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Research Article

EFFECT OF VARIOUS ANTICOAGULANTS ON AMLODIPINE BESYLATE AND VALSARTAN FOR SIMULTANEOUS ANALYSIS IN HUMAN PLASMA USING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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ABSTRACT

Amlodipine besylate and valsartan are one of the fixed combination drugs used for antihypertensive therapy. To obtain plasma from blood, addition of anticoagulant is needed. Anticoagulant citrate is usually used in the analysis of plasma *in vitro*; EDTA and heparin are used *in vivo*. This anticoagulant difference can interfere with the analysis. Thus, partial validation is needed. The study evaluated the effect of anticoagulant type (citrate, heparin, and EDTA) on the analysis of amlodipine besylate and valsartan in plasma using ultra performance liquid chromatography tandem mass spectrometry. This study involved six healthy volunteers. The chromatography used an Acquity®bridged ethylene hybrid C18 column (2.1 × 100 mm; 1.7 µm). The mobile phase consisted of 0.1 % formic acid in water–acetonitrile with gradient elution. The flow rate was 0.2 mL/minute and irbesartan was the internal standard. Aliquots were obtained by liquid-liquid extraction with ammonium acetate and ethyl acetate as the extraction solvent. The accuracy and precision of the analysis of citrate, heparin and EDTA plasma met the requirements of linear calibration curves at concentrations range of 0.2 to 10 ng/mL for amlodipine besylate and 5 to 6000 ng/mL for valsartan. Stability and recovery did not differ significantly ($p > 0.05$, ANOVA). The peak area ratio displayed significant differences ($p < 0.05$, Kruskal Wallis test) in the three types of plasma. Overall, the analysis using heparin produced better results than analyses using EDTA and citrate plasma.

Keywords: Amlodipine besylate; citrate; EDTA; heparin; validation; valsartan

INTRODUCTION

Anti-hypertensive therapy is done to reduce blood pressure, which significantly increases cardiovascular risk[1]. Anti-hypertensive therapy by controlling drugs from different classes in fixed dose combinations improves patient safety, reduces side effects, simplifies the calculation of the daily amount needed and achieves greater success in controlling blood pressure[2]. One of the fixed dose combination options that is commercially available is the combination of amlodipine besylate and valsartan[3]. This combination has proven better at reducing blood pressure than amlodipine or valsartan monotherapy[4]. Amlodipine belongs to the class of calcium ion (Ca^{2+}) dihydropyridine channel blockers[1]. Valsartan is an angiotensin II receptor type 1 (AT1) antagonist that has an effect on the reduction of blood pressure[5]. In this study, irbesartan was used as internal standard. Irbesartan is an drug in angiotensin II receptor blocker (ARB)[2,6].

In the development of a bio analysis method, full validation is required to establish the reliability of the method in determining the concentration of analytes on bio analytical matrices that include blood, urine, serum, plasma, and saliva[7]. Plasma is a biological matrix that is commonly used in determining the levels of systemically circulating drugs. This is because plasma is easier and less time-consuming to separate compared to other biological matrices. Additionally, there is an anticoagulant in the plasma that can inhibit blood clotting. The benefits of using anticoagulants in bio analysis include minimal interference, improved drug stability and the minimized formation of fibrinogen clots that can block the pipette tip during pipetting of plasma[8].

Anti-coagulants, commonly termed blood thinners, can also delay the formation of blood clots, so that before the analysis process begins, the analyte does not change significantly during the analysis process. Anticoagulants that are commonly analytically used are EDTA, heparin, and citrate[9]. In our country, during the full validation and development of analytical methods, the plasma used is obtained from the Indonesian Red Cross, which uses citrate anticoagulants. By contrast, *in vivo* studies tend to use EDTA and heparin as anticoagulants to isolate plasma from the blood[10]. According to EMEA, if there is minor change in the method that has been fully validated, such as a change in the anticoagulant used for *in vivo* sample analysis, partial validation must be done to determine the accuracy, precision, and stability of the method using the particular anticoagulant[7].

Analysis of amlodipine besylate and valsartan using liquid chromatography mass spectrometry (LC-MS) has been described[11]. The current study is a modification of the method with respect to the anticoagulant. The study aims were to obtain validated methods for the analysis of amlodipine besylate and valsartan in plasma using three different types of anticoagulants—citrate, EDTA and heparin-using LC-tandem MS (LC-MS/MS). The analyses included multiple analysis parameters, such as accuracy and precision, recovery, chromatogram, stability and matrix effects.

MATERIAL AND METHODS

Chemicals and materials

Amlodipine besylate was purchased from Cadila Pharmaceuticals (Ahmadabad, India), valsartan from Zhejiang Second Pharma Co (Zhejiang Sheng, China), and irbesartan from Zhejiang Huahai Pharmaceutical (Zhejiang, China), aquabidest from Ikapharmindo (Jakarta, Indonesia). Acetonitrile pro HPLC, formic acid, ethyl acetate, methanol and chloroform were purchased from Merck Co., Ltd. (Darmstadt, Germany). Plasma with citrate anticoagulant was obtained from Indonesia Red Cross.

LC-MS/MS

The analysis was performed using the Acquity™ ultra performance liquid chromatography (UPLC) MS/MS system (Waters Corp., Milford, MA, USA). The optimized analysis conditions included an Acquity BEH C18 column (100 mm × 2.1 mm, 1.7 µm; Waters), a mobile phase of 0.1 % formic acid solution in water (A) - acetonitrile (B) with gradient elution (Table 1), flow rate of 0.2 mL/min and column temperature of 45ºC. MS detection using the electro spray ionization positive ionization method and multiple reaction monitoring was performed at 50 kV, desecration gas temperature of 450ºC and dissolved gas flow rate of 700 L/h. Based on the results of the optimization, the m/z values were 436.22 > 291.15 for valsartan, 409.16 > 238.06 for amlodipine besylate and 429.22 > 207.1 for irbesartan.

