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NEW METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF ASPIRIN, ATORVASTATIN AND CLOPIDOGREL IN CAPSULE DOSAGE FORM BY HPLC

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Abstract:

A simple, accurate, rapid and precise isocratic reverse phase high performance liquid chromatographic method has been developed and validated for simultaneous determination of aspirin, atorvastatin and clopidogrel in capsule dosage form. The chromatographic separation was carried out on an Inertsil ODS analytical column (250×4.6mm,5µm) with a mixture of solvents phosphate buffer (pH 3.15 adjusted with o-phosphoric acid), acetonitrile and methanol (40:40:20 v/v/v) as mobile phase, at a flow rate of 1.0 ml/minute maintaining the temperature at 30ºc. UV detection was performed at 240 nm. The retention times were 2.4, 3.5 and 4.5 for atorvastatin, aspirin and clopidogrel respectively. The method was validated according to ICH guidelines and the acceptance criteria of results for accuracy, precision, linearity, robustness, limit of detection, limit of quantification and ruggedness were met in all cases. The % RSD values for atorvastatin, aspirin and clopidogrel were found to be 0.101%, 0.547% and 0.515% respectively. The linearity of the calibration curve for each analyte in the desired concentration range was good (r^2>0.999). The high recovery and value of low relative standard deviation confirm the suitability of the method for routine evaluation of aspirin, atorvastatin and clopidogrel in pharmaceutical dosage forms.

Keywords: *Aspirin, atorvastatin, clopidogrel, simultaneous, HPLC.*

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INTRODUCTION:

Low dose of Aspirin[1] (ASP) prevents blood clots. This effect may reduce the risk of complications like stroke, heart attack etc. ASP, a derivative of salicylic acid and a non-steroidal anti-inflammatory drug (NSAID), works by blocking certain natural substance in our body. It is also used to reduce fever, mild to moderate pain. Therefore, it is indicated commonly in conditions such as muscle aches, headaches, toothaches, swelling in gout arthritis.

Chemically the compound is known as 50-78-2; 2-Acetoxybenzoic acid; 2-(Acetyloxy)benzoic acid; O-Acetoxybenzoic acid. Figure 1 represents the structure of ASP.

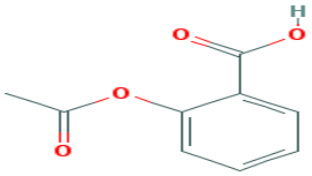

Fig. 1: Structure of Aspirin

Atorvastatin[2] (ATR)is a drug of choice to modify lipid profile in elderly patients when administered orally. Among all the statins available this molecule is used extensively the medical fraternity. Chemically the compound is known as Calcium (βR,δR)-2-(p-fluorophenyl)-β,δ-dihydroxy-5- isopropyl-3- phenyl-4-(phenylcarbamoyl)pyrrole-1-heptanoic acid (1:2) trihydrate. Figure 2 represents the structure of Atorvastatin.

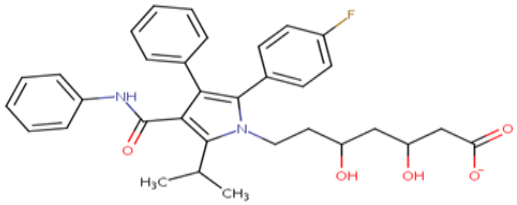

Fig. 2: Structure of Atorvastatin

Clopidogrel[3] (CLP)is a most common co-prescription along with aspirin to inhibit blood clotting. This molecule has been proved as a co-therapy in patients with cardiac complications. Chemically it is known as methyl (2S)-2-(2-chlorophenyl)-2-{4H,5H,6H,7H-thieno[3,2-c]pyridin-5-yl}acetate. Fig 3 represants the Structure of Clopidogrel.

