

# Method development and validation of liquid chromatography-tandem/mass spectrometry for aldosterone in human plasma: Application to drug interaction study of atorvastatin and olmesartan combination

Rakesh Das, Subhasis Dan,  
Tapan Kumar Pal

Department of Pharmaceutical  
Technology, Bioequivalence Study  
Center, Jadavpur University, Jadavpur,  
Kolkata, West Bengal, India

*J. Adv. Pharm. Technol. Res.*

## ABSTRACT

In the present investigation, a simple and sensitive liquid chromatography-tandem mass spectrometry (LC/MS/MS) method was developed for the quantification of aldosterone (ALD) a hormone responsible for blood pressure in human plasma. The developed method was validated and extended for application on human subjects to study drug interaction of atorvastatin (ATSV) and olmesartan (OLM) on levels of ALD. The ALD in plasma was extracted by liquid-liquid extraction with 5 mL dichloromethane/ethyl ether (60/40% v/v). The chromatographic separation of ALD was carried on Xterra, RP-Column C18 (150 mm × 4.6 mm × 3.5 μm) at 30°C followed by four-step gradient program composed of methanol and water. Step 1 started with 35% methanol for first 1 min and changed linearly to 90% in next 1.5 min in Step 2. Step 3 lasted for next 2 min with 90% methanol. The method finally concluded with Step 4 to achieve initial concentration of methanol that is, 35% thus contributing the total method run time of 17.5 min. The flow rate was 0.25 mL/min throughout the process. The developed method was validated for specificity, accuracy, precision, stability, linearity, sensitivity, and recovery. The method was linear and found to be acceptable over the range of 50-800 ng/mL. The method was successfully applied for the drug interaction study of ATSV + OLM in combination against OLM treatment on blood pressure by quantifying changes in levels of ALD in hypertensive patients. The study revealed levels of ALD were significantly higher in ATSV + OLM treatment condition when compared to OLM as single treated condition. This reflects the reason of low effectiveness of ATSV + OLM in combination instead of synergistic activity.

**Key words:** Aldosterone, atorvastatin, drug interaction, liquid chromatography-tandem mass spectrometry, olmesartan

## Address for correspondence:

Prof. Tapan Kumar Pal,  
Department of Pharmaceutical Technology, Bioequivalence  
Study Center, Jadavpur University, Kolkata - 700 032,  
West Bengal, India.  
E-mail: tkpal12@gmail.com

## Access this article online

Quick Response Code:



Website:

www.japtr.org

DOI:

10.4103/2231-4040.137402

## INTRODUCTION

Primary aldosteronism (PAL) is a specifically treatable and potentially curable form of hypertension. The exact measurement of circulating aldosterone (ALD) is essential for the correct diagnosis of PAL. The importance of ALD measurement has greatly increased with the recent recognition that PAL is a more frequent cause of hypertension.<sup>[1]</sup> The current mainstay for measuring ALD is by antibody-based methods. These methods can be direct or require initial extraction of plasma or serum using liquid-liquid extraction, solid-phase extraction, or chromatographic methods.<sup>[2-6]</sup> Overall, immunoassays have problems with varying selectivity and poor interlaboratory

reproducibility, requiring each laboratory to establish its own reference range for the diagnosis of PAL.<sup>[7]</sup> For example, Schirpenbach *et al.*<sup>[8]</sup> recently reported a 2- to 3-fold difference in ALD concentrations measured by 4 currently used methods. Such discrepancies in ALD measurement between laboratories suggest a need for improved ALD measurement for both screening and confirmation of PAL.<sup>[9,10]</sup>

Gas chromatograph-mass spectrometer (GC-MS) has been used to measure ALD in biological fluids.<sup>[11-13]</sup> Although this technique is considered a reference method that provides both accurate results and excellent specificity, these methods in general require extensive sample preparation, including chemical derivatization. The lack of automation and complexity of sample preparation has relegated GC-MS to specialty clinical laboratories and is not used in routine clinical services.