Blood sampling and plasma preparation

Plasma containing the citrate anticoagulant citrate phosphate dextrose adenine (CPD-A) was obtained from the Indonesia Red Cross (Palang Merah, Indonesia). Plasma containing EDTA and heparin anticoagulant was obtained from the separation of blood samples from six healthy volunteers who provided informed consent. Each blood sample was split into two vacuum tubes containing Li-Heparin and K_3EDTA anticoagulant. The blood sample in each vacuum tube was inverted ten times to thoroughly mix the blood with anticoagulant in the tube, and then centrifuged at 1149 g for 20 min at room temperature. The sample was then frozen at -20°C until analyzed. Ethical clearance number: KET.188/UN2.FI/ETIK/PPM.00.02/2019

Sample preparation

Sample was prepared using liquid-liquid extraction (LLE). A total of 250 µL of plasma containing a mixture of amlodipine and valsartan plus 25 µL irbesartan as internal standard 100 ng/mL was vortexed for 10 s. Each plasma sample then received 200 µL of 0.05 M ammonium acetate (pH 4.83) and was vortexed for 10 s. The extraction was continued with the addition of 1 mL of ethyl acetate and 2-min vortex mixing. Then, the solution was centrifuged at 2043 g for 10 min. Eight hundred microliters of the organic phase was dispensed in a clean test tube and evaporated for 10 min at a temperature of 50ºC. The dry residue was reconstituted with 100 µL of the mobile phase (mixture of 0.1 % acetonitrile and formic acid, 95:5), vortexed for 10 s, sonicated for 10 s, and vortexed again for 10 s. The final solution was transferred to the vial insert and injected into the chromatographic system.

Method validation

According to the European Medicines Agency in 2011 and the Food and Drug Administration[12], full validation of new analytical methods or methods developed based on the literature is necessary. Analysis parameters for full validation are accuracy and precision, lower limit of quantitation (LLOQ), selectivity, carry over, linearity, matrix effects, dilution integrity and stability. The full validation was conducted on plasma citrate, covering all the validation parameters. If minor changes are made to an analytical method that has already been validated, such as a change in the anticoagulant, a partial validation is needed[6]. Partial validation parameters performed on heparin and EDTA plasma included linearity, within run accuracy and precision, stability, and matrix effects.

LLOQ

LLOQ was determined by injecting 10 µL of each sample into the LC-MS/MS system containing amlodipine and valsartan that had been extracted from plasma by selected methods (0.2 ng/mL and 5.0 ng/mL, respectively). LLOQ concentrations were eligible if the accuracy (% diff) and precision (% CV) of the five sample replicas obtained was not ± 20 %[6].

Linearity

The calibration curve consisted of a minimum of six points of calibration concentration along with blank samples (matrices without analytes and standards) and zero samples (matrices without analytes but added standard). The standard solution of amlodipine besylate and valsartan was diluted in blood plasma until a series of stepwise concentrations were obtained. The series for amlodipine was 0.4, 0.6, 0.8, 1.0, 2.0, 5.0, 7.5, and 10.0 ng/mL. The series for valsartan was 5.00, 10.00, 15.00, 20.00, 50.00, 200.00, 1000.00, 4500.00, and 6000.00 ng/mL. Blank and zero blank samples were prepared using the selected method, but the blank samples did not contain irbesartan standard during preparation. Each final solution was injected (up to 10 µL) into the LC-MS/MS system with selected analysis conditions[6].

Selectivity

Blank plasma from six different sources was prepared in accordance with the selected method. A total of 10 µL of each final solution was injected into the chromatographic system under the selected analytical conditions. The selectivity test was carried out in two replicas[6].

Carry over

The standard working solution of amlodipine and valsartan was diluted in blood plasma until the upper limit of quantification (ULOQ) was obtained, and then prepared according to the chosen method. A total of 10 µL of each final solution was injected into the chromatographic system using the selected analytical conditions. The test was carried out using as many as five replicates and results were observed from the blank sample area. Requirements for carry over were that the blank sample area not exceeds 20 % of the LLOQ in the analyte and 5 % of the internal standard[6].

Accuracy and precision

Accuracy describes the closeness of the value of the closeness of the value obtained with the actual value (expressed as a percentage). Accuracy is shown as % diff. Precision describes the closeness of the repeat value of an analyte produced between one replica with another replica, which is expressed as the coefficient of variation (CV). Accuracy and precision determinations were carried out intra-day or within run. In one analysis, LLOQ, quality control low (QCL), quality control medium (QCM), and quality

control high (QCH) are performed on each of the five replicas. Testing accuracy and precision met the requirements if the value of the % diff did not exceed ± 15 % unless the concentration of LLOQ % diff did not exceed ± 20 %[6].

Recovery

Recovery compares the added analyte extracted from the biological matrix with the actual concentration of the analyte in the solvent. Recovery value is related to how efficiently the extraction method has been optimized. The value does not have to reach 100 %, but both analytes and standards in the recovery value must be consistent, precise and reproducible[11]. The parameters of this test were presently carried out using the extraction method and without extracting each of the three replicates. The requirements for recovery were % recovery ± 100% and % CV not more than 15 %[6].

Matrix effect

Matrix effect testing was carried out on six matrix blanks from different sources with concentrations of QCL and QCH, where each concentration was determined in a maximum of three replicates[6]. Quantitative assessment of the effects of matrices is usually obtained by calculating the value of matrix factor (MF) by comparing the relative peak responses of the analyte in the presence of matrix ions to responses in the absence of matrix ions.

