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RESEARCH ARTICLE

Development and Validation of Simultaneous Analysis of Amlodipine Besylate and Valsartan in Spiked Human Plasma Using Liquid Chromatography Tandem Mass Spectrometry

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Abstract

Amlodipinebesylate, a dihydropyridine calcium channel blocker, and valsartan, an angiotensin II receptor blocker, are antihypertensive agents. Fixed dose combination of amlodipine and valsartan can reduce blood pressure (BP) effectively than amlodipine or valsartan monotherapy. Amlodipine and valsartan have low concentration in blood, so a highly selective and sensitive method is required. This research is aimed to obtain an optimum and validated method for determining amlodipinebesylate and valsartan in plasma using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). Mass detection was performed by Waters Xevo TQD with Electrospray Ionization source at positive ion mode in the Multiple Reaction Monitoring. Amlodipine besylate, valsartan, and irbesartan were detected at *m/z*409.16 > 238.06; 436.22 > 291.15; and 429.22 > 207.1; respectively. The optimum analysis condition was obtained using Waters Acquity™ UPLC C18 1.7 µm (2.1 x 100 mm); the column temperature was45°C; eluted under a gradient of mobile phase of 0.1% formic acid in water and acetonitrile at a flow rate 0.2 mL/min within 6 minutes; and irbesartan as an internal standard. Sample preparation was carried out by liquid-liquid extraction with ammonium acetate and ethyl acetate; mixed with vortex for 2 minutes; centrifugated at 2043 G-force for 10 minutes; evaporated with nitrogen gas at 50°C for 10 minutes; and reconstituted with 100 µL of mobile phase. This method fulfilled the acceptance criteria of validation based on Bioanalytical Method Validation Guidance by Food and Drug Administration in 2018. This method was linear at 0.20 – 10.00 ng/mL with r ≥ 0.997357 for amlodipine and 5.00 – 6000.00 ng/mL r ≥ 0.998476 for valsartan.

Keywords: *Amlodipine, valsartan, hypertention, LC-MS/MS, liquid-liquid extraction, validation.*

Introduction

Hypertension is a non-communicable cardiovascular disease that threatens the health of every individual in the world, where one-third of the world's population is affected [1]. According to the World Health Organization, cardiovascular disease causes 17 million deaths annually in the world, and around 9.4 million deaths are caused by hypertension. Hypertension causes 45% of deaths due to ischemic heart disease and 51% of deaths due to stroke [2].

Hypertension can be treated with single therapy or combination therapy [3]. In patients with uncontrolled blood pressure, increasing the strength of the dose in monotherapy is a rational alternative therapy, however, some anti-hypertensives have a dose-dependent tolerability profile. Thus, increasing the strength of the dose can increase the risk of side effects that can reduce patient compliance with therapy [5].

One of fixed combination antihypertensive drugs is the combination of amlodipine besylate (AML) and valsartan (VAL) in tablet [5]. Amlodipinebesylate, a dihydropyridine calcium channel blocker, and valsartan, an angiotensin II receptor blocker, are antihypertensive agents. Fixed dose

combination of amlodipine and valsartan can reduce blood pressure (BP) effectively than amlodipine or valsartan monotherapy [6].

The monitoring of drug therapy is based on the pharmacological and pharmacokinetic knowledge of a drug obtained by determining the concentration of the drug and its metabolite using bioanalysis method. Bioanalysis is performed on biological samples, one of which is plasma obtained by taking blood from a syringe vein (venipuncture) [7].The combined doses of amlodipine besylate and valsartan are very low, so a highly sensitive and selective analysis method is needed by using liquid chromatography–tandem mass spectrometry (LC-MS/MS) [8].

The validated method for analyzing amlodipine besylate and valsartan in human plasma by LC-MS/MS has been done by several researchers using internal standard labeled radioisotopes [4,9,10]. This study aims to obtain optimum conditions and validated methods for determining amlodipinebesylate and valsartan in human plasma simultaneously with irbesartan as internal standard using LC-MS/MS. Irbesartan as internal standard was used because it isanalog of valsartan and has similar physical chemistry properties.

This study has some advantages than the previous methods, such as a simpler method of extraction and lower cost of internal standard.Validation parameters of analysis methods tested refer toFood and Drug Administration (FDA), 2018, which are selectivity, linearity, lower limit of quantification (LLOQ), accuracy and precision, recovery, carry over, dilution integrity, matrix effect, and stability [11].