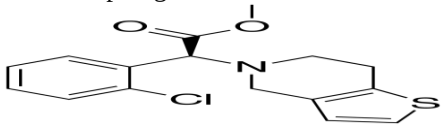

Fig. 3: Structure of Clopidogrel

Literature survey [4-10], helps us to get motivated and go for the present research work. There are certain assay methods available for this combination. Pawan K. Porwal, Akhalaque Ahmad, R. A. Santosh, S. ChhajedVivekanand, and A. Chatpalliwar developed a Liquid Chromatographic Method for Simultaneous Quantitation of CLP, ASP and ATR in Rat Plasma and Its Application to the Pharmacokinetic Studies. Keerthisikha Palur, Bharathi Koganti , Sreenivasa Charan Archakam , Sridhar Chenchugari , Bhavana Nagireddy , Mahesh Babu Devabhaktuni et al developed chemometric assisted spectrophotometric method for simultaneous estimation of all the three drugs in bulk and capsule dosage form. AnasRasheed, SumanPattanayak, T. Rama Rao developed an UPLC method for simultaneous estimation of all three drugs in tablet dosage form. H. O. Kaila1, M. A. Ambasana and A. K. Shah also developed an UPLC procedure. Jinesh Bahubali Nagavi, Bannimath Gurupadayya also invented one UPLC method. S. V. Londhe, R. S. Deshmukh, S. V. Mulgund and K. S. Jain developed a Reversed-phase HPLC Method for Simultaneous Determination of ASP, ATR and CLP in Capsules. Abdullah Al Masud and Iris Begum developed a RP HPLC method for simultaneous estimation of ASP and CLP in combined tablet dosage form. But the extensive use of this combination makes a scope to work further so that a more simple method will be available for the estimation purpose.

MATERIALS AND METHODS:

Instruments: HPLC make-waters 2690 with detector PDA-2996. Column as ODS C18 (250mm x 4.6 mm, 5m). Balance analytical- ER-180A, Sartorius Microbalance-M500P, Thermo scientific pH Meter, Sar torius Sonicator, Empower V 1.2.2.1 Software.

Chemicals: Ortho phosphoric acid (AR) make- SDF, HPLC grade Water, HPLC grade Acetonitrile, HPLC grade Methanol, Potassium di-hydrogen phosphate. All these three solvents are products of Merck.

Preparation of mobile phase: 400 ml of phosphate buffer having pH 3.15 (pH was adjusted using AR-grade ortho phosphoric acid) and 400 ml of HPLC grade Acetonitrile was taken in a 1000 ml volumetric flask containing 200 ml of HPLC grade Methanol. This solution was filtered through a 0.45 µ membrane filter under vacuum, degassed in ultrasonic water bath for around 5 minutes.

Preparation of working stock solution of Aspirin, Atorvastatin and Clopidogrel: 20 Capsules were weighed and finely powdered. Capsule powder equivalent to 10mg of ATR, 75mg of ASP and 75mg of CLP was weighed accurately and transferred into

100 ml volumetric flask, diluted up to the mark with mobile phase to get the concentration of 100 µg/ml ATR, 750 µg/ml of ASP and 750 µg/ml of CLP.

Preparation of working standard solution of Aspirin, Atorvastatin and Clopidogrel: From the above stock solution 1 ml was pipetted out into a 10 ml volumetric flask and diluted up to the mark with the mobile phase to get a concentration of 100 % and considered it as a standard. This solution was filtered through 0.45 µm membrane filter.

Label Claim: 10 mg of atorvastatin 75mg of Aspirin and 75mg of Clopidogrel

Method development

To develop a new method[11,12] for estimation work several trials were conducted so that we can achieve most suitable chromatographic condition and the best results. The initial attempt was to use as much low part of organic solvents for the purpose of elution. But increased part of aqueous solvents in our mobile phase resulted in extending of retention time for all the compounds. But reasonable retention time, value of tailing factors, number of theoretical plates and all were found to be within the validation limit while using optimized chromatographic condition.

Method validation

The method was evaluated [13,14] as per protocol designed by ICH[15].The evaluation parameters took into consideration were system suitability parameters, precision accuracy, intermediate precision, linearity, limit of quantification, limit of detection, robustness studies etc.