Dilution integrity

Dilution integrity is an important analysis, because the dilution of the sample can affect accuracy and precision. Dilution integrity is done by mixing the analyte at ULOQ concentration, then diluting in a blank matrix. At least five replicates were done in this study. Requirements for the integrity of taste are accuracy and precision with conditions not exceeding ± 15 %[6].

Stability

Stability testing is carried out to ensure that during sample preparation and analysis, storage conditions do not affect the concentration of the analyte[6]. The usual stability tests are stability test of stock solution, short term stability (stability at a minimum room temperature for 24 h), long term stability (stability of the analyte at -20°C for a long time), post-preparation stability (autosampler; whether the analyte is stable when stored in an autosampler for 24 h), and stability in the freeze-thaw cycle in which the analyte is frozen first in the freezer at -20ºC for 12 h, completely thawed at room temperature, then frozen again. This test was carried out using QCL and QCH samples, with three replicates of each. Stability test requirements for short term and long term stock solutions (% CV and % diff, respectively) were ± 15 %.

RESULTS AND DISCUSSION

LC-MS/MS was used because according to the literature, amlodipine besylate levels in plasma are very small. A determination method with high sensitivity and selectivity for small concentrations of analytes was needed[13,14]. Positive electro spray ionization (ESI) detection was used. This was because amlodipine besylate, valsartan, and irbesartan contain alkaline groups, so they could be protonated by the addition of H+. In addition, ESI is able to ionize compounds having a broad range molecular weight[15]. A triple quadrupole mass analyzer in the MRM) mode with a parent and daughter scan system was used. MRM was chosen because of its' selectivity, high sensitivity, and ability to quantify several compounds simultaneously[16]. The C18 bridged ethylene hybrid (BEH) column (100 mm × 2.1 mm; 1.7 µm) was used. It is a combination of silica and polymer particles. The combination column is mechanically strong, has a high efficiency, and can operate over a broad pH range[17].

The irbesartan internal standard was used because it has similar physicochemical properties to valsartan, especially acidity and solubility. Thus, it can be detected and eluted using the same analytical method. The analytical conditions resulted in a good separation between the analyte and the standard with retention times of 3.49 min for amlodipine besylate, 3.89 min for valsartan, and 3.64 min for irbesartan (Figure 1). The analyses were performed simultaneously, so that the retention time obtained for the three analytes was 6 min. The LLE sample preparation method was chosen since the sample separation by LLE was cleaner as compared to other sample preparation methods, with an increased detection signal due to the evaporation process and concentration of the sample. In addition, LLE also uses simpler steps and is less expensive compared to solid phase extraction[18].

Method validation of amlodipine besylate and valsartan in plasma

Calibration curve and LLOQ

The calibration curve was linear with a correlation coefficient of ($r > 0.9800$ in the concentration range from 0.20 to 7.50 ng/mL for amlodipine besylate and 5 to 4500 ng/mL for valsartan. LLOQ concentration of amlodipine besylate was 0.20 ng/mL with a CV value of 3.67 % and a % diff between 5.06 and 13.61 %. The LLOQ concentration of valsartan was 5 ng/mL with a CV value of 4.25 % and % diff between -17.15 and -7.33 %. When compared with a previous study[10], the LLOQ produced amlodipine was lower at 0.02 ng/mL. The difference was due to the different preparation methods, where the method used in the study used solid phase extraction, which was able to separate analytes based on acidity. Different analytes can be separately more efficiently and cleanly because they are more selective[12].

Accuracy, precision, and recovery

Accuracy and precision testing can be said to meet the requirements if the % diff value does not exceed ± 15 % unless the LLOQ % diff concentration does not exceed ± 20 %. Plasma citrate, EDTA and heparin met the requirements.

Carry over

The analytical area on the blank must not exceed 20 % of the LLOQ area and not more than 5 % of the inner standard area[12]. The result of % carry over test was 11.81 % to 16.52 % for amlodipine besylate, 14.28 % to 16.83 % for valsartan and 0.19 % to 0.56 % for irbesartan. The analytical and standard capabilities in subsequent injections met the requirements.

Selectivity

Plasma blank samples from six different sources were analyzed. A method was considered selective if the blank sample was free of disturbing components during the analytic retention time and of internal standards. Based on the results, the method developed was selective because it distinguished analytes and raw standards from other components in the sample (Figure 1).

Dilution integrity

In this parameter analysis, standard was defined with concentration above the ULOQ. The dilution fulfilled the requirement because the % diff and % CV did not exceed 15 %.

Table 1: Gradient elution profile of mobile phase

Time (min)	0.1 % formic acid in water (%)	Acetonitrile (%)
0,00	90	10
1,00	5	95
3,00	5	95
3,50	90	10
6,00	90	10

Table 2: Within run and between run accuracy and precision of amlodipine besylate (AML) and valsartan (VAL) in citrate plasma

Nominal conc AML (ng/mL)	Within run		Between run	
	Mean accuracy (% diff)	Precision (% CV)	Mean accuracy (% diff)	Precision (% CV)
0.20	-15.89 to 7.16	10.59	-16.25 to 16.74	10.71
0.60	-11.40 to 5.39	6.67	-11.85 to 12.15	6.86
5.00	-6.45 to 12.59	7.52	-6.45 to 14.04	5.78
7.50	-14.42 to 10.61	12.29	-14.98 to 11.16	9.62
Nominal conc VAL (ng/mL)	**Within run**		**Between run**	
	Mean accuracy (% diff)	**Precision (% CV)**	**Mean accuracy (% diff)**	**Precision (% CV)**
5	-16.68 to 17.37	13.82	-16.68 to 17.37	12.40
15	-14.47 to -3.62	5.44	-14.47 to 14.68	9.96
3000	-13.74 to 10.39	9.63	-13.74 to 10.39	7.11
4500	-11.23 to 1.15	4.77	-14.68 to 1.36	5.70