Experimental

Chemical and Reagents

Amlodipine besylate (Cadila Pharmaceuticals, India), valsartan (Second Pharma Co., China), and irbesartan as internal standard (Zhejiang Huahai Pharmaceutical, China). The mobile phase was formic acid and acetonitrile were obtained from Merck Co. Ltd. (Darmstadt, Germany). Human plasma with citrate anticoagulant was obtained from the Indonesian Red Cross (Jakarta, Indonesia). Reagents such as methanol, ethyl acetate, ammonium acetate, and glacial acetic acid were obtained from Merck Co. Ltd. (Darmstadt, Germany).

Chromatography Conditions

The experiment was performed on an ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA) and a Xevo TQD triple quadrupole mass spectrometer (Waters Corp., Manchester, UK) equipped with positive electrospray ionization (ESI^+). All data were acquired in centroid mode by the Masslynx™ NT4.1 software and analyzed by QuanLynx™program (Waters Corp., Milford, MA, USA).

The analyte was separated on Acquity® UPLC BEH C_{18} column (1.7 µm, 2.1 mm × 100 mm, Waters, Milford, MA, USA). The mobile phase was composed of 0.1% formic acid solution and acetonitrile; flow rate of 0.2 mL/min; autosampler temperature at 8ºC; and the injection volume was 10 µL. The gradient elution was used within 6 minutes was shown in Table 2.

The mass spectrometric detector parameters were optimized and set as follows: nitrogen desolvation temperature as 450°C with a flow rate of 700 L/h, and capillary voltage of 3.50 kV. The cone voltage was 16 V for amlodipine besylate; 30 V for valsartan; and 26 V for irbesartan as IS, and collision energy was 10 V for amlodipine besylate; 16 V for valsartan and 24 V for irbesartan as IS. The detector was performed in positive ion mode obtained by positive mode of electrospray ionization (ESI^+) technique and quantification was acquired with multiple reaction monitoring (MRM) with ion transition at 409.16→238.06 for amlodipine besylate; m/z 436.22→291.15 for valsartan; and m/z 429.22→207.1 for irbesartan as IS, respectively.

Preparation of Stock and Working Standard Solution

The stock solution of amlodipine besylate and valsartan was prepared at 1.0 mg/mL in methanol and then serially diluted with methanol to obtain tshe working solution of amlodipine besylatewith concentration of 500 ng/mL and valsartan was 10µg /mL. The stock solution of irbesartan as IS was prepared at 1.0 mg/mL in methanol and then

serially diluted with methanol to obtain the working solution of irbesartan with concentration of 100 ng/mL. All solutions were stored at 4°C and brought to room temperature before use.

Calibration standards and quality control (QC) samples were used to estimate precision and accuracy of the method and were prepared from two separate sets of solutions in plasma. Calibration standard samples of amlodipine besylate (0.2, 0.6, 0.8, 1, 2.5, 5, 7.5 and 10 ng/mL) and valsartan (5, 15, 20, 50, 200, 1000, 4500 and 6000 ng/mL) were obtained by spiking 25µL of the appropriate working solutions to 250 µL plasma (calibration curve in plasma).

The QC samples of amlodipine besylate were prepared separately in the same procedure at concentration of 0.6 ng/mL, 5.0 ng/mL, and 7.5 ng/mL for low, medium, and high quality control (QCL, QCM, and QCH). The QC samples of valsartan were prepared separately in the same procedure at concentration of 15.0 ng/mL, 3000.0 ng/mL, and 4500.0 ng/mL for low, medium, and high quality control (QCL, QCM, and QCH), respectively. All aliquot plasmas were stored at freezer -20°C and brought to room temperature before use.

Preparation of Sample in Human Plasma

A 250 µL aliquot plasma was added with 25µL IS solution (100 ng/mL) and vortex-mixed for 10 s. Then 200 µL of ammonium acetate pH 4.8 (adjusted with glacial acetic acid) wasadded in the centrifugation tube. The mixture was vortex-mixed for 10 s. Then1 mL ethyl acetate was added, vortex-mixed for 2 min and the mixture was centrifuged at 4000 rpm for 10 min at 4°C. The supernatantphasewasevaporated to dryness under nitrogen at 50°C for 10 min. The residue was reconstituted with 100 µL mobile phase. Then 10 µL aliquot was injected into the LC-MS/MS system for analysis.

