Method Development and Validation of Liquid Chromatography-Tandem Mass Spectrometry for Angiotensin-II in Human Plasma: Application to Study Interaction Between Atorvastatin & Olmesartan Drug Combination

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Abstract Simple and sensitive Liquid Chromatography-Tandem Mass Spectrometry (LCMS/MS) method was developed and validated, then was implicated on hypertensive human subjects to study drug interaction of atorvastatin (ATVS) and Olmesartan (OLM) on status of Angiotensin-II (ANG-II). The ANG-II in plasma was extracted with 5 mL methanol containing 5 % formic acid through C₁₈ (cartridges) liquid-liquid extraction, dried and reconstituted with 1 mL of 16 % acetonitrile in 0.1 % formic acid in water. The chromatographic separation of ANG-II with a Agilent technology 6410 Triple quadrupole was carried multiple reaction monitoring scan mode with a Agilent 1290 Infinity LC system for UHPLC. The sample were separated on a (Thermo Scientific) Hy-Purity advance (50 × 4.6 mm, 5 µm) using Mobile Phase A: 16 % acetonitrile in 0.1 % formic acid in water and Mobile Phase B: 0.1 % formic acid in methanol at a flow rate of 0.3 mL/min, performed at ambient temperature. The mobile phase gradient of 16 % acetonitrile in water was linearly increased to 38 % acetonitrile over 10 min and subsequently the mobile-phase was increased to 100 % acetonitrile over 15 min. The developed method was validated for specificity, accuracy, precision, stability, linearity, sensitivity and recovery. The method was linear between peak area ratio of standard and internal

standard over the range of 50–800 ng/mL. The method was successfully applied for the drug interaction study revealed levels of ANG-II were significantly higher in ATV-S + OLM treatment condition as compared to individual treatment of OLM. This reflects the reason of low effectiveness of ATVS + OLM in combination instead of synergistic activity.

Keywords Angiotensin-II · Angiotensin-III · LCMS/MS · Drug interaction · Olmesartan · Atorvastatin

Introduction

Angiotensin II (ANG-II) is the biologically active product of the renin-ANG system [1, 2]. The octapeptide ANG-II (molecular weight 1,046) is the strongest physiological vasoconstrictor known. From a large protein precursor (pre-proangiotensinogen) synthesized in the liver it is liberated in a series of proteolytic steps catalyzed by enzymes from various tissues [1–3]. ANG-II is very short-lived in the plasma: Once generated from ANG-I, it is degraded further into physiologically inactive peptides by various plasma peptidases, at a plasma half life of less than a minute [4]. Olmesartan (OLM) medoxomil is the latest ANG-II receptor blocker approved for use in US. Potential advantages of this drug include once-daily dosing, an absence of significant adverse reactions, a well-tolerated side-effect profile, and a cost-effective. OLM medoxomil is currently being used as an alternative therapeutic antihypertension agent for patients intolerant of ANG-converting enzyme inhibitors [5]. Peak plasma concentrations of OLM occurred 1–3 h after administration, after which concentrations decreased quickly. The elimination half-life was 10–15 h. Bioavailability increased proportionally with

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dose, after single and multiple daily oral doses, over the therapeutic dose range, up to 40–80 mg daily [6].

The OLM peak concentration in blood determines the aldosterone (ALD) secretion as well as concentration of ANG-II too, for an instantaneous time interval, which later converted into ANG-III and ALD. So, the tracing of ANG-II high peak level with its control study is very crucial at that stage.

Virtually all of the biological actions of the principle effector peptide ANG-II have been attributed to an action at the type 1 (AT1) ANG receptor. Evidence has now accumulated that the AT2 receptor opposes functions mediated by the AT1 receptor. Whereas the AT1 receptor stimulates cell proliferation, the AT2 receptor inhibits proliferation and promotes cell differentiation [7–12]. Until now, there have been many reports of peptides with respect to analysis of drugs in plasma using column switching techniques [13–15]. Analysis of ANG peptides has been performed using HPLC combined with radioimmunoassay [16–19]. Liquid chromatography–mass spectrometry (LC–MS) features high selectivity and high sensitivity. LC–MS is a useful tool for biological analysis and is widely used for identification and quantification of proteins and peptides. Usually, LC–MS is used to provide initial identification based on molecular weight, LC–MS/MS provide further confirmation via structural specific fragmentation. The combination of LC–MS and LC–MS/MS analysis allows sensitive and unambiguous analysis of peptides in complex sample matrices.