Table 3: Within run accuracy and precision of amlodipine besylate (AML) and valsartan (VAL) in heparin and EDTA plasma

Nominal conc AML (ng/mL)	Within run heparin		Within run EDTA	
	Mean accuracy (% diff)	Precision (% CV)	Mean accuracy (% diff)	Precision (% CV)
0.20	-0.99 to 6.88	2.94	-0.21 to 11.21	4.72
0.60	1.17 to 10.22	3.91	-7.55 to 6.12	6.36
5.00	-1.88 to 0.36	0.83	-5.67 to 2.59	3.56
7.50	-7.94 to -4.74	1.57	-12.03 to 6.81	2.97

Nominal conc VAL (ng/mL)	Within run heparin		Within run EDTA	
	Mean accuracy (% diff)	Precision (% CV)	Mean accuracy (% diff)	Precision (% CV)
5.00	2.68 to 11.08	3.28	5.51 to 14.36	3.69
15.00	9.81 to 13.16	1.37	2.12 to 5.63	1.26
3000.00	4.65 to 8.71	1.54	0.31 to 6.54	2.42
4500.00	-8.28 to -5.27	1.84	-8.46 to 6.81	2.97

Table 4: Short term stability test results of amlodipine besylateat room temperature 25ºC plasma citrate, EDTA and heparin

Time, h	Conc.	Measured Conc					
		Citrate		EDTA		Heparin	
		CV (%)	% diff	CV (%)	% diff	CV (%)	% diff
0	QCL	8.36	1.81	6.82	-4.17	3.18	5.72
			-11.40		-10.41		2.02
			3.60		2.69		8.71
	QCH	12.22	-9.40	4.66	-3.41	1.04	-9.16
			-10.38		-11.25		-10.78
			10.61		-10.25		-9.15
6	QCL	2.55	7.22	4.38	6.90	10.06	4.18
			12.46		13.04		3.77
			8.17		3.75		-13.15
	QCH	3.78	-5.11	3.72	10.12	7.08	-0.41
			-10.76		6.63		7.30
			-11.32		14.84		-6.84
24	QCL	2.04	12.59	2.90	5.62	5.20	-3.19
			9.65		11.26		5.10
			8.17		6.12		6.82
	QCH	4.55	-4.46	1.00	-6.02	2.95	-0.12
			-1.84		-6.81		3.34
			-10.25		-4.93		-2.55

Table 5: Short term stability test results of valsartan at room temperature 25ºC plasma citrate, EDTA and heparin

Time, h	Conc.	Measured Conc					
		Citrate		EDTA		Heparin	
		CV (%)	% diff	CV (%)	% diff	CV (%)	% diff
0	QCL	5.73	-3.62	6.58	-6.60	3.49	10.98
			-13.47		-2.03		4.23
			-5.13		6.28		10.58
	QCH	0.88	-6.67	2.10	-12.91	2.16	1.36
			-5.54		-11.13		-2.10
			-7.15		-9.16		-2.55
6	QCL	6.52	-5.63	7.21	-13.39	6.54	-2.23
			-14.47		-1.77		11.40
			-2.97		-12.77		6.14
	QCH	6.78	-11.23	1.08	-2.35	4.80	-1.55
			1.15		-3.80		0.90
			-1.54		-4.37		-8.11
24	QCL	8.28	-12.10	4.21	10.32	2.92	1.79
			-4.40		11.48		1.44
			3.76		3.07		6.83
	QCH	4.89	4.78	1.91	4.03	2.27	7.81
			14.36		2.91		11.49
			5.66		6.80		6.81

Table 6: Long term stability test results of amlodipine besylate at freezer temperature 20ºC plasma citrate, EDTA and heparin

Time, days	Conc.	Measured Conc					
		Citrate		EDTA		Heparin	
		CV (%)	% diff	CV (%)	% diff	CV (%)	% diff
0	**QCL**	8.36	1.81	5.68	6.12	2.34	9.78
			-11.40		4.56		10.22
			3.60		-4.61		5.62
	QCH	12.22	-9.40	3.88	-4.93	0.39	-5.42
			-10.38		-12.03		-4.74
			10.61		-8.09		-4.82
14	**QCL**	5.08	12.97	3.69	-6.46	8.94	-3.54
			5.52		0.16		-8.98
			2.39		-0.70		8.26
	QCH	7.47	-14.66	1.88	-2.19	4.62	1.86
			-2.78		-0.48		5.73
			-14.03		-4.15		-3.59
30	**QCL**	5.59	-8.30	0.87	-6.12	2.20	13.09
			1.78		-5.68		11.37
			0.45		-7.26		8.27
	QCH	3.37	-4.57	2.66	-10.59	1.60	12.43
			-2.18		-7.01		8.89
			-8.51		-11.57		10.76

Table 7: Long term stability test results of valsartan at freezer temperature 20ºC plasma citrate, EDTA and heparin

Time, days	Conc.	Measured Conc					
		Citrate		EDTA		Heparin	
		CV (%)	%diff	CV (%)	% diff	CV (%)	% diff
0	**QCL**	5.73	-3.62	0.38	3.07	1.51	10.17
			-13.47		3.14		9.81
			-5.13		3.78		12.88
	QCH	0.88	-6.67	4.13	6.80	1.70	-7.63
			-5.54		-1.68		-5.27
			-7.15		2.68		-8.28
14	**QCL**	5.45	1.87	5.41	-9.01	10.52	5.01
			-8.67		-4.44		-13.90
			-3.17		1.36		-9.38
	QCH	3.85	-4.34	0.95	-10.65	3.68	-2.78
			-10.49		-11.24		-8.57
			-10.39		-9.57		-8.84
30	**QCL**	1.94	-13.93	2.62	-13.65	5.68	-8.94
			-11.46		-10.28		-2.39
			-14.67		-9.18		-12.71
	QCH	7.00	-14.07	1.97	-10.14	3.12	12.21
			-13.97		-6.89		11.92
			-3.16		-9.82		6.12

