ORIGINAL RESEARCH ARTICLE

INTERNATIONAL JOURNAL OF BIOASSAYS
ISSN: 2278-778X
CODEN: IJBNHY
OPEN ACCESS

Development of RP-HPLC method for simultaneous estimation of Aspirin, Ramipril, Atenolol and Atorvastatin

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Received for publication: September 20, 2015; **Accepted**: October 13, 2015

Abstract: Polypill is envisaged to be a combination anti CVDs contained in the same dosage form unit. In that a polypill or Multicomponent cardiovascular pill (MCCP) is closest to 'Fixed dose combinations (FDC)' or its synonym 'Fixed dose Combination-Finished pharmaceutical product' (FDC-FPP). Developing a single analytical method for estimation of individual drug from a multidrug composition is a very challenging task. A simple, rapid, precise, and reliable reverse phase HPLC method was developed for the separation and estimation of four drugs Aspirin, Ramipril, Atenolol and Atorvastatin in cardiovascular polypill based synthetic mixture. The estimation was carried out using Inertsil ODS-3V (250 mm × 4.6 mm, 5 μm) column; mobile phase consisting of acetonitrile, methanol and buffer (pH=2.5); flow rate of 0.8 mL/min and ultraviolet detection at 225 nm. All the drugs were properly eluted within run time of 10 min with retention times about 4.1 min for Aspirin; 5.9 min for Atorvastatin; 7.2 min for Atenolol; and 8.3 min for Ramipril 8.333 min, respectively. The method was validated as a final verification of method development with respect to precision, linearity, accuracy, ruggedness, and robustness. This validated method was successfully applied to the commercially available pharmaceutical dosage form, yielding very good and reproducible result.

Key words: Simultaneous Estimation; Aspirin; Ramipril; Atenolol and Atorvastatin

INTRODUCTION

Polypill[5-6] is envisaged to be a combination anti CVDs contained in the same dosage form unit. In that respect a polypill or Multicomponent cardiovascular pill (MCCP) is closest to 'Fixed dose combinations (FDC)' or its synonym 'Fixed dose combination-Finished pharmaceutical product' (FDC-FPP). Aspirin[7] also known as acetylsalicylic acid is an analgesic, anti-pyretic, anti-rheumatic and anti-inflammatory agent. Acetyl salicylic acid's mode of action as an anti-inflammatory and anti-rheumatic agent may be due to inhibition of synthesis and release of prostaglandins. Ramipril[8] is a pro-drug belonging to the angiotensin-converting enzyme (ACE) inhibitor class of medications. Chemical name of Ramipril is (2S, 3aS, 6aS)-1-[(2S)-2-{[(2S)-1-ethoxy-1-oxo-4-phenylbutan-2-yl] amino} propanoyl]-octahydrocyclopenta[b]pyrrole-2-carboxilic acid. Atenolol[9] is a beta-blocker beta1-selective (cardioselective) adrenoreceptor blocking agent, may be chemically described as benzene acetamide, 4-[2'-hydroxy-3'-[(1-methylethyl) amino] propoxy. Atorvastatin Chemically known as (3*R*, 5*R*)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid. Atorvastatin[10], a selective, competitive hydroxyl methyl glutaryl coenzyme-A (HMG-CoA) reductase inhibitor, is used to lower serum total and LDL cholesterol, Apolipoprotein B (apoB), and triglyceride levels while increasing HDL cholesterol. High LDL-C, low HDL-C and high triglycerides concentrations in the plasma are associated with increased risk of atherosclerosis and cardiovascular disease.

A literature survey revealed that spectrophotometric, chromatographic methods have been reported for determination of Aspirin, Ramipril, Atenolol and Atorvastatin in single and combinations[11-15] with other drugs. However, there were no HPLC methods reported for simultaneous estimation of Aspirin, Ramipril, Atenolol and Atorvastatin. Analysis of Aspirin, Ramipril, Atenolol and Atorvastatin has been carried out by isocratic method, hence is simpler.

