry. Blood were collected from six healthy subjects until 72 hours after drug administration. Plasma samples were analyzed using LC-MS/MS with Waters Acquity TM BEH C18 column (100.0 mm × 2.1 mm, 1.7 µm). Mass detection was performed by ESI positive in MRM mode. Amlodipine besylate (AML), Valsartan (VAL), and Irbesartan (IRB) were detected at m/z 409.16 > 238.06; 436.22 > 291.15; and 429.22 > 207.1; respectively. Sample was extracted using ethyl acetate. The mobile phase consisted of 0.1% formic acid and acetonitrile with gradient elution, the flow rate was 0.2 mL/min. The obtained samples were reanalyzed until 30 days. The mean values of Cmax, tmax and AUC0-t for amlodipine besylate were 5.68 ng/mL, 5.83 h and 149.2 h.ng/mL respectively, while the mean values of Cmax, tmax, and AUC0-t for valsartan were 4172.44 ng/mL, 4.17 h, and 31952.45 h.ng/mL respectively. The results of incurred sample stability in plasma samples from six healthy subjects who were administered 10 mg of AML and 160 mg of VAL stored for 30 days were ranged -21.01 until 17.48% for AML and -12.98 until 14.67% for VAL. The incurred sample stability of AML and VAL in human plasma was stable until 30 days after drug administration, with more than 67% incurred samples had %diff value not more than ± 20%.

Keywords: Amlodipine besylate, hypertension, incurred sample, pharmacokinetic, LC-MS/MS, valsartan.

INTRODUCTION

Hypertension or high blood pressure is a condition with abnormal high arterial blood pressure[1]. According to the Joint National Committee 7 (JNC7), normal blood pressure is a systolic BP < 120 mmHg and diastolic BP < 80 mm Hg. Hypertension is defined as systolic BP level of ≥140 mmHg and/or diastolic BP level ≥ 90 mmHg[2]. Hypertension can increase the risk of cardiovascular disease such as coronary heart disease, heart failure and peripheral arterial disease, stroke and kidney failure[3,4]. If it is not treated for a long time, hypertension can develop complications such as intracranial bleeding, left ventricular hypertrophy, heart failure, myocardial infarction, thrombosis, and angina pectoris[5]

Treatment for hypertension is an approach to reduce the risk of cardiovascular disease, such as pharmacological treatment using amlodipine besylate and valsartan[6]. Amlodipine besylate belongs to Calcium Channel Blocker class and valsartan belongs to Angiotensin II Receptor Blocker class[7]. These drugs have different mechanisms of action, so therapy using fixed-dose combination of amlodipine besylate and valsartan are more effective than administration of amlodipine besylate or valsartan alone[8,9]. According to regulation of The National Agency of Drug and Food Control of Republic of Indonesia, antihypertensive agents are drugs for serious condition that need bioequivalence test[10]. Bioequivalence test is carried out in long period of time, so it is necessary to determine the stability of amlodipine besylate and valsartanby analyzing the incurred sample stability.

Evaluation of incurred sample stability needs to be done because the long-term stability test at the time of validation of the bioanalytical method does not describe the *in vivo* stability. *In vivo* stability of the analyte is affected by drug-protein binding, the back conversion of metabolites into the main form, biological matrix interference and the presence of other drugs consumed[11]. According to the Global CRO Council for Bioanalysis, evaluation of incurred sample stability is a re-analysis of several study samples in a certain time frame to see the stability and reproducibility an analyte concentration[12]. Incurred sample stability evaluation was performed on several samples with concentrations near C_{max} and near the elimination phase[13]. The number of samples evaluated were 10% of the total sample if the total sample less than 1000 and if the total sample is more than 1000 then the total sample to be analyzed is 5% of the total sample[13,14]. Analytes in plasma can be considered stable if the %diff value is within ±20% and at least 67% of the results meet these requirements[13,14].

In a previous study by Kim *et al.* in 2013, only the pharmacokinetic profile comparison of two fixed-dose combination tablets of amlodipine besylate and valsartan in healthy subjects was performed[15]. There was no evaluation of incurred sample stability of amlodipine besylate and valsartan in plasma was carried out. Until now, the study related to evaluate incurred sample stability of amlodipine besylate and valsartan has not been done before, so it can be said that this research is quite new.

The aimed of this study was to evaluate ISS of AML and VAL in six healthy subjects after administration FDC tablets of AML/VAL 10/160 mg. ISS evaluation was performed on days 7th, 14th and 30th after storing the samples in -20°C. The sample used in ISS evaluation were near Cmax phase and the elimination phase, so before evaluated the ISS it was necessary to determine the pharmacokinetic profile to determine the maximum concentration and the half-life of the analyte.