Method

Chemicals and Reagents

Standard 5 mg human ANG-II (catalog number 002-12, lot number 429391) from Phoenix Pharmaceuticals, Inc., 330 Beach Road, Burlingame, CA, 94010, USA. The Bestatin hydrochloride (B-8385) 5 mg standard powder and ANG-III (internal standard, IS) of Sigma Aldrich through (Sintex Chemical Company, Lokangan, Ganganagar, Kolkata-700125). The acetonitrile from Sigma Aldrich, formic acid and methanol from Merck.

Instrumentation, Chromatographic Conditions and MS/MS Condition

Instrumentation

The LCMS/MS system consisted of Agilent technology 6410 Triple quadrupole, positive polarity switching, multiple reaction monitoring (MRM) scan mode with a Agilent 1290 Infinity LC system for UHPLC. The mass spectrometer is equipped with multimode source [simultaneous

Table 1 Gradient program of mobile phase for the determination of ANG-II by LCMS/MS

Steps	Acetonitrile	Water (%)	Time (min)	Flow rate (mL/min)
Step 1	16 %	84	0–5	0.3
Step 2	Up to 38 %	62	5–10	0.3
Step 3	Up to 100 %	0	10–15	0.3

electrospray ionization (ESI) and APCI] and is controlled by MassHunter Quantitative Analysis software.

Chromatographic Conditions

The sample were separated on a (Thermo Scientific) Hypurity advance (50 × 4.6 mm, 5 µm) using Mobile Phase A: 16 % acetonitrile in 0.1 % formic acid in water and Mobile Phase B: 0.1 % formic acid in methanol at a flow rate of 0.3 mL/min. All HPLC separations were performed at ambient temperature. The mobile phase gradient of 16 % acetonitrile in water was linearly increased to 38 % acetonitrile over 10 min and subsequently the mobile-phase was increased to 100 % acetonitrile over 15 min (Table 1).

MS/MS Conditions

The positive ion ESI mass spectrometric conditions were: capillary voltage 6 kV, cone voltage 20–25 V, desolvation temperature 350 °C, desolvation gas flow 600 L/h and the source temperature is 120 °C. LC–MS/MS was performed with MRM of transition of precursor ion → product ion m/z 349.6 → 255.1 and 523.8 → 263.2. The collision energies were set at 20 eV.

Preparation of Calibration Standards and Quality Control Samples

The calibration standards were prepared by spiking human plasma with standard ANG-II with corresponding IS, ANG-II (ANG-III) to obtain resultant concentrations of 5, 10, 25, 50, 100, 200 ng/mL. The developed method was validated using three different quality control (QC) samples by spiking ANG-II in human plasma at 5, 50 and 200 ng/mL to represent low QC (LQC), middle QC (MQC) and high QC (HQC), respectively.

Sample Extraction Procedure

From the separated plasma, 250 µL of sample was taken and 500 µL of 0.5 % formic acid was added to it. Cartridges (Rida, C₁₈) were used, the cartridges was pre-conditioned

with 5 mL methanol and 5 mL of Milli Q water. The sample was then loaded into the cartridge, thereafter the cartridge was washed with 5 mL of water. Finally, the sample was eluted with 5 mL methanol containing 5 % formic acid. Then the sample was made evaporate up to dryness under nitrogen and reconstituted with 1 mL of 16 % acetonitrile in 0.1 % formic acid in water and was transferred to a sample vial for LCMS and LCMS/MS analysis.

Method Validation

Selectivity and Specificity

ANG-II is an endogenous compound present in humans, as a part of specificity studies plasma devoid of ANG-II was obtained from patients suffering from severe hypotensive disorders which are having no circulating ANG-II in them. The obtained plasma sample was processed by the liquid–liquid extraction procedure. The samples were chromatographed to determine to which extent endogenous plasma components may contribute to the peak interference at retention times of ANG-II. Also the eqi-volume of spiked plasma of standard ANG-III and ANG-III (IS) represent the exact location of peaks and ratio of extracted ANG-III from plasma with this methodology.

Linearity

Calibration curves were prepared by adding a known amount of ANG-II as well as ANG-III, IS (5, 10, 25, 50, 100 and 200 ng/mL) to 0.5 mL of blank plasma. The samples were extracted as described previously. The standard curves were constructed by plotting the peak area ratio of ANG-II concentration and of ANG-III (IS) upon X and Y-axis concentration ranges. Linearity was assessed by observing the values of coefficient of correlation (r^2) of linearity plots, nearness of values near to 1 suggested linearity of responses. Also, the linearity of each standard curve was confirmed by plotting the peak area of ANG-II. The unknown sample concentrations were calculated from the weighted ($1/x^2$) linear regression analysis of the standard curve.

