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Simultaneous Estimation of Enalapril Maleate, Hydrochlorothiazide, Aspirin and Atorvastatin in pure and Its Simulated Dosage form Using Isocratic RP-HPLC

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ABSTRACT

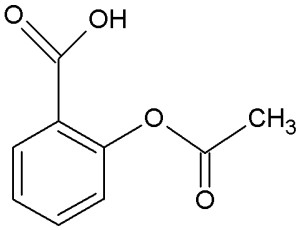
A simple and reliable isocratic reversed-phase high-perfomance liquid chromatographic (RP-HPLC) method has been developed and validated for the simultaneous estimation of Enalapril Maleate(ENL), hydrochlorothiazide(HCT), aspirin(ASP) and atorvastatin(ATR) in bulk and its simulated dosage form. All the drugs were separated on a 250mm×4.6mm C18 column packed with 5 µm particles. The mobile phase, optimized through an experimental design, was a 50:25:25 (v/v/v) mixture of acetontrile, methanol and triethylammonium phosphate buffer (pH 2.5), pumped at a flow rate of 0.6 ml/min. UV detection was performed at 225 nm. The retention time of ENL, HCT, ASP and ATR was found to be 3.667 min, 4.725 min, 5.508 min and 8.282 min respectively. The method was validated in the sample concentration ranges of 25-500 µg/ml for ENL, 12.5-250 µg/ml for HCT, 125-250 µg/ml for ASP and 50-1000 µg/ml for ATR, where it demonstrated good linearity with correlation coefficient values of 0.999 (n = 3) for all the drugs in the study. The method demonstrated to be robust, resisting to small deliberate changes in pH and flow rate of the mobile phase. The LOD values were 1.474µg/ml, 0.737µg/ml, 0.545µg/ml and 1.641µg/ml, while the LOQ values were 4.466µg/ml, 2.233µg/ml, 1.653µg/ml and 4.972µg/ml for ENL, HCT, ENL and ATR respectively. The recoveries for all four compounds were above 98%. The applicability of the method was demonstrated by determining the drug content in simulated pharmaceutical formulation, where it exhibited good recovery. Hence the method can easily applicable for routine quality control laboratories.

Keywords: Aspirin, Enalapril Maleate, Hydrochlorothiazide, Atorvastatin, Simultaneous estimation.

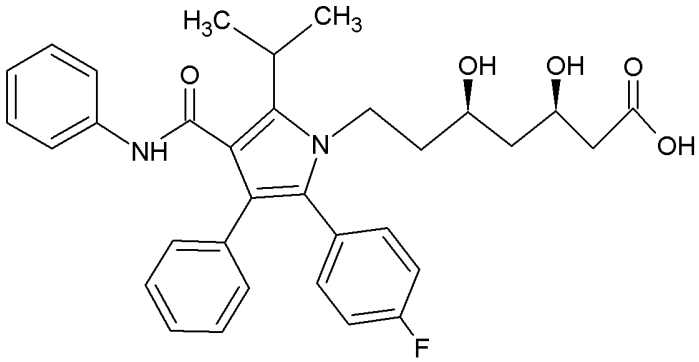
INTRODUCTION

Aspirin (ASP) is an analgesic and antipyretic, anti-inflammatory, anti-arthritic and anti-platelet drug[1-4]. Chemically aspirin is 2-acetoxybenzoic acid. Enalapril Maleate (ENL) is a potent and

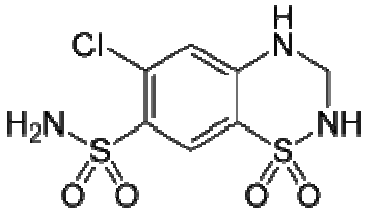
specific angiotensin converting enzyme (ACE) inhibitor[1-4]. Chemically ENL is 2*S*)-1-[(2*S*)-2-{[(2*S*)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino}propanoyl]pyrrolidine-2-carboxylic acid. Hydrochlorothiazide (HCT) is a is a first-line diuretic drug of the thiazide class that acts by inhibiting the kidneys ability to retain water[1-4]. Chemically HCT is 6-chloro-1,1-dioxo-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide. Atorvastatin calcium (ATO) is an anti-hyperlipoproteinemic agent that acts by inhibiting HMG-CoA Reductase[1-4]. Chemically atorvastatin is (βR,δR)-2-(4-fluro phenyl)-β, δ-dihydroxy-5-(1-methyl ethyl)-3-phenyl-4-[(phenyl amino)carboxyl]-1H-pyrrole-1-heptanoic acid , calcium salt (2:1) trihydrate . These drugs are used in the treatment of cardiovascular diseases.