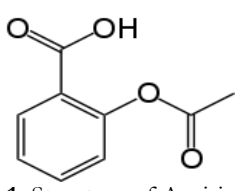

Figure 1: Structure of Aspirin

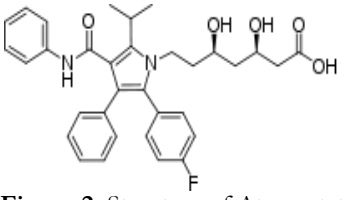

Figure 2: Structure of Atorvastatin

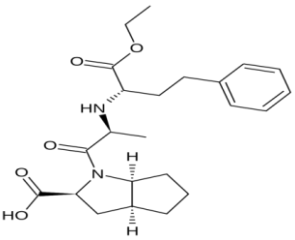

Figure 3: Structure of Ramipril

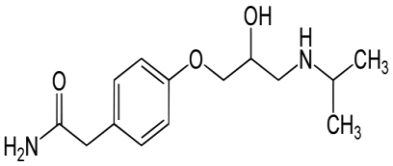

Figure 4: Structure of Atenolol

MATERIALS AND METHODS

Agilent technologies 1100 model HPLC with U.V detector and running on chemstation software equipped with a Lichrosphere 100 RP-18 reverse phase C18 column (250x4.6 mm, 5µm) was employed for the study. Sample injection was done with a Rheodyne 7725 injection valve via a 20µL loop. Detection of the drug was done by using a UV-2075 detector.

The reference samples of Aspirin, Ramipril, Atenolol and Atorvastatin were obtained from Aurobindopharma and Smilax Laboratories Limited.

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Purified water was prepared by using 0.45 Millipore Milli-Q water purification system. HPLC grade acetonitrile (Merck, Mumbai) was used for preparing the mobile phase and the diluent.

Preparation of buffer solution

0.1% orthophosphoric acid was prepared with HPLC grade water and pH was adjusted to 2.5 with triethyl amine. It was filtered through 0.45 µ Nylon membrane filter and sonicated before use of this buffer.

Preparation of standard drug solutions

Various dilutions employed in the study were made by using mobile phase as diluent. The stock solution was prepared by dissolving about 1.5gm of Aspirin, 1gm of Atenolol, 200mg Atorvastatin and 100mg of Ramipril in 10 ml of acetonitrile initially taken in a 100ml volumetric flask and the solution was sonicated for 15 minutes. The volume was made up to the mark with further quantity of acetonitrile to get 15mg/ml Aspirin, 10mg/ml Atenolol, 2mg/ml Atorvastatin and 1 mg/ml Ramipril solution. The working standard solution was made by diluting 10ml of the respective standard stock solution to 100 ml in volumetric flask to get 1500µg/ml Aspirin, 1000µg/ml Atenolol, 200µg/ml Atorvastatin and 100µg/ml Ramipril respectively. Further dilutions were made from the working standard solution in the required concentration range in 10ml volumetric flasks for the calibration curve.

Optimization of the Method

A number of eluting systems were examined for optimization of the mobile phase for separation of the drugs. Mixtures containing acetonitrile, methanol and buffer were examined at different proportions like 40:25:35, 50:15:35 and 50:25:25 (% v/v) at flow rates of 0.5ml/min to1.0 ml/min. A mixture of acetonitrile, methanol and buffer in the ratio of 50:25:25v/v provided an efficient separation of the drugs with good peak shapes and retention times. A flow rate of 0.8ml/min was found to be optimum in the range of 0.5 to 1.0 ml/min which gave retention times of 4.117min for Aspirin, 5.900min for Atorvastatin, 7.158min for Atenolol and 8.333 min for Ramipril respectively with baseline stability.