High-performance liquid chromatography (HPLC)-MS/MS is a powerful analytical technique that is, becoming increasingly used in the clinical setting.<sup>[14]</sup> HPLC-MS/MS offers the opportunity to provide more reliable measurement of ALD than immunoassays.<sup>[15]</sup> Of late, it has been shown that online solid-phase extraction coupled to HPLC-MS/MS was suited to plasma free metanephrine analysis and the diagnosis of pheochromocytoma.<sup>[16]</sup> Using this general approach, we report the development and validation of an HPLC-MS/MS method of measuring ALD that uses online semi-automated sample preparation. The given research study deals with the development and validation of a method for the determination of ALD in human plasma and apply the given method for drug interaction study in hypertensive patient.

## MATERIALS AND METHODS

### Chemicals and reagents

The standard powder of 5 mg ALD (A-9477) with internal standard (IS) ALD d-7 was procured from Sigma Aldrich. Dichloromethane, diethyl ether, methanol were of HPLC grade and were procured from Merck (Darmstadt, Germany). HPLC-grade organic solvents were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade. Distilled water was prepared as required using an aqua MAX ultra, Younglin Instrument Co., Ltd. 60, Anyangcheondong-ro, Dongan-gu, Anyang-si, Gyeonggi-do, 431-836, The Republic of Korea, ultrawater purification system.

### Instrumentation, chromatographic conditions and (MS)/MS condition

#### Instrumentation

The HPLC system consisted of Waters Alliance 2695 low pressure gradient separation module (Waters, Milford, MA) with the configuration of quaternary solvent delivery pump,

sampler cooler, column heater. Mass spectra data were acquired using a Micromass ZQ mass detector (Micromass, Ltd., United Kingdom).

#### Chromatographic conditions

The chromatographic separation of ALD was carried on Xterra, RP-Column C18 (150 × 4.6 × 3.5 μm) at 30°C. Optimized method was a four-step gradient program composed of methanol and water. Whole chromatographic separation was carried at flow rate of 0.25 ml/min, Step 1 started with 35% methanol for first 1 min and changed linearly to 90% in next 1.5 min in Step 2. Step 3 lasted for next 2 min with 90% methanol. The method finally concluded with Step 4 to achieve initial concentration of methanol that is, 35% thus contributing the total method run time of 17.5 min. The sample volume was 20 μL as shown in Table 1.

#### Mass spectrometry/MS conditions

Mass spectrometry/MS detector was a Micromass ZQ mass detector (Micromass, Ltd., United Kingdom). The column was directly connected to the electrospray ionization probe operating at 350°C (disolvation temperature was 348-350°C and source temperature was 119-120°C). The sample temperature was 20°C with sample volume of 20 μL.

### Preparation of calibration standards and quality control samples

The calibration standards were prepared by spiking human plasma with standard ALD with corresponding IS (ALD-d-7) to obtain resultant concentrations of 50, 100, 200, 400, and 800 ng/mL, respectively. The developed method was validated using three different quality control samples by spiking ALD in human plasma at 50, 200, and 800 ng/mL to represent low quality control (LQC), middle and high quality controls (HQC), respectively.

### Sample extraction procedure

Sample extraction was done by liquid-liquid extraction using dichloromethane and ethyl ether in the ratio 60:40. For the same 1 mL of collected frozen plasma was added with 500 mL methanol/L of water was added before extraction. Thereafter, the extraction was carried out with 5 mL dichloromethane/ethyl ether (60/40) in a multi-tube vortexer. After centrifugation the upper organic phase was

**Table 1: Gradient program of mobile phase for the determination of aldosterone by liquid chromatography-tandem mass spectrometry**

Steps	Methanol	Water	Time (min)	Flow rate (mL/min)
1	35	65	0-1	0.25
2	90	10	1-2.5	0.25
3	90	10	2.5-4.5	0.25
4	35	65	4.5-17.5	0.25

separated out in a conical flux and allowed to pass through nitrogen gas. Finally, the residue was dissolved in 350  $\mu$ L of 350 mL/L methanol/water.