Table 8: Comparison of analyses of amlodipine besylate in plasma citrate, plasma EDTA and heparin plasma

Parameter Analysis	Plasma Type			p-value	Differences
	Citrate	Heparin	EDTA		
Peak Area Ratio (± SD)					
LLOQ	0.0040 ± 0.0002	0.0049 ± 0.0003	0.0064 ± 0.0002	< 0.05	Significant difference
QCL	0.0088 ± 0.0005	0.0166 ± 0.0011	0.0223 ± 0.0009	< 0.05	Significant difference
QCM	0.0623 ± 0.0045	0.1435 ± 0.0052	0.1829 ± 0.0015	< 0.05	Significant difference
QCH	0.0864 ± 0.0104	0.2022 ± 0.0061	0.2600 ± 0.0041	< 0.05	Significant difference
Recovery (% ± SD)					
QCL	86.05 ± 3.7419	68.47 ± 4.6967	79.94 ± 3.0469	> 0.05	No significant difference
QCM	80.98 ± 4.0348	69.98 ± 2.5353	78.21 ± 4.4813	> 0.05	No significant difference
QCH	79.23 ± 6.3327	69.70 ± 3.3302	78.09 ± 6.2654	> 0.05	No significant difference
Matrix Effect					
QCL	0.9704 ± 0.0105	0.9015 ± 0.0269	0.9938 ± 0.0364	< 0.05	Significant difference
QCH	0.9905 ± 0.0069	0.9466 ± 0.0212	1.0125 ± 0.9934	< 0.05	Significant difference
Short term stability (± 25˚C)					
QCL	Minimum 24 hours	Minimum 24 hours	Minimum 24 hours	-	-
QCH	Minimum 24 hours	Minimum 24 hours	Minimum 24 hours	-	-
Auto sampler stability					
QCL	Minimum 24 hours	Minimum 24 hours	Minimum 24 hours	-	-
QCH	Minimum 24 hours	Minimum 24 hours	Minimum 24 hours	-	-
Freeze-thaw stability					
QCL	Minimum 3 cycles	Minimum 3 cycles	Minimum 3 cycles	-	-
QCH	Minimum 3 cycles	Minimum 3 cycles	Minimum 3 cycles	-	-
Long term stability (-20˚C)					
QCL	Maximum 30 days	Maximum 30 days	Maximum 30 days	-	-
QCH	Maximum 30 days	Maximum 30 days	Maximum 30 days	-	-

Table 9: Comparison of valsartan analyses results in plasma citrate, plasma EDTA and heparin plasma

Parameter Analysis	Plasma Type			p-value	Differences
	Citrate	Heparin	EDTA		
Peak Area Ratio (± SD)					
LLOQ	0.0104 ± 0.0005	0.0065 ± 0.0002	0.0045 ± 0.0001	< 0.05	Significant difference
QCL	0.0167 ± 0.0005	0.0184 ± 0.0003	0.0139 ± 0.0002	< 0.05	Significant difference
QCM	2.1381 ± 0.2051	3.6349 ± 0.0880	2.6387 ± 0.0408	< 0.05	Significant difference
QCH	3.0272 ± 0.1440	5.4602 ± 0.1625	3.4469 ± 0.0635	< 0.05	Significant difference
Recovery (% ± SD)					
QCL	54.25 ± 3.2235	71.70 ± 1.4797	80.41 ± 2.9677	> 0.05	No significant difference
QCM	55.48 ± 1.7668	71.26 ± 0.3262	79.04 ± 7.9226	> 0.05	No significant difference
QCH	55.58 ± 1.1802	75.88 ± 3.3713	72.67 ± 1.4331	> 0.05	No significant difference
Matrix Effect					
QCL	0.9905 ± 0.0156	0.8868 ± 0.0255	1.0025 ± 0.0246	< 0.05	Significant difference
QCH	0.9982 ± 0.0071	0.9981 ± 0.0201	1.0624 ± 0.0254	< 0.05	Significant difference
Short term stability (± 25˚C)					
QCL	Minimum 24 h	Minimum 24 h	Minimum 24 h	-	-
QCH	Minimum 24 h	Minimum 24 h	Minimum 24 h	-	-
Auto sampler stability					
QCL	Minimum 24 h	Minimum 24 h	Minimum 24 h	-	-
QCH	Minimum 24 h	Minimum 24 h	Minimum 24 h	-	-
Freeze-thaw stability					
QCL	Minimum three cycles	Minimum three cycles	Minimum three cycles	-	-
QCH	Minimum three cycles	Minimum three cycles	Minimum three cycles	-	-
Long term stability (-20˚C)					
QCL	Maximum 30 days	Maximum 30 days	Maximum 30 days	-	-
QCH	Maximum 30 days	Maximum 30 days	Maximum 30 days	-	-

LLOQ = Lower Limit of Quantification; QCL = Quality Control Low; QCM = Quality Control Medium; QCH = Quality Control High