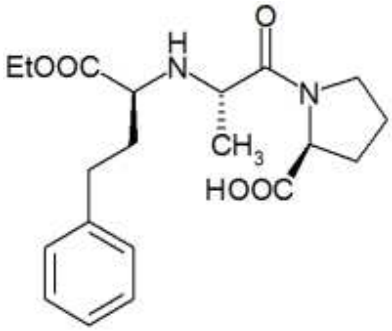
ASPIRIN



ATORVASTATIN



HYDROCHLOROTHIAZIDE



ENALAPRIL

A survey of literature revealed that few chromatographic and spectrophotometric methods are reported for determination of ENL, HCT, ASP and ATR individually [5-16]. However no RP-HPLC method is reported till date for simultaneous determination of these drugs it is a combination of poly drugs under clinical trials. In this communication we report a new, simple, precise and accurate isocratic RP-HPLC method for the simultaneous estimation of ENL, HCT, ASP and ATR in pure and simulated dosage form. The proposed method is optimized and validated as per the International Conference on Harmonization (ICH) guidelines.

MATERIALS AND METHODS

Drug samples

The reference samples of ENL, HCT, ASP and ATR were obtained from Aurobindo Pharma, Dr. Reddy's Laboratories and Smilax Laboratories Limited, Hyderabad, India.

Chemicals and reagents

Purified water was prepared by using 0.45 Millipore Milli-Q water purification systems. Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Merck (India) Ltd. Mumbai, India. Orthophosphoric acid and triethylamine (HPLC grade) were purchased from Sigma-Aldrich (India) Bangalore, India.

Preparation of triethylammonium phosphate buffer

0.1% orthophosphoric acid was prepared with HPLC grade water and its pH was adjusted to 2.5 with triethyl amine. This solution was used as a buffer. Water was previously filtered through a 0.45 µ membrane filter.

Preparation of mobile phase

Mobile phase was freshly prepared by mixing acetonitrile, methanol and buffer in the ratio of 50:25:25v/v respectively. It was filtered through 0.45µ Nylon membrane filter and sonicated. This solution was also used as diluent.

Preparation of standard drug solutions

The stock solution was prepared by dissolving about 500 mg of aspirin, 250 mg of hydrochlorothiazide, 250 mg of enalapril and 1000 mg of atorvastatin in 10 ml of acetonitrile initially and the solution was sonicated for 15 minutes. The volume was made up to the mark with further quantity of acetonitrile to get 5 mg/ml aspirin, 2.5 mg/ml hydrochlorothiazide, 2.5 mg/ml enalapril and 5mg/ml atorvastatin solution. The working standard solution was made by diluting 10 ml standard stock solution to 100 ml in volumetric flask to get 500µg/ml aspirin, 250µg/ml hydrochlorothiazide, 250µg/ml enalapril and 1000µg/ml atorvastatin respectively. Further dilutions were made from the working standard solution in the required concentration range in 10 ml volumetric flasks for the calibration curve.

Instrumentation

A JASCO 2080 model chromatograph equipped with a Lichrosphere 100 RP-18 reverse phase C_{18} column (250x4.6mm I.D: particle size 5 µm) was employed for the study. Sample injection was done with a Rheodyne 7725 injection valve via a 20 µl loop. Detection of the drug was done by using a UV-2075 detector (JASCO) and the output signal was monitored and integrated by JASCO -BORWIN software. Solubility of the compound was enhanced by sonication on an ultrasonicator.

The flow rate of mobile phase was maintained at 0.6 ml/min. The detection of the drugs in the eluates was carried out at 225nm.

TABLE-1: System suitability parameters for the proposed method

Parameter	Result			
	ASP	**HCT**	**ENA**	**ATR**
THEORETICAL PLATES	9708.70	5997.14	5362.77	10276.21
HETP	$2.6x10^{-5}$	$4.17x10^{-5}$	$4.66x10^{-5}$	$2.43x10^{-5}$
ASYMMETRY	1.22	1.15	1.30	1.13
LOD(µg/ml)	1.474	0.737	0.545	1.641
LOQ(µg/ml)	4.466	2.233	1.653	4.972

TABLE-2: Linearity parameters for calibration curve (n=3)