### Method validation

#### *Selectivity and specificity*

Aldosterone is an endogenous compound present in humans, as a part of specificity studies plasma devoid of ALD was obtained from patients suffering from Addison's disease which are having no circulating ALD 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 ALD. Furthermore, the eqi-volume of spiked plasma of standard ALD and aldo d-7 (IS) represent the exact location of peaks and ratio of extracted ALD from plasma with this methodology.

#### *Linearity*

Calibration curves were prepared by adding a known amount of ALD as well as aldo d-7, IS (50, 100, 200, 400, and 800 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 ALD Standard concentration and of ALD d-7 (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 one suggested linearity of responses. Furthermore, the linearity of each standard curve was confirmed by plotting the peak area of ALD. 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 quality control dilutions (50, 200 and 800 ng/mL) as described earlier ( $n = 12$ ) in developed chromatographic method. Peak areas were calculated for % relative standard deviation (RSD) values.

#### *Extraction recovery*

To calculate recovery of the extraction procedure, 12 replicates of quality control samples of ALD (50, 200, and 800 ng/mL) were extracted and analyzed. The peak area was compared with the same concentrations of unextracted standards of ALD reconstituted in methanol.

#### *Stability*

In bench-top stability, twelve replicates of low and HQCs of ALD (50 and 800 ng/mL) were analyzed at 0 and 6 h at room temperature and the deviation was calculated. In freeze-thaw stability, twelve replicates of LQC and HQC samples of ALD were prepared, frozen at  $-20^\circ\text{C}$  and analyzed after two and three freeze-thaw cycles.

Long-term stability was examined for 14 days by taking twelve replicates of HQC and LQC samples. The mean concentration was taken into consideration, which was compared with 0-day sample concentration.

#### *Limits of detection and limits of quantification*

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  $3.3 \sigma/S$  and  $10 \sigma/S$ , respectively, where  $\sigma$  is the standard deviation 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, noninfected, 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 that is, olmesartan (OLM) and atorvastatin (ATSV), according to their therapeutic regimens.

The collection of blood plasma was basically done 3 times per individual for 7-14 days-in first stage without any drug administration. Second, along with combination of OLM + ATVS and third with only single hypertensive drug that is, 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 BP followed by withdrawal of blood to quantify the levels of ALD.

## RESULTS AND DISCUSSIONS

### Method validation

#### *Selectivity and specificity (matrix effect)*

Blank plasma obtained from patients suffering from Addison's disease was used for studies. The plasma was used to mark any endogenous interference. A representative chromatogram of the plasma blank is shown in Figure 1a. No additional peaks of endogenous substances were observed. Figure 1b shows the chromatograms of calibration standard containing ALD spiked in plasma with aldosterone d-7 (IS).

#### *Linearity*

Linear calibration curves Figure 2a with correlation

coefficients near to 0.999399 were obtained over the concentration range of ALD (50, 100, 200, 400, and 800 ng/mL) to 0.5 mL of blank plasma. The coefficient of regression that is,  $r^2 = 0.998$  was obtained indicating linearity of results and an excellent correlation between peak area ratio for each concentration of ALD. The plasma spiked ALD standard versus ALD-d7 (IS) calibrated curve Figure 2b represents regression line linearity equation of  $y = 2.7917837018877 + 1.0014117703309x$ .

**Table 2: Accuracy study of developed method for aldosterone in human plasma**

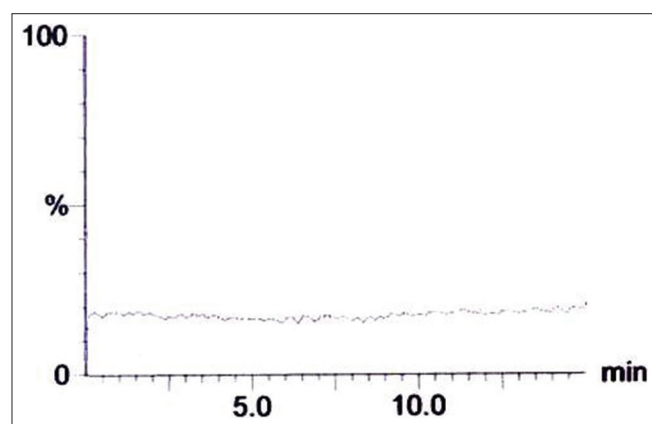
Quality control samples of ALD	Recovered amount (ng/mL)	Accuracy (%)
LQC	48.2±1.2	96.4
MQC	195.8±3.1	97.9
HQC	789.8±12.4	98.73