Method validation

Validation of the method was assessed including selectivity, linearity, lower limit of quantitation (LLOQ), accuracy and precision, recovery, carry over, dilution integrity, matrix effect, and stability of the analytes in biological matrix according to the Food and Drug Administration (FDA) 2018 guidelines on bioanalytical method validation Committee for Medicinal Products for Human Use [11].

Selectivity

The selectivity of the method was evaluated by analyzing six blank plasma and spiked plasma at the LLOQ. The peak areas of the endogenous interference co-eluted with the analytes should be less than 20% of the peak area of the LLOQ standard and less than 5% of the peak area of the IS [11].

Linearity

Calibration standards were prepared and analyzed using a blank, a zero, and samples at eight concentration levels and prepared at scalar concentrations in triplicate. Calibration curves were considered acceptable when the correlation coefficient (r) was greater than 0.98 for the biological matrix and the bias of the calculated concentrations was within ± 15% of the nominal concentrations, except the LLOQ with an allowed deviation of ± 20% [12].

Lower Limit of Quantification

LLOQ was established by analyzing blank plasma samples spiked with ½ or ¼ of the lowest concentration of amlodipine besylate and valsartan in the sample. The analyte response should be identifiable, discrete, and reproducible with acceptable precision and accuracy (less than 20% for each criteria) [11].

Accuracy and Precision

Accuracy and precision were evaluated by assessing five replicates of the QC samples at four concentrations levels (LLOQ, low, medium, and high) on three consecutive validation days. Intra and inter-day precision were required % coefficient of variance (%CV) not to exceed 15%, and accuracy (%diff) should be within ± 15% and except the LLOQ with an allowed deviation of ± 20% [11].

Recovery

Recovery values (%) were calculated at three QC levels (QCL, QCM, and QCH) by comparing the peak areas of the regularly processed QC samples with those of spiked post-extraction samples. The % coefficient of variance (%CV) of the recovery values should be less than 15% [2].

Carry Over

Carry over was assessed by injecting blank samples after calibration standard at the upper limit of quantification. The measured peak area should not be greater than 20% of the peak area of the analyte at lower limit of quantification (LLOQ) and 5% of the peak area of the internal standard, respectively [8].

Dilution Integrity

The standard work solution of amlodipine besylate and valsartan was diluted in plasma until the concentration above ULOQ and 2 times concentration of QCH. Then diluted to half the concentration and a quarter by using blank plasma. The test is performed in five replicas. Dilution should not affect accuracy and precision with the requirements of %diff and %CV not more than ± 15% [8].

Matrix Effect

Blank plasma from six lots was extracted and then spiked with analyte at concentration of QCL and QCH to evaluate the matrix effect (% ME) of the analyte. The peak area in spiked plasma post-extraction samples was then compared with those of standard solutions containing the analyte at equivalent concentrations. The %CV of the ME should not be more than ± 15% [8]. The standardized matrix factor values with internal standard should obtain the acceptance range of 0.80 to 1.20 [18].

Stability

Stock solution stability of amlodipine besylate, valsartan, and irbesartan were evaluated in short term at 0, 6, and 24 hours at room temperature (25°C) and long term day to 1, 15, and 30 at storage temperature at (-20°C). The test was performed in two replicates and the %diff value should not more than 10% [10-11]. Sample stability was tested by analyzing the QCL and QCH after short-term storage (kept at room temperature for 0, 6, and 24 hours) and long-term storage (at freezer -20°C for day to 1, 10, and 15).

It was also tested by analyzing the QCL and QCH after three freeze-thaw cycles and autosampler stability (kept at autosampler temperature for 0 and 24 hours). The test is performed in three replicas and the %diff and %CV value should not more than 15% [8].

Result and Discussion

The LC-MS/MS is currently considered as the best choice for supporting bioanalytical studies due to high specificity, sensitivity, and rapidity. There we're not some methods for determining amlodipine besylate and valsartan by LC-MS/MS that was usingananalog compound such as irbesartan as internal standard yet.This study described the development and validation of LC-MS/MS method for simultaneous quantitative analysis of amlodipine besylate and valsartan in human plasma.