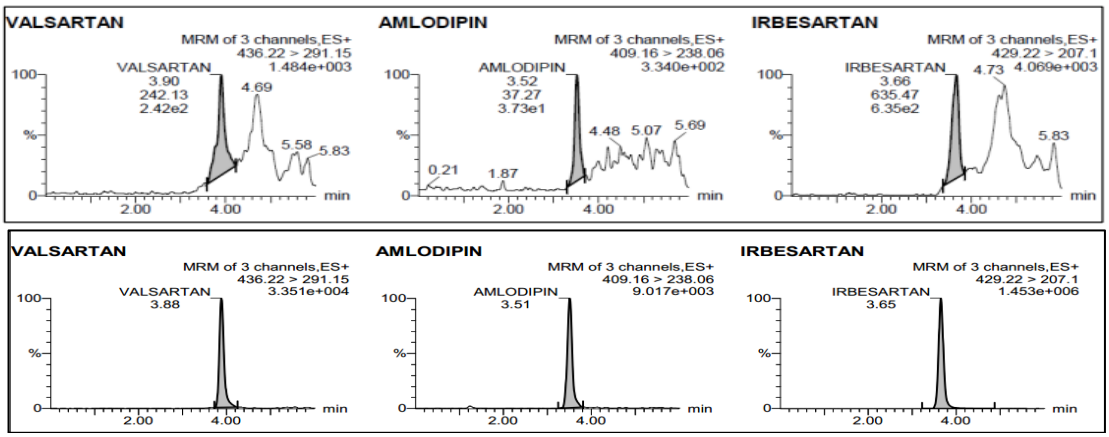

Figure 1: Chromatogram of amlodipine besylate, valsartan and irbesartan in blank plasma (A) and plasma spiked with analyte at LLOQ (B)

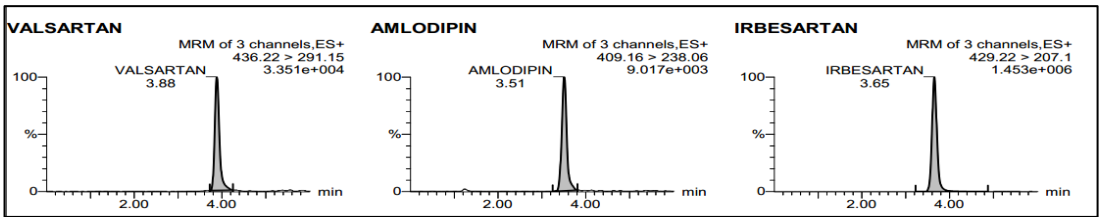

Figure 2: Chromatograms of amlodipine besylate, valsartan and irbesartan in plasma citrate

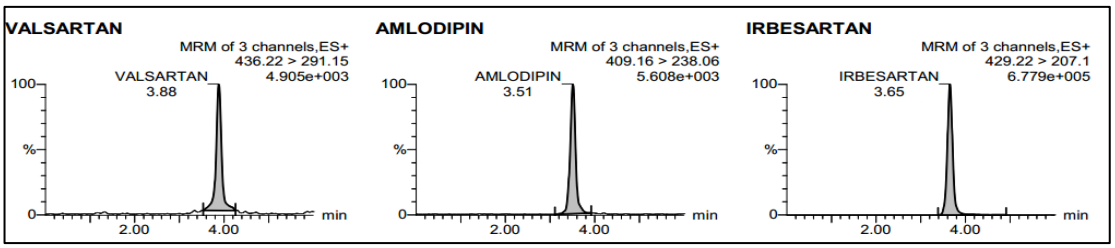

Figure 3: Chromatograms of amlodipine besylate, valsartan and irbesartan in plasma EDTA

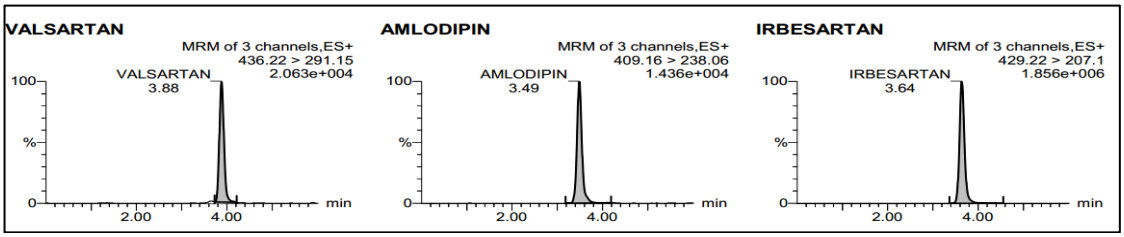

Figure 4: Chromatograms of amlodipine besylate, valsartan and irbesartan in plasma heparin