LQC: Low quality control, MQC: Middle quality control, HQC: High quality control

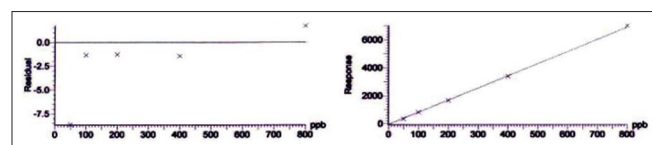
**Table 3: Precision study of developed method for aldosterone in human plasma (inter- and intra-day)**

Quality control samples of ALD	Inter-day precision		Intra-day Precision	
	Recovered amount (ng/mL)	% RSD	Recovered amount (ng/mL)	% RSD
LQC	47.90±0.86	1.79	48.82±0.93	1.90
MQC	194.6±3.7	1.90	196.1±3.2	1.63
HQC	790.00±13.9	1.75	792.3±14.2	1.79

LQC: Low quality control, MQC: Middle quality control, HQC: High quality control, ALD: Aldosterone



**Figure 1a:** Liquid chromatography-tandem mass spectrometry trace of without aldosterone spike to human plasma (quality control standard: 50 ng/mL)



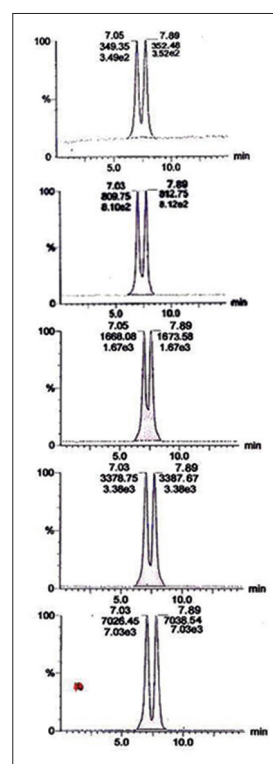
**Figure 2a:** Standard curve of calibrators' concentration of aldosterone

**Accuracy and precision**

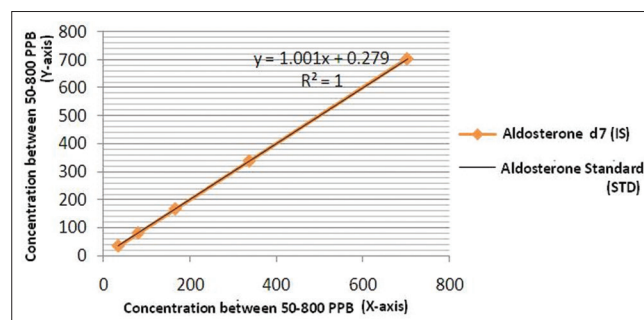
Method performance was evaluated as accuracy and precision as shown in Tables 2 and 3, determined by 12 replicate analyses for ALD at three concentration levels that is, LQC (50 ng/mL), MQC (200 ng/mL) and HQC (800 ng/mL), each on the same analytical run.

**Stability**

In bench-top stability, six replicates of LQC and HQCs of ALD (50 and 800 ng/mL) analyzed at 0 and 6 h at room temperature resulted in recovery in acceptable ranges, at 0 h the recovery of ALD from plasma was 96.5% for LQC and 103.1% for HQC, whereas after 6 h the extraction recovery of ALD was 93.4% for LQC and 100.2% for HQC. For LQC sample mean recovery for second and third freeze-thaw



**Figure 1b:** Liquid chromatography-tandem mass spectrometry trace of standard aldosterone (ALD) and ALD-d7 (internal standard) spiked in human plasma (blanked plasma sample)



**Figure 2b:** Linearity curve plotting the peak area ratio of aldosterone (ALD)-d7 (internal standard) versus ALD (standard) concrete

stability cycle were 98.3% and 96.8%, respectively, for HQC samples the mean recoveries for second and third freeze-thaw stability cycle were 105.2% and 102.8%, respectively, which were well inside the acceptable ranges as per the guidelines (i.e.  $\pm 15\%$ ). The method exhibited excellent sensitivity by demonstrating LOD of 0.13 ng/mL and LOQ of 0.432 ng/mL.