Method Development

Selection of IS

In LC-MS/MS, an IS should be an isotropically labeled compound or be as similar in physicochemicalproperties to the target analyte as possible and elute as close to it as possible [12]. Irbesartan was chosen as IS because it has similarity charateristic and including classes of valsartan.

Optimization of Mass Condition

In order to optimize ESI conditions for amlodipine besylate, valsartan, and irbesartan as IS, the MS parameters were tuned in positive ionization mode. This positive ionization related to their basic properties. The spectra showed a high intensity signal at m/z 409.16; 436.22; and 429.22 for amlodipine, valsartan, and irbesartan, respectively as protonated molecular ions $[M+H]^+$.

The product ions of mass spectra for amlodipine, valsartan, and irbesartan resulting from the fragmentation process were observed at m/z 171.08; 245.10 and 147.04, respectively (Figure 1a, b, c). Following the optimization of mass spectrometry conditions, the quantification was acquired with multiple reaction monitoring (MRM) with ion transition at 530.16 → 171.08 for amlodipine besylate; m/z 313.16 → 245.10 for valsartan; and m/z 359.10 → 147.04 for irbesartan as IS, respectively.

Optimization of Mobile Phase Combination

It tested three types of mobile phase combinations of 0.1% formic acid in water

with acetonitrile; 0.1% formic acid in water with ammonium formate pH 3.57; and 0.1% formic acid in water with 0.1% formic acid in acetonitrile. This mobile phase was tested using isocratic methods with aqueous phase and acetonitrile as an organic phase (30:70). Based on the results, a combination of 0.1% formic acid in water with acetonitrile was chosen, because it producedthe best chromatogram with the largest area.

Optimization of Mobile Phase Composition

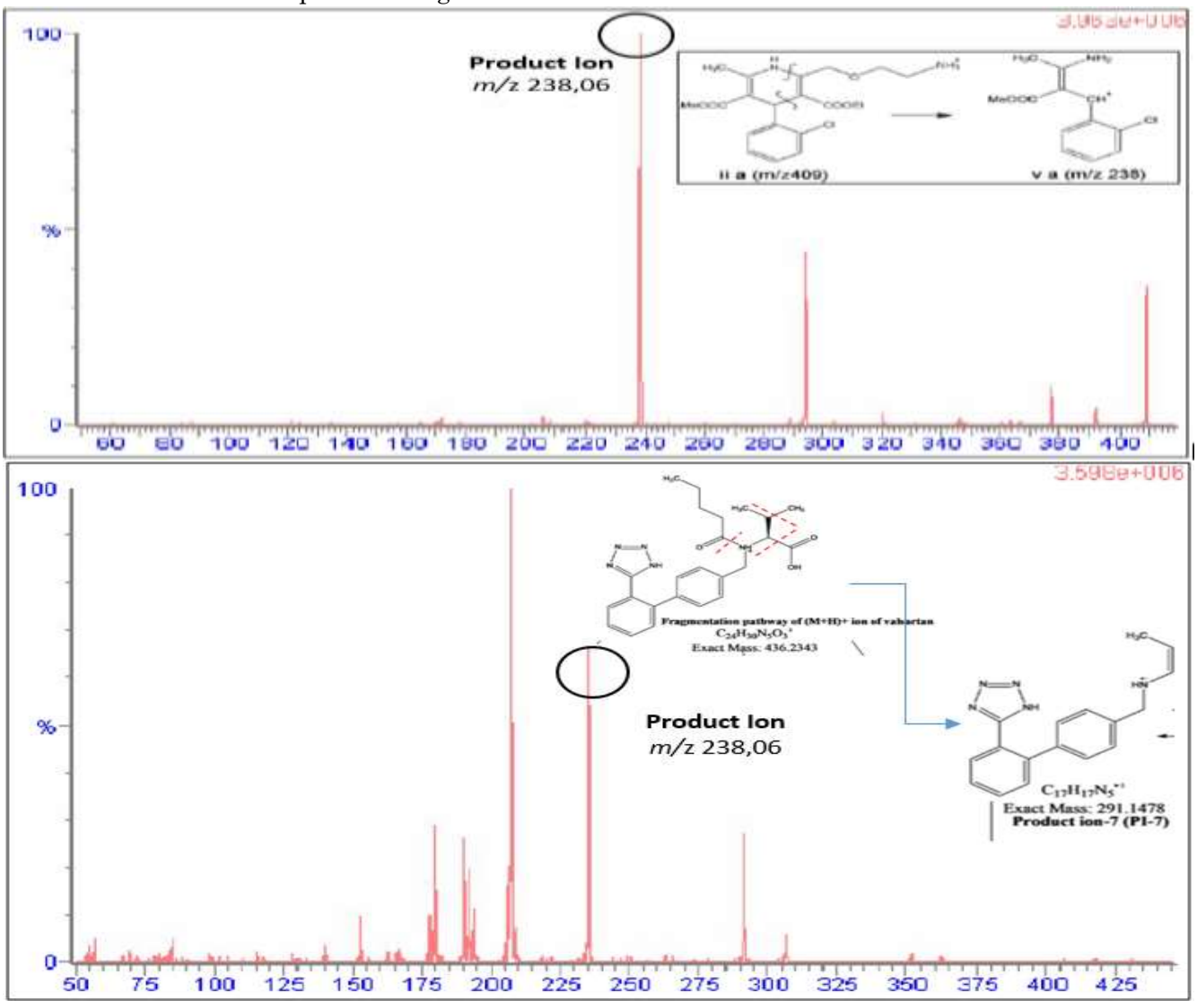
It tested two types of mobile phase compositions between formic acid 0.1% in water (A) and acetonitrile (B) such as: 20:80 and 30:70. Based on the area, the amlodipine besylate and valsartan produced at 0.1% formic acid in water with acetonitrile (20:80) was greater than that of other mobile phase compositions.