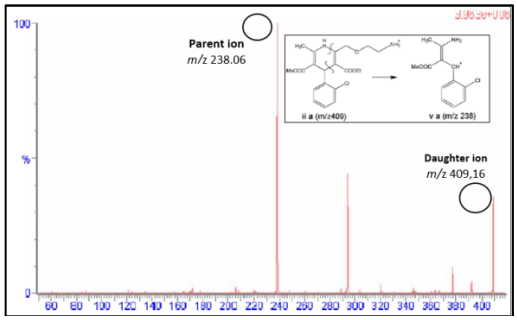

Figure 5: Amlodipine besylate mass spectrum fragmentation in parent ion

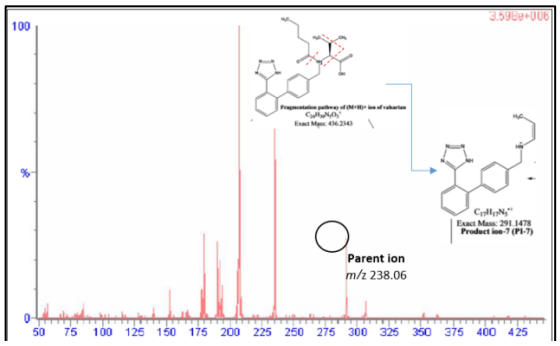

Figure 6: Valsartan mass spectrum fragmentation in parent ion

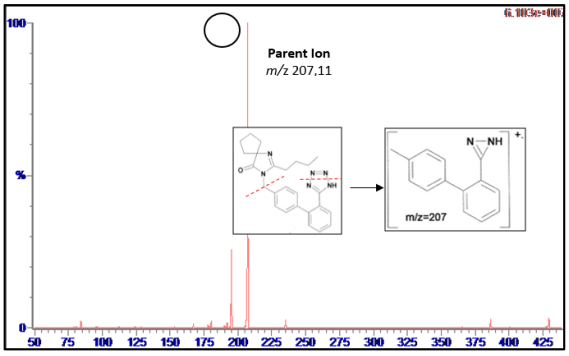

Figure 7: Irbesartan mass spectrum fragmentation in parent ion

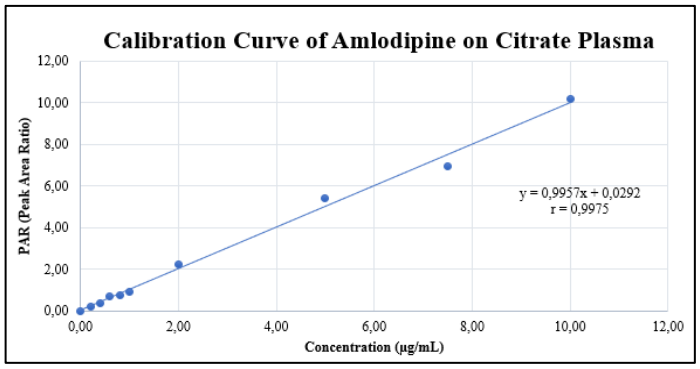

Figure 8: Calibration curve of amlodipine in citrate plasma

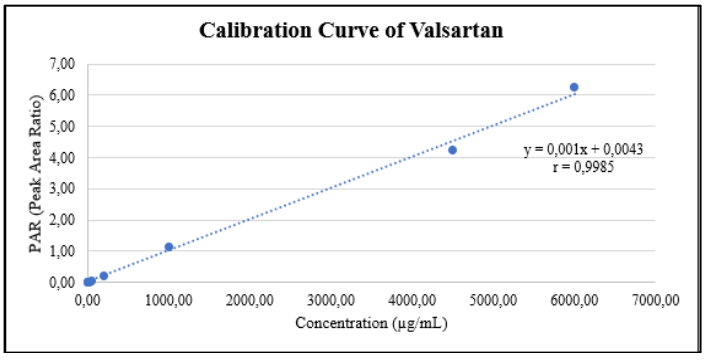

Figure 9: Calibration curve of valsartan in citrate plasma

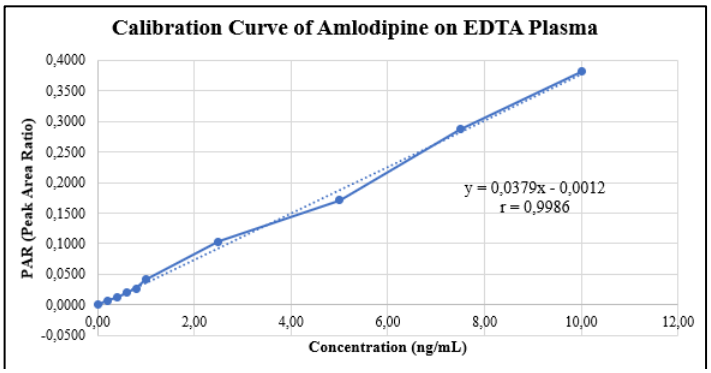

Figure 10: Calibration curve of amlodipine in EDTA plasma

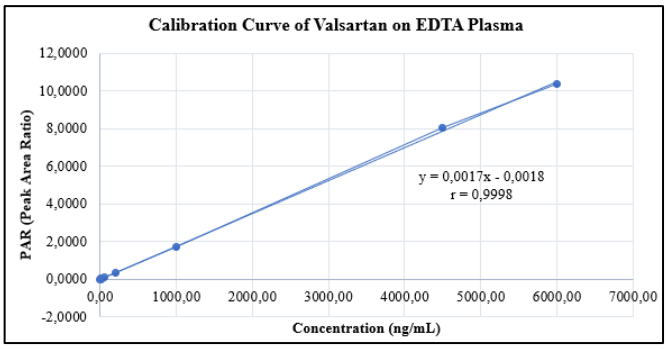

Figure 11: Calibration curve of valsartan in EDTA plasma

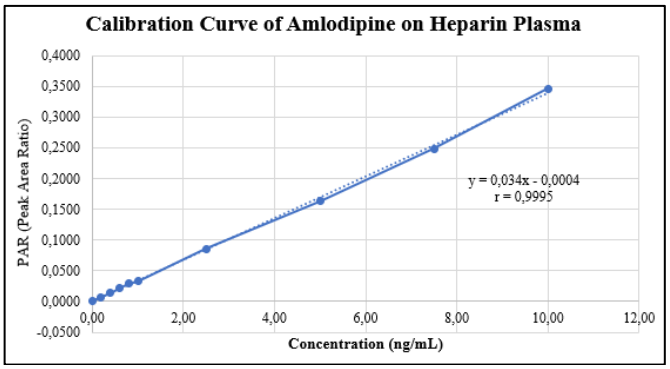

Figure 12: Calibration curve of amlodipine in heparin plasma

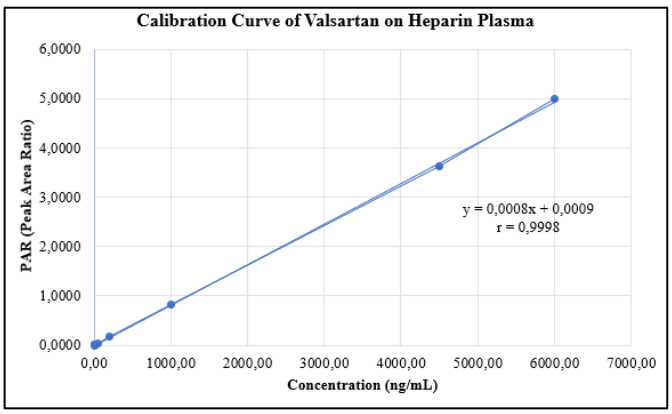

Figure 13: Calibration curve of valsartan in heparin plasma

Stability

Amlodipine besylate and valsartan were stable at room temperature for at least 24 h in each plasma. The long term stability test was determined by analyzing the concentrations of QCL and QCH and then storing at -20ºC before being analyzed on days 0, 14 and 30. The long term stability tests indicated that amlodipine besylate and valsartan were stable for up to 30 days in each plasma. The samples were also tested for freeze-thaw stability. Amlodipine besylate and valsartan were stable after three freeze-thaw cycles for each plasma. To evaluate autosampler stability of amlodipine besylate and valsartan sample, QCL and QCH in triplicate were maintained for 24 h immediately after preparation at the autosampler temperature used during the analysis for each plasma.

Matrix effect

The matrix effect test on the analytes and internal standard fulfilled the criteria of % CV value (within ± 15 %) for both internal standard normalized MF and MF. The average internal standard normalized MF for amlodipine besylate on QCL citrate, heparin and EDTA met the requirement. Moreover, the average of internal standard normalized MF for valsartan on QCL citrate, heparin and EDTA were 1.02, 1.06 and 0.92, respectively, and were 1.01, 1.12, and 1.00 on QCH citrate, heparin, and EDTA, respectively. There was a significantly different matrix effect on each plasma; but heparin had the highest ion enhancement. The finding indicated that plasma heparin sensitivity in detecting analytes was better than that of EDTA and citrate. Heparin plasma has increased ions, so the sensitivity of heparin plasma in detecting analytes is better than EDTA and citrate. In addition, heparin has a saline chemical structure similar to amlodipine besylate, so that the MF value of heparin against amlodipine is high. Meanwhile, based on the literature, Li-heparin anticoagulants have high salt and lipid content, which can cause an increase in ions[8]. Data are presented Table 8 and Table 9.

Comparison of amlodipine besylate and valsartan in the three types of anticoagulants

Comparative analysis of the three types of anticoagulants was done because there were differences in the properties of these anticoagulants, such as physicochemical properties, pH differences in ion types, and plasma pH. It was necessary to analyze the three anticoagulants by observing chromatograms and parameters produced by each plasma, including peak area ratio (PAR), recovery value, stability of the analyte, and matrix effect. Data are presented in Table 8 and Table 9.

Comparison of chromatogram and spectra for the three plasmas

The shape of the chromatograms of the three different plasmas did not differ significantly. There was no interference in each plasma for either the compound retention time or the total time of analysis (Figure 2-4).