System suitability parameters: For one analytical method validation system suitability parameters to be determined by preparing standard solutions of the compounds of specific concentration and the solutions to be injected six times and the parameters like peak tailing, theoretical plate count, retention time etc to be determined.

Specificity: Checking of interference if any in the optimized method. We should not find any interfering peak in blank in this method so that the method can be considered as specific.

Accuracy: The accuracy for a developed HPLC method is to be examined by calculating the extant of recoveries of all the compounds by a procedure called standard addition. Correct amount of drug solutions (standard) of that particular project (each drug 50%, 100%, and 150%) to be added and injected to pre-quantified solution of sample. The quantity of each substances recovered to be determined.

Precision: The experimental repeatability as well as intermediate precision to be examined by repeatedly applying six injections containing the compounds with specific concentration at two subsequent days. Number of theoretical plates, retention time, peaks resolution, peak symmetry etc must be the subject of observation.

Linearity: A series of gradually increased concentration for the entire range of compounds to be designed to conduct linearity test. To build up calibration curve, concentration and area should be considered at X and Y axis respectively.

LOD and LOQ: Calculation for Limit of detection as well as Limit of quantification to be been done by using standard Equations. LOD = $3.3\times\sigma/S$, LOQ = $10\times\sigma/S$. Here σ denotes for standard deviation of intercepts of regression lines, S denotes for slope.

Robustness: Evaluation for robustness to be conducted by making alteration in different chromatographic parameters. These parameters included flow rate, temperature, mobile phase composition etc.

Assay of marketed formulation: Assay of marketed product must be carried by injecting sample corresponding to equivalent weight into HPLC system, percentage purity to be found out by the following formula

$$Conc_{unknown} = \left(\frac{Area_{InternalStd.in\ known}}{Area_{InternalStd.in\ unknown}}\right) \times \left(\frac{Area_{unknown}}{Area_{known}}\right) \times (Conc_{known})$$

RESULTS AND DISCUSSION:

Method development: A unique method of assay was innovated by using columns of different length and make. Mobile phases containing various compositions with different proportions were tried by taking standard as well as sample in individual. Column or oven temperature, flow rate, different buffers (salt and acid combination) with slightly varying pH value and solvents were applied. Whichever the different mobile phases were prepared, subjected for filtration through membrane filters prior of their use. The mobile phase containing a mixture of solvents phosphate buffer (pH 3.15 adjusted with o-phosphoric acid), acetonitrile and methanol (40:40:20 v/v/v) was considered as the best to obtain peaks of ATR, ASP and CLP at 2.4, 3.5 and 4.5 minutes.

Validation Results

Results of system suitability: The optimized chromatographic procedure as developed resulted in the elution of ATR, ASP and CLP at 2.4, 3.5 and 4.5 minutes. Figure 4 is the representative chromatogram of standard solution of ATR, ASP and CLP. System suitability results were evaluated by taking six replicates of standard solution at 10µg/ml, 75µg/ml and 10µg/ml for the compound as mentioned respectively. Table 1 narrates about the results of system suitability parameters.