## DISCUSSION

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 standard deviation to mean in percent. %RSD values were small indicating good accuracy of results. Inter- and intra-day results also were good as the %RSD values were low [Table 4]. The recovery of ALD for freeze-thaw stability studies was found to be within the limits as per the guidelines. Long-term stability results for extraction recovery of LQC and HQC samples resulted in acceptable recoveries concluding the method suitable to stability

**Table 4: The percentage extraction recovery of aldosterone from plasma**

Quality control samples of ALD	Mean % recovery
LQC	91.2
MQC	93.4
HQC	89.7

LQC: Low quality control, MQC: Middle quality control, HQC: High quality control, ALD: Aldosterone

**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	Blood pressure (systolic and diastolic pressure) measurement in three stages		
					Without drug	ATVS+OLM	OLM
					124/82 (control)	122/82 (control)	120/79 (control)
1	Male	28	84	5.8 m	176/117	169/113	160/110
2	Female	29	65	5.3 m	182/123	175/120	164/112
3	Male	23	83	5.7 m	185/126	180/119	168/102
4	Male	27	86	5.9 m	165/119	162/115	156/104
5	Male	27	79	5.6 m	171/128	167/122	161/111
6	Male	26	81	5.6 m	157/110	150/106	143/100
7	Female	32	82	5.8 m	179/119	170/110	145/89
8	Male	29	77	5.7 m	184/130	173/124	152/112
9	Male	26	73	5.6 m	174/121	170/115	147/92
10	Male	28	85	5.10 m	169/118	166/114	156/100
11	Female	28	66	5.2 m	172/122	165/112	142/88
12	Female	30	69	5.5 m	177/125	168/117	137/113
Mean $\pm$ SEM			78.27 $\pm$ 2.21	5.57 $\pm$ 0.0721	174.25 $\pm$ 2.35 /121.5 $\pm$ 1.57	167.91 $\pm$ 2.134 /115.58 $\pm$ 1.484	152.58 $\pm$ 2.82** /102.75 $\pm$ 2.66**

Foot notes: Values are Mean $\pm$ SEM ( $n=12$ ), \*\* $P<0.0001$  when compared to without drug treated condition. ATVS: Atorvastatin, OLM: Olmesartan, SEM: Standard error mean, ND: Not detected

studies for long periods. The method was confirmed for sensitivity by estimating LOD and LOQ. The method exhibited excellent sensitivity by demonstrating LOD of 0.13 ng/mL and LOQ of 0.432 ng/mL.

The developed liquid chromatography (LC)/MS/MS method for quantification of ALD 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 blood pressure 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 reductions ( $P < 0.001$ ) in the blood pressure of volunteers with OLM when compared to without treatment stage.

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

## CONCLUSION

This study was undertaken to develop a sensitive method for quantification of ALD in human plasma. The developed LC/MS/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 0.13 ng/mL and 0.432 ng/mL, respectively.

The validated sensitive method was applied for pharmacokinetic studies to quantify the levels of ALD in human volunteers under therapy of ATSV, OLM, and combinations of both. The study concluded that the concentration of ALD in human volunteers treated

with ATSV + OLM was higher than the OLM treated hypertensive human volunteer also the demographic characters revealed decrease in blood pressure of OLM treated patients when compared to ATSV + OLM treated ones. Thus, this study concludes failure of combination therapy for fulfilling the objective of synergistic activity in treating ALD mediated blood pressure as cardiovascular therapeutics, rather than the individual drug treatment [Figure 3].