MATERIALS AND METHODS

Chemical and reagents

Amlodipine besylate was purchased from Cadila pharmaceutical (Ahmedabad, India), Valsartan was purchased from Zhejiang Second pharmaca (Zhejiang, China) and Irbesartan was purchased from Zhejiang Hua Hai pharmaceutical (Zhejiang, China). Fixed dose combination (FDC) was purchased from PT. Novartis Indonesia (Jakarta, Indonesia), acetonitrile and formic acid HPLC grade, ethyl acetate, acetic acid, ammonia for analysis were purchased from Merck (Darmstadt, Germany) and ultra-pure water.

Stock and working solution preparation

Stock solution of amlodipine besylate, valsartan and irbesartan were prepared at concentration of 1,000 µg/mL in methanol. The stock solution was then diluted with methanol to obtain working solution of AML with 1 ng/mL concentration, VAL with 10 ng/mL concentrations and IRB with 0.1 ng/mL concentrations. All solutions were stored at 4^0C.

Chromatographic Condition

This study was validated using UPLC-MS/MS (Waters Corp, Milford, MA, USA) and Xevo TQD triple quadrupole mass spectrometer (Waters Corp, Manchester, UK) equipped with electrospray ionization in positive mode. All data were processed by MasslynxTM software. Separation was conducted using UPC BEH C18 column (1.7 µm, 2.1 mm × 50 mm, Waters, Milford, MA, USA). The mobile phase was composed of acetonitrile and 0.1% formic acid solution; flow rate was set at 0.2 mL/min with total run time 6 minutes. The injection volume was 10 µl. The column was maintained at temperature 45°C. In this study AML, VAL and IS were separated using gradient elution.

Sample preparation

The sample preparation method used liquid-liquid extraction. A 250 µL plasma was added to the sample cup followed by the addition of irbesartan (25 µL; 100 ng/mL) and vortex-mixed for 10 seconds. Then 200 µL of 100 mM ammonium acetate (pH = 4.85) was added and vortex-mixed for 10 seconds. After that 1 mL of ethyl acetate was added to the mixture and vortex-mixed for 2 min and centrifuged at 2043 g for 10 min at 4^0C. The supernatant was evaporated under a stream of nitrogen gas at 50^0C and the residue was reconstituted with acetonitrile – 0.1% formic acid (75:25 v/v). Then 10 µL aliquot was injected into the LC-MS/MS system for analysis.

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Linearity

Calibration curves were prepared by spiking an appropriate volume of methanol for resulting various concentrations of 0.2; 0.4; 0.6; 0.8; 1.0; 2.5; 5.0; 7.5; 10.0 ng/mL for amlodipine besylate and 5.0; 10.0; 15.0; 20.0; 50.0; 200.0; 1,000.0; 4,500; 6,000 ng/mL for valsartan. The calibration curve was also consisted of blank and zero samples; which was used to confirm the absence of interferences. All the calibration samples were prepared with the selected method. As much as 10 µL was injected into the chromatographic system for analysis. According to the FDA 2018 guidelines criteria for calibration curve are the %diff of each calibrator should be within ± 15%, except for LLOQ where the %diff should be within ± 20% and a minimum of six calibrator levels should meet the above criteria in each validation run[13].

Accuracy and precision

Working solutions of AML and VAL were diluted with blank plasma to obtain four concentrations (LLOQ, QCL, QCM, and QCH). Each of these concentrations was prepared with the selected method then injected into the UPLC-MS/MS system for analysis. The validation was replicated five times. Accuracy and precision were considered acceptable when the bias of the calculated concentrations was within ±15% of the nominal concentrations, except the LLOQ was within ±20%[14].

Sampling procedure

Blood samples were taken at 0; 0.5; 1; 1.5; 2; 2.5; 3; 3.5; 4; 5; 6; 7; 8; 10; 12; 24; 48 and 72 hours following the administration of 10/160 mg of amlodipine besylate and valsartan. Blood samples were collected in 5 mL vacuum tube containing K_3EDTA anticoagulant. The blood samples were centrifuged to obtain the plasma then transferred to a new sample tube to be analyzed**.**

Pharmacokinetics parameter analysis of subject's plasma

The validated method was successfully applied to quantify AML and VAL concentration in human plasma samples after oral administration. In this study, the calculated pharmacokinetic parameters were C_{max}, t_{max}, t½, AUC0–t, and AUC0-∞. C_{max} and t_{max} were obtained directly from the observed plasma concentration–time data. The area under the plasma concentration–time curve from time 0 to the last measurement ($AUC_{0–t}$) was calculated using the linear trapezoidal method.