Precision and Accuracy

Inter and intra-day precision studies were done by injecting QC dilutions (5, 50 and 200 ng/mL) as described earlier ($n = 12$) in developed chromatographic method. Peak areas were calculated for %RSD values.

Extraction Recovery

To calculate recovery of the extraction procedure, 12 replicates of QC samples of ANG-II (5, 50 and 200 ng/mL) were extracted and analyzed. The peak area was compared with the same concentrations of unextracted standards of ANG-II reconstituted in methanol.

Stability

In bench-top stability, 12 replicates of LQC and HQCs of ANG-II (5 and 200 ng/mL) were analyzed at 0 and 6 h at room temperature and the deviation was calculated. In freeze–thaw stability, 12 replicates of LQC and HQC samples of ALD were prepared, frozen at -20°C and analyzed after two and three freeze–thaw cycles. Long-term stability was examined for 14 days by taking 12 replicates of HQC and LQC samples. The mean concentration was taken into consideration, which was compared with zero-day sample concentration.

LOD and LOQ

The limits of detection (LOD) and limits of quantification (LOQ) were determined by injecting progressively low concentrations of the standard solution under the chromatographic conditions. LOD and LOQ were calculated directly from the calibration plot. LOD and LOQ were calculated as 1.3 and $10 \sigma/S$, respectively, where σ is the standard deviation (SD) of intercept and S is the slope of the calibration plot. The LOD was defined as a signal-to-noise (S/N) ratio of 3:1 and the LOQ was defined as an S/N ratio of 10.

Study Design

The study on human volunteers was carried under approval of ethics committee “HURIP Independent Bioethics”, Ibrahimpur Road, Kolkata, India. The study was performed along with patients consent and under supervision of Doctor. Twelve hypertensive patients aged between 21 and 30 years, non-infected, not under any antibiotics, steroid and other medicines therapy for a month, except specific cardiovascular drug provided by experts for this study. The patients were routinely supervised and also prescribed with single category of medicines, i.e., OLM and atorvastatin (ATVS), according to their therapeutic regimens.

The collection of blood plasma was basically done three times per individual for 7–14 days in first stage without any drug administration. Secondly, along with combination of OLM + ATVS and thirdly with only single

hypertensive drug, i.e., OLM. First stage was for 1 week in which the patients were devoid of any antihypertensive drugs. The patients were evaluated for demography and measurement of blood pressure (BP) followed by withdrawal of blood to quantify the levels of ANG-II.

Results and Discussion

Method Validation

Selectivity and Specificity (Matrix Effect)

Blank plasma obtained from patients suffering from severe hypotension was used for studies. The plasma was used to mark any endogenous interference. A representative chromatogram of the plasma blank is shown in Fig. 1. No additional peaks of endogenous substances were observed. Figure 2 shows the chromatograms of calibration standard containing ANG-II spiked in plasma with ANG-III (IS).

Fig. 1 LCMS/MS trace of angiotensin-II in human plasma (blood plasma sample) of hypotensive patient

Fig. 2 LCMS/MS trace of angiotensin-II spiked in human plasma (Quality control standard: 5 ng/mL)

Linearity

Linear calibration curves (Fig. 4) with correlation coefficients near to 0.99806448 were obtained over the concentration range of ANG-II (5, 10, 25, 50, 100 and 200 ng/mL) to 0.5 mL of blank plasma. The coefficient of regression, i.e., $r^2 = 0.998$ was obtained indicating linearity of results and an excellent correlation between peak area ratio for each concentration of ANG-II. The chromatograms of peaks area of ANG-II and ANG-III on concentration 5, 10, 25, 50, 100 and 200 ng/mL (Fig. 3) as comparative peak area ratio. The plasma spiked ANG-II (std) and ANG-III (IS) calibrated curve (Fig. 5) represents regression line linearity equation of $y = 0.985x + 0.861$ and coefficient of regression $r^2 = 0.999$, which is also an excellent correlation between area ratio of ANG-II (std) and ANG-III (IS) linearity.