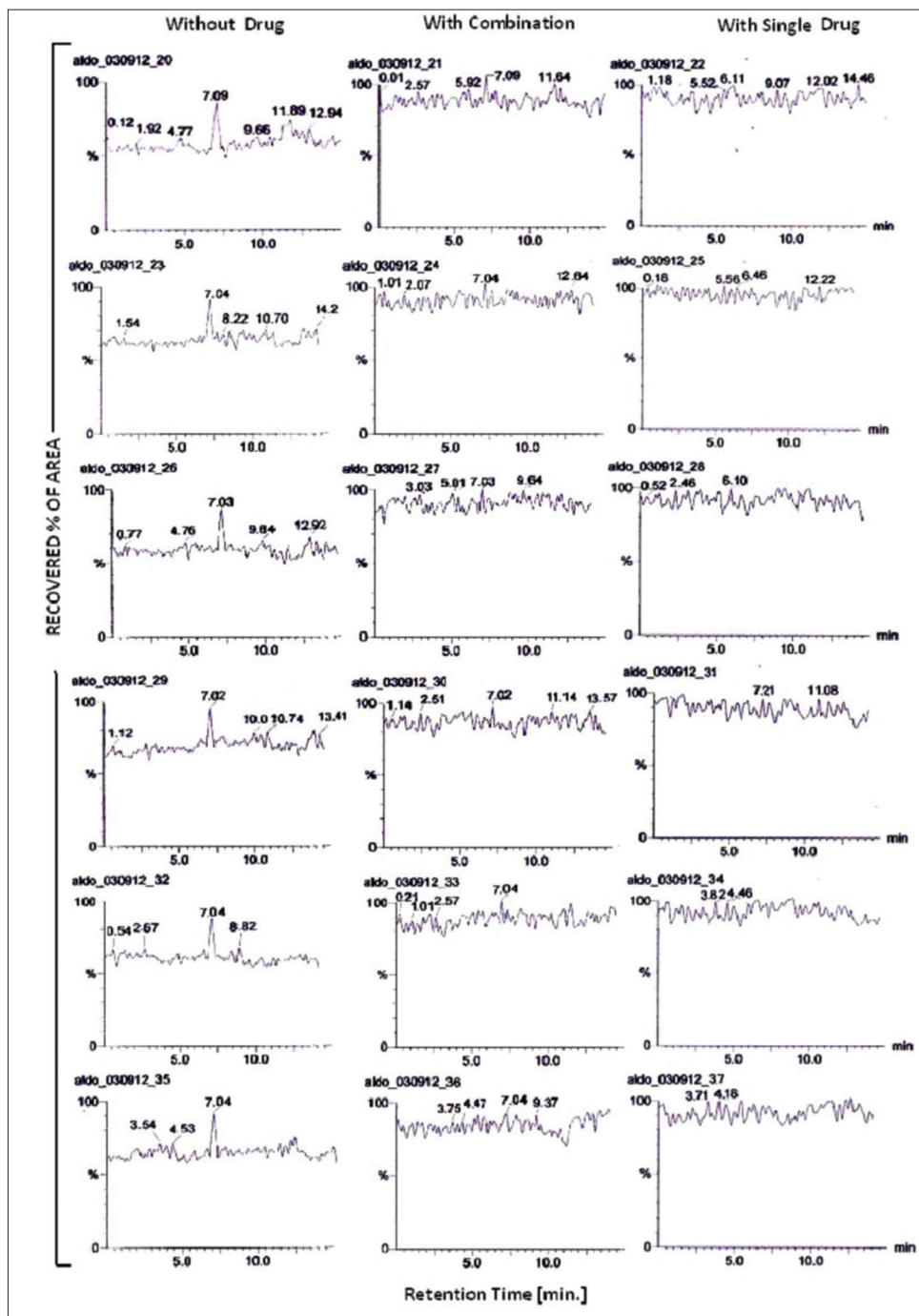


Figure 3: Chromatograms of 12 blood plasma aldosterone trace in three different stages

**Table 6: The effect of different antihypertensive treatment stage over the plasma concentration of aldosterone of hypertensive human volunteers**