Drug	Concentration (µg/ml)	Equation of regression line	R^2
ASP	25-500	y= 20912x + 45828	0.999
HCT	12.5-250	y = 20912x + 22914	0.999
ENA	12.5-250	y = 18084x – 19048	0.999
ATR	50-1000	y=24000x + 21765	0.999

TABLE-3: Accuracy and recovery studies

Drug	Added concentration(µg/ml)	Measured concentration	% Recovery	Mean % recovery ±RSD
ASP	200	198.957	99.478	99.661 ± 0.184499
	250	249.23	99.692	
	300	299.53	99.844	
HCT	100	100.567	100.567	100.639 ± 0.136141
	125	125.996	100.797	
	150	150.831	100.553	
ENA	100	100.313	100.313	100.1377 ± 0.163043
	125	125.142	100.11	
	150	149.982	99.99	
ATR	400	396.908	99.23	99.33333± 0.18018
	500	496.505	99.23	
	600	597.226	99.54	

TABLE-4: Intraday and interday precisions

Drug	Conc. (µg/ml)	Intraday precision			Interday precision		
		Found (µg/ml)	**±SD**	**% RSD**	**Found** (µg/ml)	**±SD**	**%RSD**
ASP	250	249.2283	0.032476	0.01303	249.2283	0.032476	0.01303
HCT	125	126.1317	0.324386	0.25718	125.994	0.069596	0.055238
ENA	125	125.5895	0.287244	0.228717	124.6337	0.181573	0.145685
ATR	500	496.3317	0.048878	0.009848	496.3826	0.080036	0.016124

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 used as eluting systems at different proportions like 40:25:35, 50:30:20, 50:20:30, 50:25:25, 50:15:35, 50:0:50, 60:0:40, 85:0:15 and 80:5:15 and in a pH range of 2.3-3.02. A mixture of acetonitrile, methanol and buffer of pH 2.5 in the ratio of 50:25:25 provided an efficient separation of the drugs with good peak shapes and retention times. A flow rate of 0.6 ml/min was found to be optimum in the range

of 0.5-1.0 ml/min which gave retention times of 3.667 min for enalapril, 4.725 min for hydrochlorothiazide, 5.508 min for aspirin and 8.283 min for atorvastatin respectively with baseline stability.

TABLE-5: Robustness Study

Parameters	Change in conditions		ENA	HCT	ASP	ATR
Retention time (min)	flow rate	0.5ml/min	3.69	4.82	5.60	8.48
		0.8ml/min	3.51	4.78	5.59	8.34
	Concentration of OPA used in buffer	0.05%	3.51	4.78	5.59	8.34
		0.2%	3.51	4.78	5.59	8.34
	pH of buffer	2.4	3.61	4.48	5.62	8.65
		2.6	3.50	4.65	5.78	8.85
Asymmetry Factor	flow rate	0.5ml/min	1.30	1.18	1.20	1.10
		0.8ml/min	1.38	1.16	1.12	1.14
	Concentration of OPA used in buffer	0.05%	1.38	1.16	1.12	1.14
		0.2%	1.38	1.16	1.12	1.14
	pH of buffer	2.4	1.34	1.34	1.03	1.14
		2.6	1.20	1.53	1.18	1.45
Plate count	flow rate	0.5ml/min	5365	5997	9708	10278
		0.8ml/min	5365	5997	9708	10278
	Concentration of OPA used in buffer	0.05%	5362	5987	9707	10274
		0.2%	5365	5997	9708	10278
	pH of buffer	2.4	5365	5997	9708	10278
		2.6	5365	5997	9708	10278
Resolution	flow rate	0.5ml/min	-	4.86	3.42	2.25
		0.8ml/min	-	4.86	3.42	2.25
	Concentration of OPA used in buffer	0.05%	-	4.83	3.22	2.15
		0.2%		4.86	3.42	2.25
	pH of buffer	2.4	-	4.86	3.42	2.25
		2.6	-	4.96	3.62	2.55