The mobile phase consisting a mixture of acetonitrile, methanol and buffer in the ratio of 50:25:25 (% v/v) respectively, was filtered through a 0.45µ membrane filter, sonicated, degassed and was then pumped from the solvent reservoir through the column at a flow rate of 0.8 ml/min. The column was maintained at a temperature of 22°C. The detection of the eluates was monitored at 225 nm and the run time was 15 min. The volume of injection was 20µl. Prior to injection of the drug solution, the column was equilibrated for at least 20 min by pumping the mobile phase through it.

Dilutions ranging from 75-1500µg/ml of Aspirin, 50-1000µg/ml of Atenolol, 10-200µg/ml Atorvastatin and 5-100µg/ml of Ramipril were prepared from the working standard solution in 10 ml volumetric flasks with the diluent. A volume of 20µl of the solution was injected into the column. The retention times and the areas under the peaks of the drugs were noted from the chromatogram obtained. The relevant calibration curves were constructed for each drug taking the concentration of the drug on X-axis and the peak area counts on the Y-axis. From the curve, the linearity was found to be in 75-1500µg/ml range for Aspirin, 50-1000µg/ml range for Atenolol, 10-200µg/ml for Atorvastatin and 5-100µg/ml range for Ramipril. The regression equation of the curve (y=mx+c) was computed. A typical chromatogram of the standard solution of the combination of the drugs is shown in figure5 (a) and 5(b).

Table 1: Optimized chromatographic conditions of the proposed method

S. No.	Parameter	Value
1	Mobile phase	Acetonitrile, methanol and buffer in the ratio of 50:25:25(%v/v).
2	Diluent	Acetonitrile, methanol and buffer in the ratio of 50:25:25(%v/v).
3	Stationary phase	Lichrosphere 100 RP-18
4	Flow rate	0.8 mL/min
5	Column temperature	22 ºC
6	Volume of injection	20µl
7	Detection wavelength (λ_{max})	225nm
8	Run time (min)	13 min
9	Retention times (min)	Aspirin 4.1; Atorvastatin 5.9;Atenolol 7.2; Ramipril 8.3min

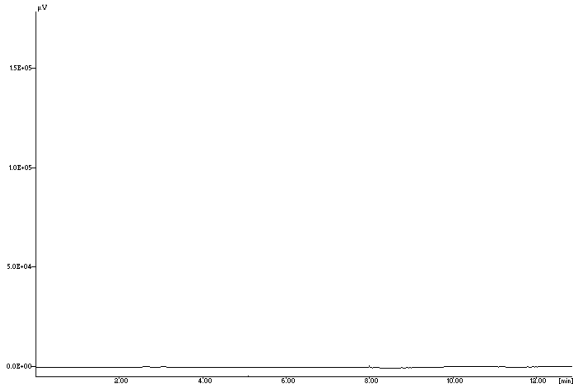

Figure 5(a): Chromatogram of Blank solution

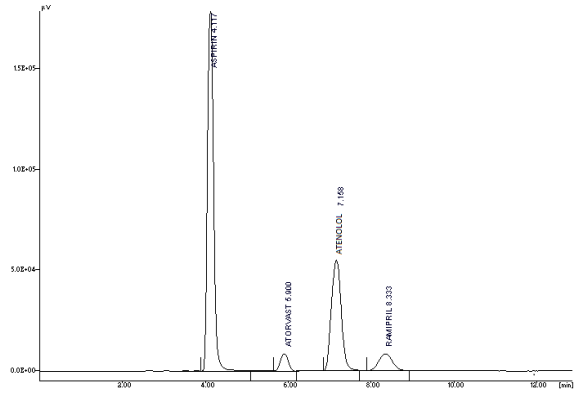

Figure 5(b): Chromatogram of standard solution of Aspirin, Atenolol, Ramipril and Atorvastatin.