Accuracy and Precision

Method performance was evaluated as accuracy and precision as shown in the Tables 2 and 3, determined by 12

Fig. 3 Chromatograms of Standard (angiotensin-II) versus Internal standard (angiotensin-III) as area peak ratio

Fig. 4 Linearity curve plotting the peaks area of standard Angiotensin-II in response versus retention time (min) graph

Fig. 5 Linearity curve plotting the peak area ratio of Angiotensin-II concentration and angiotensin-III (IS)

Table 2 Accuracy study of developed method for ANG-II in human plasma

Sr. no.	Quality control samples of ANG-II	Recovered amount (ng/mL)	Accuracy (%)
1	LQC	4.53 ± 1.3	90.6
2	MQC	47.43 ± 4.2	94.86
3	HQC	194.26 ± 9.9	97.13

replicate analyses for ANG-II at three concentration levels, i.e., LQC (5 ng/mL), MQC (50 ng/mL) and HQC (200 ng/mL), each on the same analytical run. Inter-assay precision and accuracy were calculated after repeated analysis in three different analytical runs. Results concluded the repeatability of the method, including both sample processing and chromatographic measurement. Recovery results were subjected statistically analysis and %RSD were recorded. The %RSD is a ratio of SD to mean in percent. %RSD values were small indicating good accuracy of results. Inter-day and intra-day results also were good as the %RSD values were low.

Table 3 Precision study of developed method for ANG-II in human plasma (inter and intraday)

Sr. no.	Quality control samples of ANG-II	Inter-day precision		Intra-day precision	
		Recovered amount (ng/mL)	%RSD	Recovered amount (ng/mL)	%RSD
1	LQC	4.42 ± 0.91	1.86	48.82 ± 0.93	1.60
2	MQC	47.66 ± 3.5	1.62	196.1 ± 3.2	1.73
3	HQC	194.40 ± 11.66	1.68	792.3 ± 14.2	1.90

Extraction Recovery

Extraction recovery of ANG-II was determined by comparing peak areas obtained from extracted plasma samples with those found by extracting blank matrices through the extraction procedure and spiking with a known amount of ANG-II. The results showed that the mean extraction recovery of ANG-II was >85 % at three

different QC like LQC, MQC, HQC, respectively (Table 4).

Averaged for 12 measurements at each concentration

level ($n = 12$);

$$\% \text{ Recovery} = (\text{response of extracted spike}) / (\text{response of post-extracted spike}) \times 100.$$

Stability

In bench-top stability, six replicates of LQC and HQCs of ANG-II (5 and 200 ng/mL) analyzed at 0 and 6 h at room

temperature resulted in recovery in acceptable ranges, at 0 h the recovery of ANG-II from plasma was 95.6 % for LQC and 101.2 % for HQC, whereas after 6 h the extraction recovery of ANG-II was 92.5 % for LQC and 98.2 % for HQC. The recovery of ANG-II for freeze–thaw stability studies was found to be within the limits as per the guidelines. For LQC sample mean recovery for second and third freeze–thaw stability cycle were 96.9 and 94.8 %, respectively, for HQC samples the mean recoveries for second and third freeze–thaw stability cycle were 102.3 and 101.6 %, respectively which were well inside the acceptable ranges as per the guidelines (i.e., ± 15 %). Long term stability results for extraction recovery of LQC and HQC samples resulted in acceptable recoveries concluding the method suitable to stability studies for long periods.

Sensitivity Studies (LOD and LOQ)

The method was confirmed for sensitivity by estimating LOD and LOQ. The method exhibited excellent sensitivity

Table 4 The percentage extraction recovery of ANG-II from plasma

Sr. no.	Quality control samples of ANG-II	Mean % recovery
1	LQC	93.1
2	MQC	91.7
3	HQC	88.2

Table 5 The effect of different Antihypertensive treatment over the blood pressure (systolic/diastolic) of hypertensive human volunteers

Sr. no.	Patients gender	Age	Body weight	Body height (ft)	Blood pressure (systolic and diastolic pressure) measurement and blood collection (4 mL) in three stages		
					Without drug 124/82 (Control)	ATVS + OLM 122/82 (Control)	OLM 120/79 (Control)
1	Male	28	84	5.8	176/117	169/113	160/110
2	Female	29	65	5.3	182/123	175/120	164/112
3	Male	23	83	5.7	185/126	180/119	168/102
4	Male	27	86	5.9	165/119	162/115	156/104
5	Male	27	79	5.6	171/128	167/122	161/111
6	Male	26	81	5.6	157/110	150/106	143/100
7	Female	32	82	5.8	179/119	170/110	145/89
8	Male	29	77	5.7	184/130	173/124	152/112
9	Male	26	73	5.6	174/121	170/115	147/92
10	Male	28	85	5.10	169/118	166/114	156/100
11	Female	28	66	5.2	172/122	165/112	142/88
12	Female	30	69	5.5	177/125	168/117	137/113
13	Male	33	65	5.4	172/119	166/110	149/104
14	Male	28	66	5.3	186/123	169/114	151/102
15	Male	29	72	5.8	182/131	166/119	146/90
16	Male	32	69	5.2	163/116	161/110	151/89
17	Male	32	80	5.9	179/131	170/125	166/112
18	Female	33	65	5.1	183/134	175/131	162/107
19	Male	30	69	5.5	179/122	176/114	156/85
20	Female	26	62	4.9	165/125	159/120	150/100
Mean $\pm$ SEM			78.27 $\pm$ 2.21	5.57 $\pm$ 0.0721	175 $\pm$ 1.81/ 122.95 $\pm$ 1.33	167.85 $\pm$ 1.50/ 116.50 $\pm$ 1.33	153.10 $\pm$ 1.91**/ 101.12 $\pm$ 2.09**