Optimization of Mobile Phase Gradient Elution

In the development of this method, isocratic elution has been able to produce large areas and a good chromatogram, but the resulting peak still has tailing or fronting. Therefore, the next optimization method using gradient elution. The gradient elution profile was shown in Table 1 and Table 2. The resulting area in the second elution profile was greater than the first elution profile, but the retention time on the first elution profile was faster than the second elution profile. This indicates the separation or elution process in the first elution profile (a gradual increase of the organic phase) was less suitable for the amlodipine besylate, valsartan, and irbesartan than the second elution profile (the increase in the organic phase directly). So, the second gradient elution profile is chosen.

Optimization of Sample Preparation

In the development of this method, tested based on different methods of extraction and mixing method. Sample preparation methods were tested with liquid-liquid extraction using dichloromethane, chloroform, and ethyl acetate.



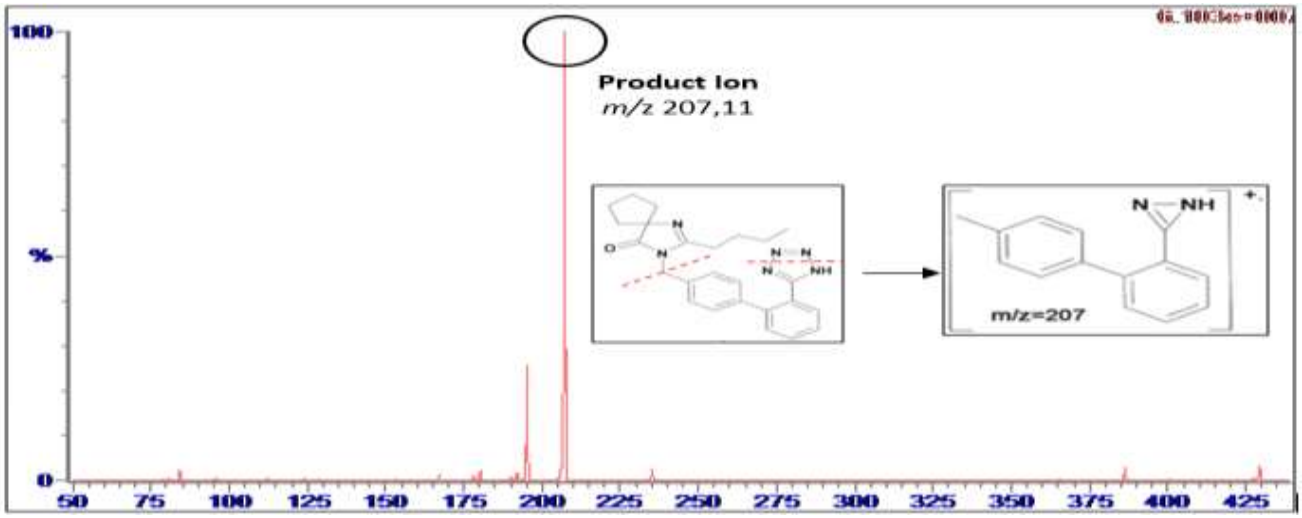


Figure 1:Fragmentation spectrum of (a) amlodipine besylate, (b) valsartan and (c) irbesartan as IS