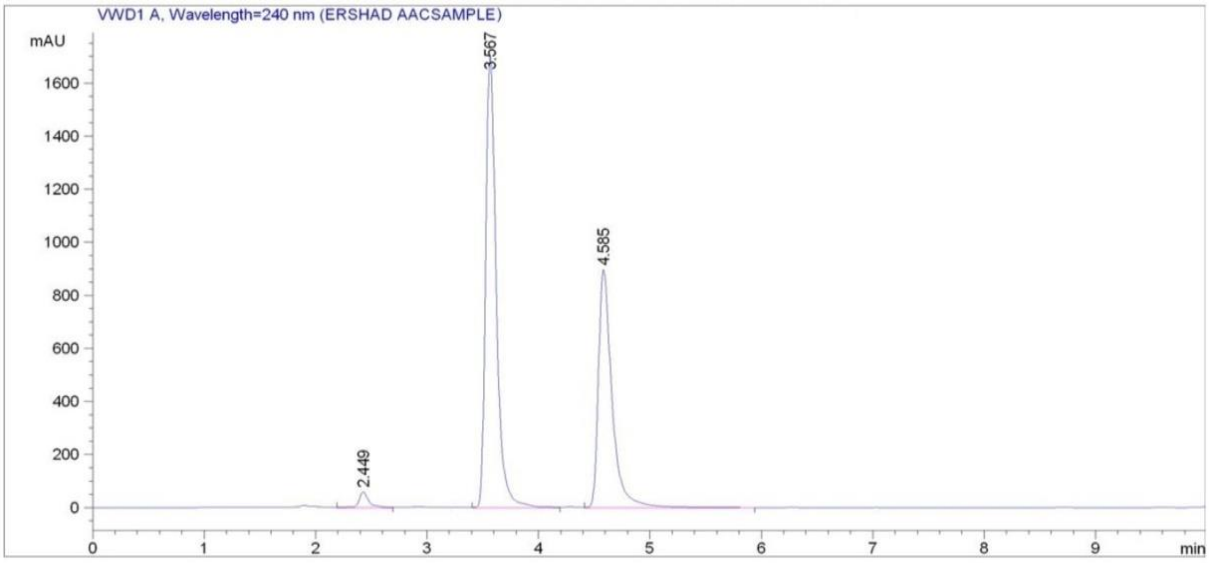

Figure. 4: Optimized chromatogram of Atorvastatin, Aspirin and Clopidogrel

Table 1: Results of system suitability parameters

Compound	R_t (minutes)	USP plate count	Tailing factor	Resolution
Atorvastatin,	2.449	11050	1.602	-
Aspirin	3.567	19748	1.143	12.18
Clopidogrel	4.585	21202	0.598	9.05

Number of trials (N) = 6

Results of accuracy studies: Accuracy of the method was well established from the results of percentage recovery. It was calculated from the amount of compounds recovered by comparing the peak average areas observed for standard and sample solutions. The percentage was found in the range of 97.93 – 99.25 % for ATR, ASP and CLP as given in table 2.

Table 2: Results of Accuracy studies

Amount of sample			Amount of standard			Amount recovered			%recovery		
ATV	ASP	CLP	ATV	ASP	CLP	ATV	ASP	CLP	ATV	ASP	CLP
10	75	75	5	37.5	37.5	4.94	37.21	36.75	98.80	99.25	98.01
10	75	75	10	75	75	9,89	74.29	73.59	98.90	99.06	98.12
10	75	75	15	150	150	14.69	147.30	146.89	97.97	98.26	97.93

N = 3 for each spiked standard.

Results of precision studies: The repeatability (intra-day trials) and intermediate precision (inter-day trials) studies for ATR, ASP and CLP revealed slight variations in the repetitive trial values (% RSD < 1.5) as narrated in table 3 indicating actual precision of the method.

Table 3: Results of precision studies

Compounds	Result	Standard area	Sample(Intraday)	Sample(Interday)	Mean Assay
Atorvastatin	Mean	36513	36129	36038	98.78
	SD	54.29	27.82	12.31	
	%RSD	0.12	0.30	0.14	
Aspirin	Mean	1137155	1114184	1131469	98.48
	SD	621.55	564.11	761.09	
	%RSD	0.44	0.43	0.35	
Clopidogrel	Mean	764329	756074	754086	99.01
	SD	111.66	119.80	106.74	
	%RSD	0.48	0.40	0.36	

N = 6

Linearity and regression analysis: Concentration range of 2.5 µg/ml -15 µg/ml for ATR , 4 µg/ml -20 µg/ml for ASP and 4µg/ml to 20µg/ml for CLP was designed for linearity test. Table 4 explains about appropriateness of the developed method. Sensitivity of the new method was good enough. With very low concentration the response in graph was sufficient to read and calculate all the results of regression analysis. Results of linearity test revealed that the RSD of Y intercept value, value of correlation coefficient and SD of slope value, LOD and LOQ for ATR, ASP and CLP was 0.233, 0.999, 0.135, 0.033, 0.101; 0.119, 0.999, 0.202, 0.045, 0.149; 0.134, 0.999, 0.168, 0.061, 0.203 respectively.