Method Validation

Linearity and range: To establish linearity and range, a stock solution containing 1500µg/ml Aspirin, 1000µg/ml Atenolol, 100µg/ml Ramipril and 200µg/ml Atorvastatin were prepared using diluent (50:25:25: Aetonitrile: Methanol: Buffer) and further diluted to yield solutions in the concentration range of75-1500µg/ml, 50-

1000µg/ml, 5-100µg/ml, 10-200µg/ml of Aspirin, Atenolol, Ramipril and Atorvastatin respectively. The solutions were prepared and analyzed in triplicate. The experiment was repeated thrice by preparing different solution and analyzed by injecting 20µl in HPLC. Linearity data for Aspirin, Atenolol, Ramipril and Atorvastatin are given in the tables 2, 3, 4 and 5 respectively. Linearity plots for Aspirin, Atenolol, Ramipril and Atorvastatin are depicted in figures6, 7, 8 and 9 respectively.

Table 2: Linearity data for Aspirin (n=3)

Concentration (µg/ml)	Mean peak area (µv/sec)	SD	%RSD
75	966440	1947.83	0.20
150	1951790	4429.09	0.23
375	4835320	3911.16	0.08
750	10076183	5205.90	0.05
1125	15621045	50250.49	0.32
1500	20054626	9724.51	0.05

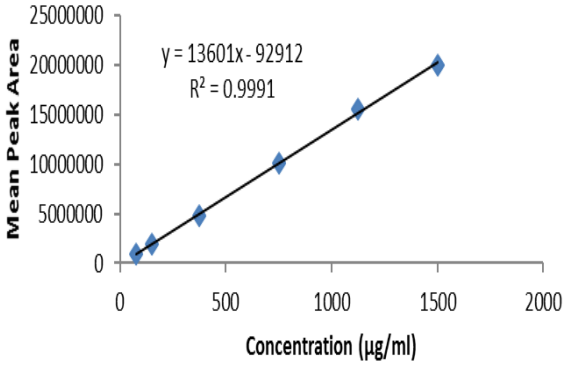

Figure 6: Linearity plot for Aspirin

Table 3: Linearity data for Atenolol (n=3)

Concentration (µg/ml)	Mean peak area (µv/sec)	SD	% RSD
50	407930	1084.18	0.22
100	816459	1148.60	0.13
250	2041148	14027.56	0.68
500	4082295	16392.65	0.40
750	6123443	6905.85	0.11
1000	8164590	13645.85	0.17

Table 4: Linearity data for Ramipril (n=3)

Concentration (µg/ml)	Mean peak area (µv/sec)	SD	%RSD
5	4970	43.10	0.87
10	9537	53.23	0.56
25	23825	305.39	1.28
50	47532	476.61	1.00
75	72393	365.96	0.51
100	93249	476.93	0.51

Table 5: Linearity data for Atorvastatin (n=3)

Concentration (µg/ml)	Mean peak area (µv/sec)	SD	%RSD
10	129634	363.58	0.28
20	258096	493.57	0.19
50	600654	4906.00	0.82
100	1291128	3970.56	0.36
150	1936692	5204.75	0.32
200	2582256	6509.96	0.25