Samplepreparation methods were tested with liquid-liquid extraction using dichloromethane, chloroform, and ethyl acetate.Sample preparation methods were also tested by ammonium acetate pH adjustment with pH 4.8 and 5.8; mixing with vortex for 1 and 2 minutes.Based on test results, liquid-liquid extractionwith ethyl acetate and ammonium acetate pH 4.8 was selected, as well as mixing with vortex 2 minutes because it produces the largest area at concentrations of LLOQ and ULOQ.

Method Validation

Selectivity

The representative chromatograms resulting from the UPLC-MS/MS analysis of 250 µL plasma from blank plasma sample and spiked LLOQ of amlodipine besylate, valsartan, and irbesartan are given in Figure 2a and b.There were no significantly interfering peaks due to the endogenous components or reagents that were observed for amlodipine besylate, valsartan, and IS.

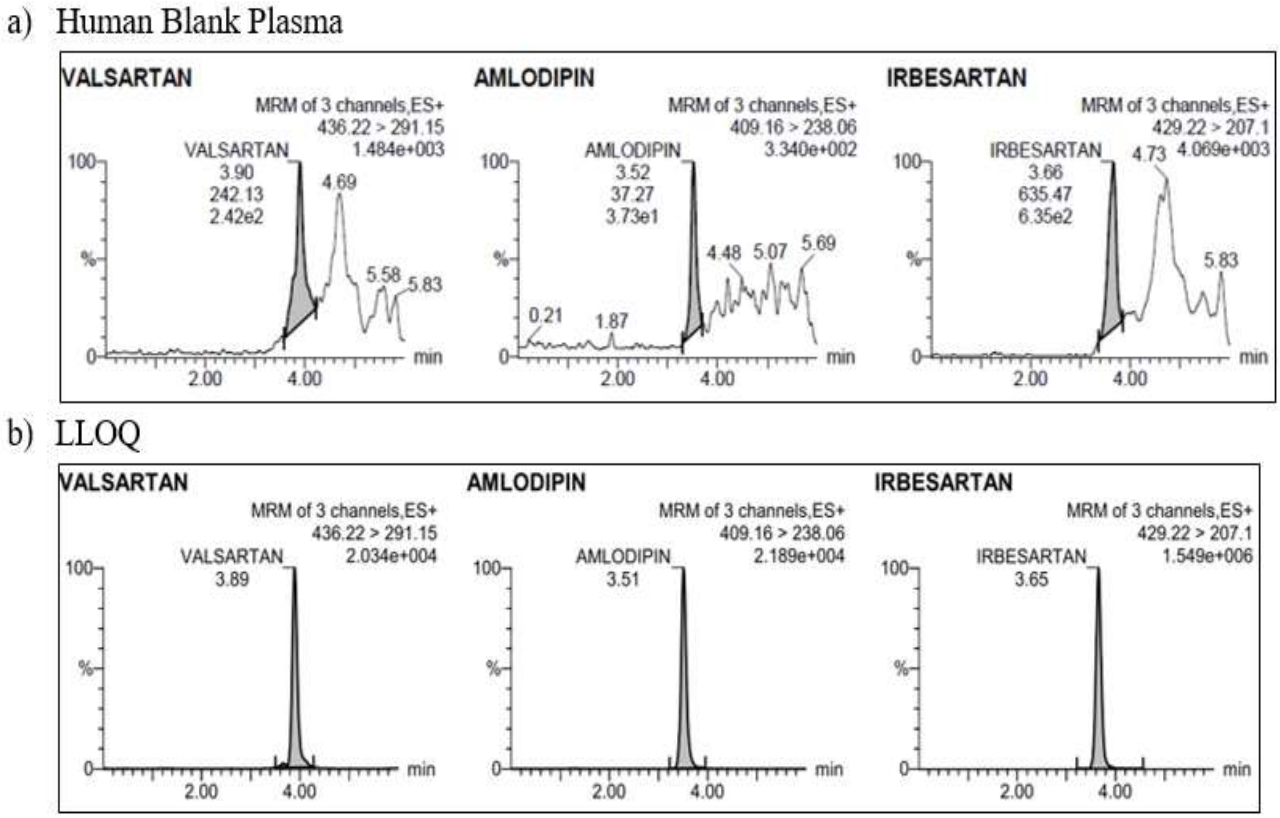


Figure 2: Representative UPLC-MS/MS chromatograms of amlodipine besylate, valsartan, and irbesartan in (a) human blank plasma; (b) human plasma spiked with analyteat LLOQ

Table 1: The first gradient elution profile

Min to-	Mobile phase A (%)	Mobile phase B (%)
0.00	75	25
1.00	5	95
3.00	5	95
3.50	75	25
6.00	75	25

Table 2: The second gradient elution profile

Min to-	Mobile phase A (%)	Mobile phase B (%)
0.00	90	10
1.00	5	95
3.00	5	95
3.50	90	10
6.00	90	10

Linearity

The calibration curve over the concentration range of 0.2 - 10 ng/mL for amlodipine besylate and 5 - 6.000ng/mL for valsartan was linear and acceptable. The calibration equation obtained was correlation coefficient (r) > 0.98 and %diff of the calculated concentrations was acceptable. Data of inter-day calibration curve of amlodipine besylate and valsartan were shown in Table 3.

Lower Limit of Quantification

The LLOQ was 0.20 ng/mL for amlodipine besylate and 5.00 ng/mL for valsartan, with %CV and %diffof back-calculated concentrations of LLOQ was 3.67% and < 20% for amlodipine besylate and 4.25% and < 20% for valsartan, respectively (Table 4). According to the 2011 EMA Guideline on Bioanalytical Method Validation, the LLOQ should not exceed 5% of the Cmax value [12].