Comparison of amlodipine besylate and valsartan responses in the three plasmas

Comparative analysis of PAR values was obtained from statistical analyses. The Kruskal Wallis test was used because the data was not normally distributed[19]. A $p < 0.05$ was evident at all concentrations, indicating significant differences between PAR values produced from the three types of plasma. These differences indicated the level of sensitivity of the type of plasma to the analyte. From the statistical data obtained, the highest PAR value of amlodipine besylate was plasma heparin. This is because amlodipine besylate and heparin are both forms of salt, so amlodipine is more sensitive to plasma types of heparin compared to plasma EDTA and citrate. The PAR value is directly proportional to the MF value. Heparin anticoagulants have high salinity so they can cause an increase in ions. By contrast, in valsartan, the highest PAR value was that of plasma EDTA. This was because EDTA and valsartan both have similar chemical structures and physicochemical properties comprising carboxylic and acidic groups. Thus, valsartan is more sensitive to plasma EDTA than are heparin and citrate. The PAR value obtained from the analysis influenced the concentration of the analyte obtained. The greater the concentration of the analyte obtained, the lower was the LLOQ value.

Comparison of recovery values of the three plasmas

Comparative recovery analysis was obtained from statistical analyses. ANOVA was used because the data was normally distributed[18]. The $p > 0.05$ obtained indicated no significant differences between the three types of plasma. The findings indicate that the use of different types of anticoagulants does not significantly influence the efficiency of analyte extraction on plasma.

Comparison of amlodipine besylate and valsartan matrix effect for the three plasma samples

Comparative analysis of matrix effects was also obtained by statistical analyses. The $p < 0.05$ obtained indicated significant differences between the three types of plasma on the matrix effect parameters. The value of the matrix effect included in the statistical program was the value of the standard normalized factor matrix. The value of matrix effects on plasma citrate and plasma EDTA did not show a matrix effect because the value of MF approached 1, in contrast to heparin, which had a value > 1 that was indicative of an increase in ion level. Thus, the sensitivity of heparin to analyte was greater than EDTA and citrate, as evidenced by the higher PAR value in heparin compared to EDTA and citrate.

Comparison of amlodipine besylate and valsartan stability for the three plasma samples

Comparative analysis of the stability in the three different types of plasma was obtained using laboratory analyses. Overall, amlodipine besylate and valsartan were stable at a minimum of 30-day long-term storage. The % diff and % CV values obtained from the stability analysis did not exceed 15 % and so met the requirements of the EMEA guidelines.

CONCLUSION

The validated method can be used to analyze the characteristics of amlodipine besylate and valsartan by applying citrate, heparin and EDTA as anticoagulants. Comparison of the analytical parameters revealed no significant differences for three plasmas concerning stability and recovery of amlodipine besylate and valsartan in plasma. However, the PAR of amlodipine besylate and valsartan in citrate, EDTA, and heparin plasma were significantly different. The collective findings demonstrate that heparin provides better results than citrate and EDTA plasma if used for analysis with LC-MS/MS.

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