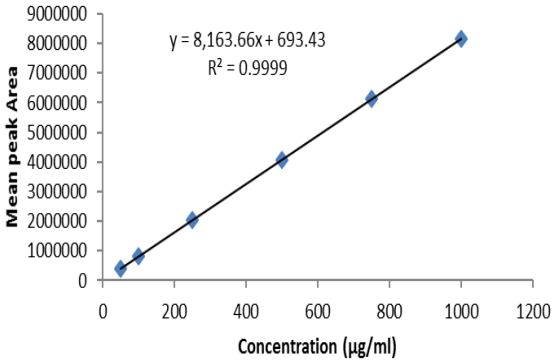

Figure 7: Linearity plot for Atenolol

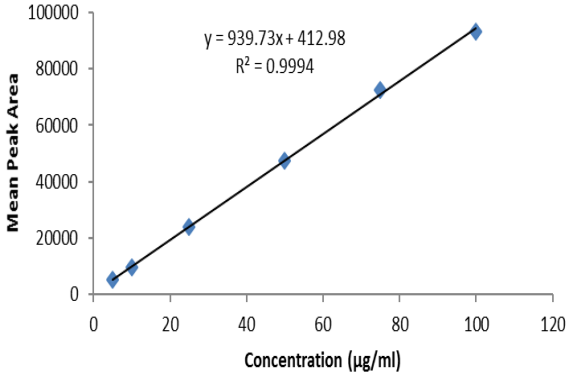

Figure 8: Linearity plot for Ramipril

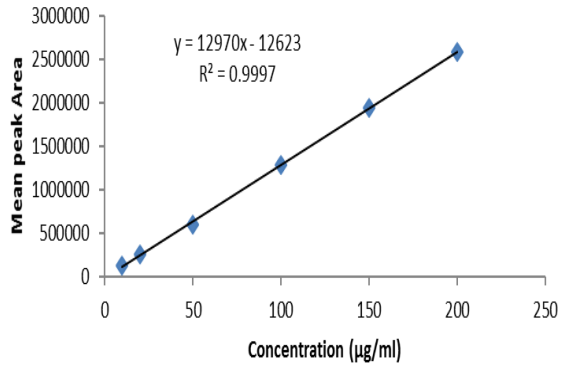

Figure 9: Linearity plot for Atorvastatin

Precision

The intra- and inter-day precisions were determined by analyzing 750µg/ml Aspirin, 500µg/ml Atenolol, 50µg/ml Ramipril, 100µg/ml Atorvastatinon same day and consecutive days, respectively. The intermediate precision was determined by changing column brand and also whole experiment was conducted by different analyst on different instrument. The intraday and inter day precision results are depicted in the table 6.

Table 6: Intraday and Interday precisions

Drug	Conc. (µg/ml)	Intraday precision			Interday precision		
		Found (µg/ml)	±SD	% RSD	Found (µg/ml)	±SD	%RSD
Aspirin	750	747.54	0.21	0.03	746.896	0.157	0.021
Atenolol	500	501.50	0.14	0.03	502.864	0.055	0.011
Ramipril	50	50.335	0.152	0.302	50.090	0.047	0.094
Atorvastatin	100	98.420	0.072	0.073	98.360	0.068	0.069

Accuracy

The accuracy of the method was determined by spiking a know mixture of the drugs corresponds to 80 %, 100 % and 120 % of Aspirin (600µg/ml,750µg/ml, 900µg/ml), Atenolol (400µg/ml, 500µg/ml, 600µg/ml), Ramipril (40µg/ml, 50µg/ml, 60µg/ml) and Atorvastatin (80µg/ml, 100µg/ml and 120µg/ml) in triplicate to a mixture solution and then determining the percent recovery by calculating differences between the peak areas obtained for fortified and unfortified solution. The results are incorporated in 7,8,9 and 10.

Table 7: Recovery of Aspirin

Drug	Added Concentration (µg/ml)	Measured Concentration (µg/ml)	% Recovery	Mean % Recovery
Aspirin	600	599.528	99.921	99.923
		599.535	99.923	
		599.552	99.925	
	750	747.69	99.692	99.694
		747.717	99.696	
		747.71	99.695	
	900	895.876	99.542	99.539
		895.837	99.537	
		895.853	99.539	

Table 8: Recovery of Atenolol

Drug	Added concentration (µg/ml)	Measured concentration (µg/ml)	% Recovery	Mean % recovery
Atenolol	400	398.548	99.637	99.640
		398.568	99.642	
		398.568	99.642	
	500	501.565	100.313	100.306
		501.526	100.305	
		501.498	100.300	
	600	599.524	99.921	99.921
		599.53	99.922	
		599.528	99.921	

Table 9: Recovery of Ramipril

Drug	Added concentration (µg/ml)	Measured concentration (µg/ml)	% Recovery	Mean % recovery
Ramipril	40	40.074	100.185	100.244
		40.106	100.265	
		40.113	100.283	
	50	50.175	100.35	100.293
		50.063	100.126	
		50.202	100.404	
	60	60.339	100.565	100.550
		60.322	100.537	
		60.328	100.547	