The mobile phase consisting of acetonitrile, methanol and buffer (50:25:25) was filtered through a 0.45µ membrane filter, sonicated and degassed and was then pumped from the solvent reservoir through the column at a flow rate of 0.6ml/min. The column was maintained at ambient temperature. The detection of the eluates was monitored at 225nm and the run time was 12 min. The volume of injection was 20 µl. 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 25-500µg/ml of aspirin, 12.5-250 µg/ml of hydrochlorothiazide, 12.5-250µg/ml of enalapril and 50-1000 µg/ml of atorvastatin were prepared from the working standard solution in 10 ml volumetric flasks with the diluent. A volume of 20 µl 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 25-500 µg/ml range for aspirin, 12.5-250 µg/ml range for enalapril, 12.5-250µg/ml of hydrochlorothiazide and 50-1000 µg/ml range for

atorvastatin. 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 fig.5

Fig 1 Linearity plot for enalapril

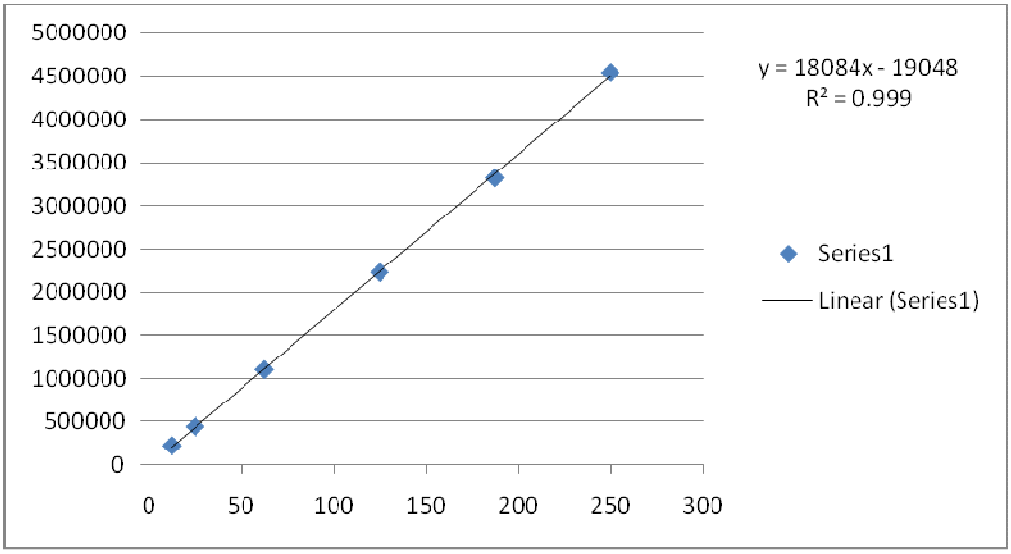
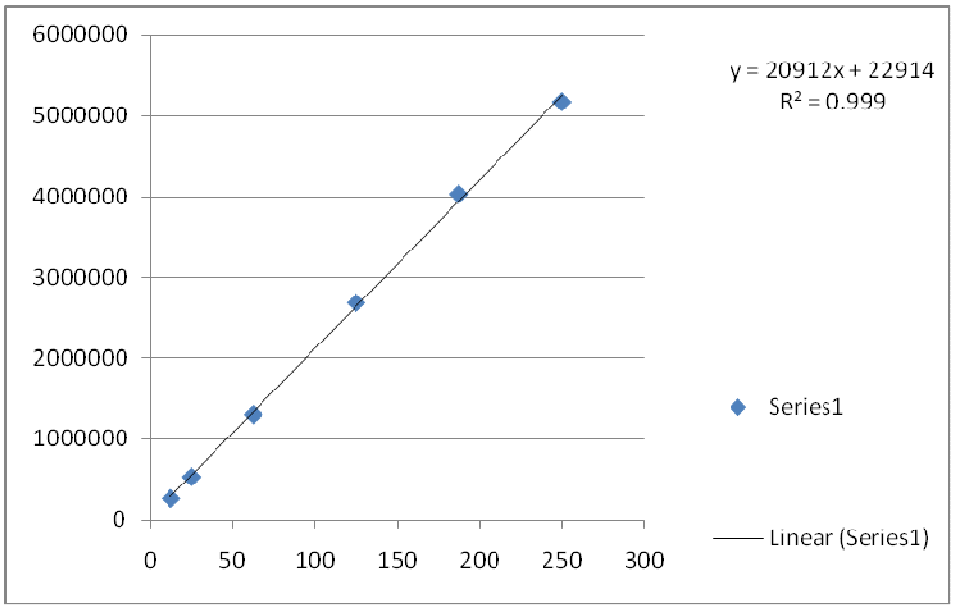


Fig 2 Linearity plot for hydrochlorothiazide



Method Validation [17-19]

Specificity and selectivity of the proposed method

Specificity is the extent to which the procedure applies to analyte of interest and is checked by examining the formulation samples for any interfering peaks. The specificity of the method was evaluated with regard to interference due to presence of excipients. The excipients used in formulation did not interfere with the drug peaks and thus the method is specific.