The Cmax value of the fixed dose combination of 10 mg amlodipine besylate and 160 mg valsartan tablet was 5,92 ng/mL and 4128,89 ng/mL, respectively [13]. The LLOQ in this study had achieved the requirement already.Another study conducted by Shah, J.V., Parekh J.M., Shah, P. A., Shah, P. V., Sanyal, M., Shrivastav, P. S. in 2017 [14], that developed the analysis of amlodipine besylate, valsartan, and hydrochlorothiazidein human plasma using solid-phase extraction (SPE), produced concentration LLOQ of 0.02 ng/mL, 5.00 ng/mL, and 0.20 ng/mL, respectively.

Another study conducted by Jangala, Vats, Khuroo, &Monifin 2014 [9], that developed the analysis of amlodipine and valsartan simultaneous in human plasma using SPE, produced concentration LLOQ of 0.302 ng/mL and 6.062 ng/mL, respectively. Compared with this study, LLOQ concentration obtained in this study has an advantage because its concentration is lower than the study that is developed by Jangala, Vats, Khuroo, &Monifand also its preparation methods used is liquid-liquid extraction, which provides cost efficiency over SPE method.

Accuracy and Precision

The intra-batch and inter-batch precision and accuracy were established from validation runs performed at QCH, QCM, QCL, and LLOQ that shown in Table 5. The data demonstrate that the accuracy and precision values are within the acceptable criteria.

Table 3: Data of inter-day calibration curve of amlodipine besylate(up) and valsartan (down)

Replica inter-day	R	Slope (b)	Intercept (a)
1	0,9974	0,0165	0,0031
2	0,9930	0,0117	0,0018
3	0,9947	0,0126	0,0017
Mean	0,9950	0,0136	0,0022

Replica inter-day	R	Slope (b)	Intercept (a)
1	0,9985	0,001007	0,0043
2	0,9970	0,000713	0,0071
3	0,9967	0,001019	0,0086
Mean	0,9974	0,000913	0,0066

Table 4: The accuracy and precision from LLOQ of amlodipine besylate (up) and valsartan (down)

LLOQ conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy (%diff)	Precision (%CV)
0.20	0.215	7.26	3.67
	0.210	5.06	
	0.228	13.87	
	0.225	12.66	
	0.227	13.61	