Sr. no.	Plasma concentration of ALD of twelve patients in three different stages of treatment (conc. in ng/mL)		
	Without drugs	ATSV+OLM	OLM
1	52.91	10.64	ND
2	52.94	10.72	ND
3	53.031	10.59	ND
4	52.66	10.57	ND
5	52.92	10.75	ND
6	66.62	13.57	ND
7	66.82	13.21	ND
8	67.22	14.75	ND
9	67.02	13.91	ND
10	66.63	13.56	ND
11	68.82	13.89	ND
12	56.21	13.30	ND
Mean±SEM	60.3167±2.095	12.455±0.4722***	ND

ATVS: Atorvastatin, OLM: Olmesartan, SEM: Standard error mean, ND: Not detected

## REFERENCES

- Mulatero P, Stowasser M, Loh KC, Fardella CE, Gordon RD, Mosso L, *et al.* Increased diagnosis of primary aldosteronism, including surgically correctable forms, in centers from five continents. *J Clin Endocrinol Metab* 2004;89:1045-50.
- Ekins RP, Newman GB, Piyasena R, Banks P, Slater JD. The radioimmunoassay of aldosterone in serum and urine: Theoretical and practical aspects. *J Steroid Biochem* 1972;3:289-304.
- Demers LM, Sampson E, Hayes AH Jr. Plasma and urinary aldosterone measurement in healthy subjects with a radioimmunoassay kit not requiring chromatography. *Clin Biochem* 1976;9:243-6.
- Kubasik NP, Warren K, Sine HE. Evaluation of a new commercial radioassay kit for aldosterone using an iodinated tracer. *Clin Biochem* 1979;12:59-61.
- Hanquez C, Rajkowski KM, Desfosses B, Cittanova N. A competitive microtitre plate enzyme immunoassay for plasma aldosterone using a monoclonal antibody. *J Steroid Biochem* 1988;31:939-45.
- Brochu M, Féthière J, Roy M, Ong H, De Léan A. Highly sensitive and rapid radioimmunoassay for aldosterone in plasma and cell culture medium. *Clin Biochem* 1989;22:289-92.
- Plouin PF, Jeunemaitre X. Would wider screening for primary aldosteronism give any health benefits? *Eur J Endocrinol* 2004;151:305-8.
- Schirpenbach C, Seiler L, Maser-Gluth C, Beuschlein F, Reincke M, Bidlingmaier M. Automated chemiluminescence-immunoassay for aldosterone during dynamic testing: Comparison to radioimmunoassays with and without extraction steps. *Clin Chem* 2006;52:1749-55.
- Gordon RD. The challenge of more robust and reproducible methodology in screening for primary aldosteronism. *J Hypertens* 2004;22:251-5.
- Stowasser M, Gordon RD. Aldosterone assays: an urgent need for improvement. *Clin Chem* 2006;52:1640-2.
- Breuer H, Siekmann L. Mass fragmentography as reference method in clinical steroid assay. *J Steroid Biochem* 1975;6:685-8.
- Siekmann L. Determination of steroid hormones by the use of isotope dilution - mass spectrometry: A definitive method in clinical chemistry. *J Steroid Biochem* 1979;11:117-23.
- Stöckl D, Reinauer H, Thienpont LM, De Leenheer AP. Determination of aldosterone in human serum by isotope dilution gas chromatography/mass spectrometry using a new heptafluorobutyl derivative. *Biol Mass Spectrom* 1991;20:657-64.
- Taylor PJ. Chromatography mass spectrometry in the clinical laboratory. *Ther Drug Monit* 2005;27:689-93.
- Fredline VF, Taylor PJ, Dodds HM, Johnson AG. A reference method for the analysis of aldosterone in blood by high-performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry. *Anal Biochem* 1997;252:308-13.
- de Jong WH, Graham KS, van der Molen JC, Links TP, Morris MR, Ross HA, *et al.* Plasma free metanephrine measurement using automated online solid-phase extraction HPLC tandem mass spectrometry. *Clin Chem* 2007;53:1684-93.

**How to cite this article:** Das R, Dan S, Pal TK. Method development and validation of liquid chromatography-tandem/mass spectrometry for aldosterone in human plasma: Application to drug interaction study of atorvastatin and olmesartan combination. *J Adv Pharm Technol Res* 2014;5:108-14.

**Source of Support:** Nil, **Conflict of Interest:** Nil.