Evaluation incurred sample stability of amlodipine besylate and valsartan in plasma

Evaluation of incurred sample stability (ISS) is a re-analysis of incurred samples over a certain time to determine the stability and reproducibility of analytes in plasma samples[12]. By evaluating the ISS, it can be described the stability of analytes *in vivo* was influenced by the presence of drug-protein binding, the back conversion of metabolites into the parent form, interference of biological matrices, concomitant medications and sample inhomogeneity[11]. Evaluation ISS of AML and VAL in plasma were performed to determine stability of the analyte. Evaluation of ISS was performed by storing plasma sample at -20^0C for a period of time. On the 0^{th}, 7^{th}, 14^{th} and 30^{th} days each sample was added 25 µL irbesartan (10 µg/mL) and the sample was prepared with the same method extraction. Then the 10 µL sample was injected into LC MS/MS. The samples which were included in ISS evaluation were near the C_{max} and the elimination phase in the pharmacokinetic profile of the drug[13].

RESULTS AND DISCUSSION

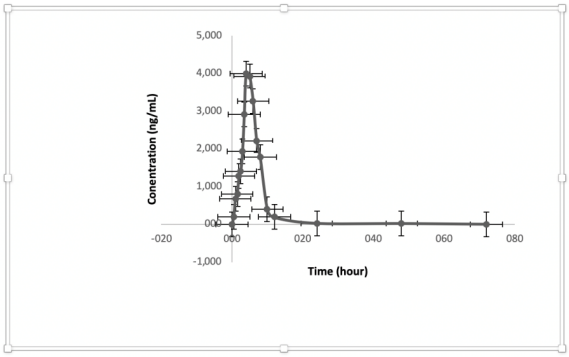

Figure 2: Pharmacokinetic profiles of VAL in six healthy human plasma

Incurred sample stability

Evaluation of ISS was performed by storing plasma sample at -20ºC for a period of time. On the 0th, 7th, 14th and 30th days each sample was reanalyzed with selected chromatographic condition. The results obtained were compared with the data obtained in initial analysis for the same sample using the same procedure. The %diff value was not more than ± 20% of the mean concentration from the initial analysis and repeated analysis in at least 67% or two-thirds of the total samples meet the requirement[4]. Evaluation incurred sample stability results were the range of %diff value for AML was -21.01% to 17.48%, while for VAL was -12.98% to 14.67%. Based on these results, the analyte in the plasma sample remained stable until the 30thday, because as much 98% of the total sample evaluated met the requirements of which the %diff values within ±20%. Based on these results it was known that not all samples meet the requirements, because the value of % AML diff on the 6th subject at 48 hours was -21.01% which indicates that the sample is unstable. Based on reported case studies in the past years, the major contributing factor on instability of analyte is issues related to instability of drug or metabolite[16]. The results for AML and VAL can be seen in Table 3 and Table 4 respectively.

Table 3: Results of evaluation incurred sample stability of AML in six healthy subjects

Subject	Time (hour)	%diff		
		Day 7	Day 14	Day 30
Subject 1	5	-3.06	9.44	0.57
	6	5.24	14.96	6.62
	48	-11.62	-14.91	-10.26
Subject 2	5	13.97	13.58	11.97
	6	8.11	14.53	12.76
	48	-2.07	-5.62	-10.84
Subject 3	5	-2.65	0.57	-7.00
	6	7.01	11.09	8.74
	48	-8.56	-8.61	-8.82
Subject 4	5	0.71	-3.97	2.57
	6	13.19	12.94	10.49
	48	5.97	-4.09	-7.90
Subject 5	4	9.31	11.87	14.31
	5	16.58	14.71	17.48
	48	-11.06	-1.28	-6.42
Subject 6	5	0.18	4.40	0.92
	6	3.59	2.45	4.93
	48	-21.01	-9.13	-13.44

Table 4: Results of evaluation incurred sample stability of VAL in six healthy subjects

Subject	Time (hour)	%diff		
		Day 7	Day 14	Day 30
Subject 1	5	9.63	4.28	-9.13
	6	-7.43	-9.08	-12.84
	48	11.39	1.43	9.84
Subject 2	5	-11.12	-8.81	-11.80
	6	7.33	-0.92	-7.98
	48	2.46	7.55	9.47
Subject 3	5	7.75	7.72	5.24
	6	14.42	10.61	7.98
	48	-3.43	-3.52	-0.59
Subject 4	5	-2.04	1.09	-5.44
	6	4.21	1.23	5.57
	48	1.38	-3.2	-4.20
Subject 5	4	4.9	11.76	14.67
	5	-6.52	-5.81	-6.04
	48	10.64	7.72	1.71
Subject 6	5	7.65	7.53	4.74
	6	8.26	12.29	8.63
	48	0.31	1.72	-6.13

CONCLUSION

The incurred sample stability of AML and VAL in six healthy human plasma was stable until 30 days after drug administration, with more than 67% incurred samples had %diff value not more than ± 20%.

The pharmacokinetics profile of AML and VAL values of C_{max} mean were 5.68 ng/mL and 4172.44 ng/mL respectively. There was no significant difference in pharmacokinetic profile of AML and VAL between in this study and previous study by Kim *et al*. (2013) with C_{max} value of AML and VAL was 5.75 ng/mL and 4128.89 ng/mL respectively.

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