LLOQ conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy (%diff)	Precision (%CV)
5.00	4.333	-13.33	4.25
	4.634	-7.33	
	4.456	-10.89	
	4.281	-14.37	
4.143	-17.15		

Table 5: The intra- and inter-day accuracy and precision of amlodipine besylate (up) and valsartan (down)

Nominal conc. (ng/mL)	Intra-day	Inter-day		
	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 to12.59	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. (ng/mL)	Intra-day	Inter-day		
	Mean accuracy (%diff)	Precision (%CV)	Mean accuracy (%diff)	Precision (%CV)
5.00	-11.73 to 17.37	13.82	-16.68 to 17.19	12.40
15.00	-14.47 to-3.62	5.44	-14.47 to 14.66	9.96
3000.00	-13.74 to 10.39	9.63	-13.74 to 6.27	7.11
4500.00	-11.23to 1.15	4.77	-14.68to 1.36	5.70

Table 6. The stability test results of amlodipine besylate and valsartan in human plasma

Stability experiments	Stable to-	
	Amlodipine besylate	Valsartan
Short-term storage (24 h, 25°C)	24 h	24 h
Long-term storage (freezer -20°C)	30 days	30 days
Autosampler (24 h)	24 h	24 h
Three freeze-thaw cycles	3 cycles	3 cycles

Recovery

The mean extraction recoveries of amlodipine besylate were 86.05%, 80.98%, and 79.23% (n = 3) at the concentration of QCL, QCM, and QCH, with %CV values of 4.35%, 4.98%, and 7.99%, respectively. The mean extraction recoveries of valsartan were 54.25%, 55.48%, and 57.03% (n = 3) at the concentration of QCL, QCM, and QCH, with %CV values of 5.94%, 3.19%, and 2.08%, respectively. While for the IS was 60.50% at concentration of 100 ng/mL with %CV value was 3.91%.

Carry Over

The measured peak area of the blank sample injected after calibration standard at the

ULOQ (10 ng/mL) was 14.576% of the peak area of the analyte at LLOQ for amlodipine besylate; ULOQ (6000 ng/mL) was 15.169% of the peak area of valsartanand 0.298% of the peak area of the IS, respectively.

Dilution Integrity

The dilution integrity testing results were acceptable because the dilution still fulfills accuracy and precision requirements with % diff and % CV not more than 15% which was diluted in human blank plasma until the concentration of QCHand a half of QCH.

Matrix Effect

Matrix effects were investigated for amlodipine besylate and valsartan using 6 lots of individual human plasma. The mean MEs of amlodipine besylate were 103.75% and 104.97% at the concentration of QCL and QCH, with %CV of 4.96 % and 3.03%, respectively. The mean MEs of valsartan were 106.93% and 105.57% at the concentration of QCL and QCH, with % CV of 4.84% and 3.91%, respectively. While for the IS, it was 104.39% with % CV of 1.98%. The mean internal standard normalized matrix effects (MEIS) were 0.99% and 1.01% for amlodipine besylate at the concentration of QCL and QCH.

The mean MEIS were 1.02% and 1.01% for valsartan at the concentration of QCL and QCH, respectively. These data indicate that the ME (ion suppression or enhancement) from human plasma was negligible under the current conditions which was closed to 100% and the MEIS was still at the range 0,8 – 1,2%. Ion suppression or enhancement within 10% indicates no interference affecting the ionization of the analyte [15].

Stability

Storage of stock solutions of amlodipine besylate, valsartan, and irbesartan in methanol at room temperature for 24 hours and in a refrigerator (-4°C) for 1 month did not alter the analyteofamlodipine besylate, valsartan, andirbesartan as IS. The stability test results of amlodipine besylate and valsartanin human plasma are given in Table 6. The data indicate that amlodipine besylate and valsartan are stable enough during sample preparation and storage conditions.

Conclusion

The LC-ESI-MS/MS method for quantitative analysis of amlodipine besylate and valsartan in human plasma was successfully developed and validated. The method provides very rapid, sensitive, and specific measurements of amlodipine besylate and valsartan concentrations.The LLOQ obtained in this study was 0.2 ng/mL for amlodipine besylate and 5.0 ng/mL for valsartan with sample preparation of liquid-liquid extraction, and rapid analysis time of 6 minutes. Irbesartan as IS can be used for amlodipine besylate and valsartan analysis and is able to control matrix effects.

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