Table 4: Sensitivity and regression analysis

Parameters	Atorvastatin	Aspirin	Clopidogrel
Linearity (µgm/ml)	2.5-15	4-20	4-20
Correlation Coefficient.(r)	0.999	0.999	0.999
Regression slope	553.2	563.8	475.8
SD of Slope	0.135	0.202	0.168
Regression Intercept (mean)	11873	15773	19917
%RSD of Intercept	0.233	0.119	0.134
LOD	0.033	0.045	0.061
LOQ	0.101	0.149	0.203

Results of robustness studies: Robustness results: This exercise had been done by bringing marginal variation in certain chromatographic parameters namely increasing and reducing flow rate, variation in the ratio or proportion of aqueous phase and organic one, temperature status of column etc. Retention time, plate counts as well as asymmetric or tailing factor etc was obtained with very marginal variation. All the observed analytical values are given in table 5 as tabular form.

Table 5: Results of robustness studies

Compounds	Chromatographic condition	Retention time (minutes)	USP plate count	Asymmetric factor	%Assay
ATR	Flowrate1.2ml/min	2.385	10445	1.521	98.77
	Flowrate0.8ml/min	2.462	11879	1.519	99.10
	Buffer 45 parts	2.466	12342	1.600	98.19
	Buffer 35 parts	2.391	09987	1.589	99.00
	Temperature(35ºc)	2.387	10107	1.601	97.99
	Temperature(25ºc)	2.455	12186	1.599	98.96
ASP	Flowrate1.2ml/min	3.488	19009	1.111	98.88
	Flowrate0.8ml/min	3.580	19927	1.152	98.67
	Buffer 45 parts	3.582	19979	1.128	99.03
	Buffer 35 parts	3.555	19276	1.109	98.48
	Temperature(35ºc)	3.549	19535	1.254	98.33
	Temperature(25ºc)	3.578	19898	1.193	99.14
CLP	Flowrate1.2ml/min	4.453	21213	0.555	99.00
	Flowrate0.8ml/min	4.599	22318	0.562	98.09
	Buffer 45 parts	4.598	22008	0.560	98.45
	Buffer 35 parts	4.521	21875	0.530	99.84
	Temperature(35ºc)	4.511	21008	0.591	99.02
	Temperature(25ºc)	4.580	22319	0.551	98.74

N = 3

Assay of marketed formulation: The formulation (Tablet- Storva trio 10, Sun pharmaceutical industries altd) was procured from local Medical store. Ten tablets had been chosen, weighed and collected in a clean and dry mortar. Tablets were triturated into powder form and then collected an equivalent quantity of 10mg of atorvastatin, 75mg of aspirin and 75mg of clopidogrel in a dry volumetric flask (100 ml). Entire quantity of powder was treated with diluent and then subjected for sonication. The volume was made with diluent. 1 ml of the solution was pipetted out into a volumetric flask (10 ml) and the volume was made with diluent. 10 µl of resultant solution was injected to the Chromatographic system and analytical result was studied as compared to that of standard preparation. Peak area response was taken into consideration.

CONCLUSION:

The present HPLC method for the simultaneous determination of atorvastatin, aspirin and clopidogrel was found to be one of the least time consuming, simple, highly accurate technique as all the validation results of all parameters were with very low value of %RSD. At the same time it also proved that the innovated technique is a precise and robust method. Therefore the above narrated novel analytical technique is a suitable one for simultaneous evaluation of bulk and combined tablet formulation of atorvastatin, aspirin and clopidogrel in laboratory.

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