Values are mean $\pm$ SEM ($n = 20$)

** $P < 0.0001$ when compared to without drug treated condition

Table 6 The effect of different antihypertensive treatment stage over the plasma concentration of ANG-II of hypertensive human volunteers

Sr. no.	Plasma concentration of ANG-II of 12 patients in three different stages of treatment		
	Without drugs Concentration (ng/mL)	ATVS + OLM Concentration (ng/mL)	OLM Concentration (ng/mL)
1	4.2145	10.4955	17.8535
2	4.3112	10.5821	17.4221
3	4.3873	10.8735	17.5532
4	4.5101	10.6655	17.6391
5	4.4552	10.5151	17.5001
6	4.2999	10.7213	17.7632
7	4.6501	10.2251	17.9531
8	4.5211	10.5189	17.9993
9	4.5982	10.3944	17.7845
10	4.3654	10.4381	17.7439
11	4.5411	10.6334	17.7569
12	4.2546	10.2422	17.6975
13	4.6623	10.9232	17.3992
14	5.2321	10.1222	17.0211
15	4.2192	11.0019	17.5111
16	5.0025	10.1010	16.2394
17	4.9332	10.4810	16.9784
18	4.2326	10.3458	17.8659
19	4.3321	11.2381	18.6681
20	4.1113	10.9123	17.9231
Mean $\pm$ SEM	4.4917 $\pm$ 0.06519	10.5715 $\pm$ 0.06820***	17.61363 $\pm$ 0.10812***

Values are in mean $\pm$ SEM
($n = 20$)

*** $P < 0.0001$ when
compared to without drug
treated condition

by demonstrating LOD of 0.16 ng/mL and LOQ of 0.520 ng/mL.

Application of the Analytical Method in Pharmacokinetic Studies

The developed LCMS/MS method for quantification of ANG-II in plasma was applied for the drug interaction study in hypertensive patients under the antihypertensive drug therapy. Blood was collected after dosing thereafter immediately the BP was observed in lying posture for same individual patient in three stages and similarly with rest of the volunteer (patients). Table 5 shows that there were significant reduction ($P < 0.001$) in the BP of volunteers with OLM when compared to without treatment stage.

The plasma concentration of ANG-II was calculated in the hypertensive human volunteers by using given developed method. There were significant decrease ($P < 0.001$) in the level of ANG-II in ATVS + OLM treated stage when compared to the without treatment stage in human volunteer. Whereas the concentration of ANG-II in the OLM treated stage was lower than the detection limit as shown in the Table 6.

Conclusion

Present study was undertaken to develop a sensitive method for quantification of ANG-II in human plasma. The developed LCMS/MS method was validated in accordance to guidelines and confirmed to be specific, selective, linear, accurate, precise and sensitive. The method exhibited acceptable recovery from spiked plasma samples also the method was proved to be sensitive demonstrating excellent LOD and LOQ levels of 0.16 and 0.520 ng/mL, respectively.

The validated sensitive method was applied for pharmacokinetic studies to quantify the levels of ANG-II in human volunteers under therapy of ATVS, OLM and combinations of both. The study concluded that the concentration of ANG-II in human volunteers treated with ATVS + OLM was higher than the OLM treated hypertensive human volunteer also the demographic characters revealed decrease in BP of OLM treated patients as compared to ATVS + OLM treated ones. Thus, the present study concludes failure of combination therapy for fulfilling the objective of synergistic activity in treating ANG-II mediated BP as cardiovascular therapeutics, rather than the individual drug treatment (Fig. 6).

Fig. 6 Chromatograms of recovered area peaks versus retention times 12 patient volunteers

Fig. 6 continued

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Conflict of interest None of the authors has, any conflict of interest for this work and manuscript publication.

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