Table 10: Recovery of Atorvastatin

Drug	Added concentration (µg/ml)	Measured concentration (µg/ml)	% Recovery	Mean % recovery
Atorvastatin	80	79.009	98.761	98.773
		79.026	98.78	
		79.019	98.774	
	100	98.353	98.353	98.354
		98.345	98.345	
		98.365	98.365	
	120	120.154	100.128	100.130
		120.162	100.135	
		120.153	100.128	

Robustness study

The robustness of the method was determined as per USP guidelines under a variety of conditions including change in flow rate, pH of buffer. The results obtained by deliberately variation in method parameters and data are summarized below in table 11. The Robustness of analytical method is a measure of its capacity to remain in effected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. Robustness examines the effect of operational parameters on analysis results.

Table 11: Robustness study

Parameter		Drug	Retention time (min)	Asymmetry	Plate count	Resolution
Flow rate (0.8ml)±10%	Flow rate 0.7 ml/min	Aspirin	4.21	1.76	415763	-
		Atenolol	7.32	1.05	360879	3.55
		Ramipril	8.52	1.34	223465	6.22
		Atorvastatin	6.01	1.25	452734	4.17
	Flow rate 0.9 ml/min	Aspirin	4.01	1.58	598454	-
		Atenolol	7.10	1.16	435052	3.45
		Ramipril	8.02	1.12	803076	6.23
		Atorvastatin	5.80	1.24	476234	4.16
Parameter		**Drug**	**Retention time (min)**	**Asymmetry Factor**	**Plate count**	**Resolution**
pH Variations ±0.1	pH -2.4	Aspirin	4.15	1.34	596794	-
		Atenolol	7.12	1.34	543052	3.34
		Ramipril	8.22	1.03	707076	6.84
		Atorvastatin	5.85	1.34	560216	4.14
	pH -2.6	Aspirin	4.20	1.18	567092	-
		Atenolol	7.32	1.16	735521	3.26
		Ramipril	8.35	1.02	658087	6.56
		Atorvastatin	5.90	1.35	592374	4.24

RESULTS AND DISCUSSION

To optimize the mobile phase, various proportions of buffer with methanol and acetonitrile were tested. Mobile phase containing a mixture of buffer, methanol and acetonitrile in the ratio of 25:25:50 resulted in peaks with good shape and resolution. A flow rate of 0.8 ml/min was found to be optimum in the 0.5-1.0 ml/min range resulting in the short retention time, baseline stability and minimum noise.

By applying the proposed method, the retention times of Aspirin, Atenolol, Ramipril and Atorvastatin were found to be about 4.1 min, 7.2 min, 8.3 min and 5.90 min respectively. Quantitative linearity was obeyed in the concentration range of 75-1500, 50-1000, 5-100 and 10-200µg/ml for Aspirin, Atenolol, Ramipril and Atorvastatin respectively. The regression equations of concentration of Aspirin, Atenolol, Ramipril and Atorvastatin over their peak areas were found to be y= 13601x-92912 (R^2=0.999), y=

8163.66x+693.43 (R^2=0.999), y= 939.7x+412.7 (R^2=0.999) and y= 12970x-12623(R^2=0.999) respectively where peak areas are on y-axis and the concentrations of Aspirin, Atenolol, Ramipril and Atorvastatin (µg/ml) are on x-aixs respectively. The numbers of theoretical plates obtained were 3779.68, 5788.58, 6702.24 and 9400.88 for Aspirin, Atenolol, Ramipril and Atorvastatin respectively, which indicates the efficiency of the column. The high percentage recovery indicates that the proposed method is highly accurate.

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CITE THIS ARTICLE AS:
N. Sanni Babu, S. Mutta Reddy. Development of RP-HPLC method for simultaneous estimation of Aspirin, Ramipril, Atenolol and Atorvastatin. *International Journal of Bioassays* 4.12 (2015): 4596-4600.

Source of support: Nil
Conflict of interest: None Declared