Fig 3 Linearity plot for aspirin

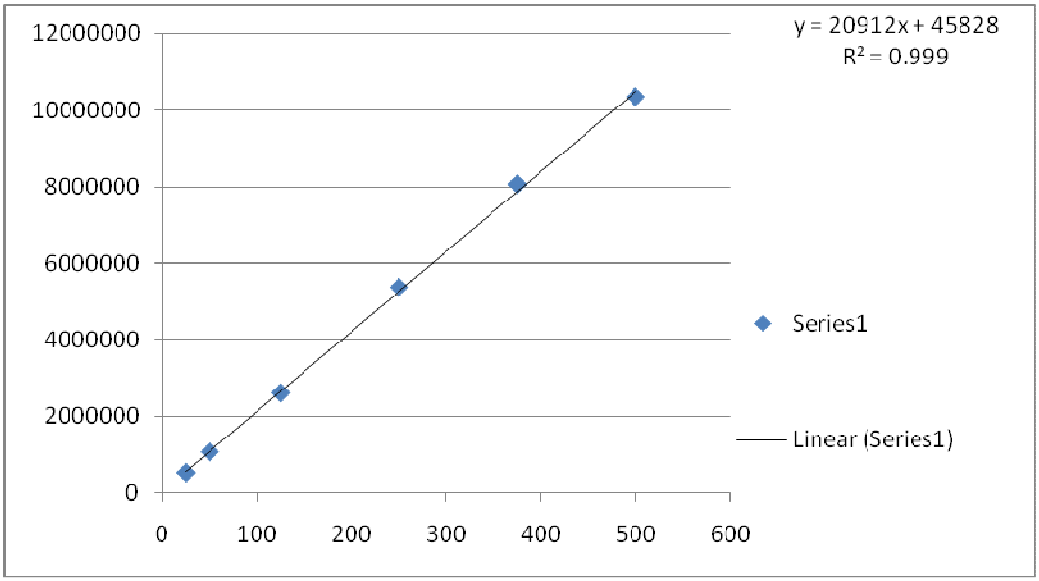
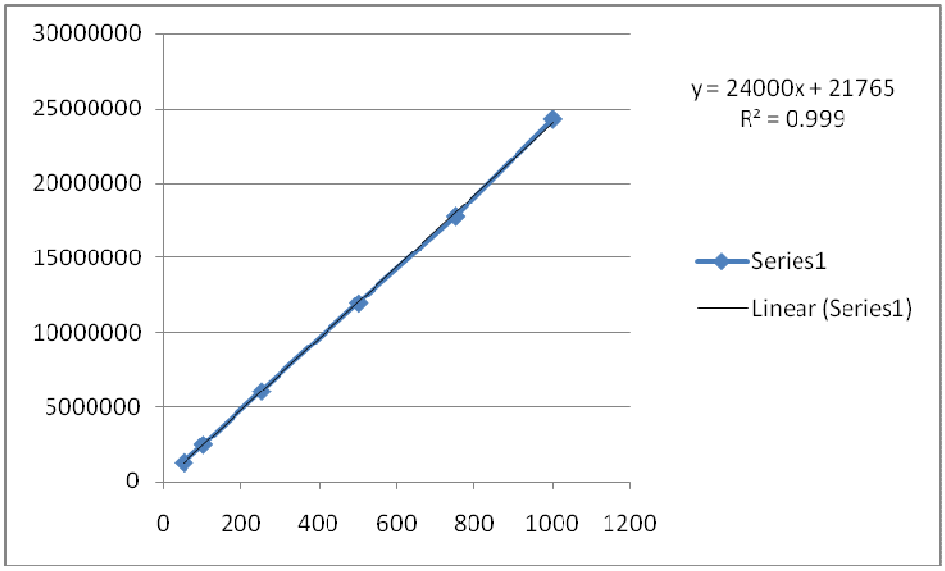


Fig 4 Linearity plot for atorvastatin



The HPLC chromatograms recorded for the drug matrix (mixture of the drug and the excipient) showed almost no interfering peaks within retention time ranges. Figures I and II show the representative chromatograms for standard and the formulation. Thus the proposed HPLC method is selective.

System suitability

For system suitability, six replicates of the working standard sample were injected and the parameters like plate number (N), HETP and peak asymmetry of samples were calculated. These results are shown in table-1.

Linearity

To establish linearity and range, a stock solution containing 1500 µg/ml aspirin, 1000 µg/ml metoprolol, 100µg/ml ramipril and 200 µg/ml atorvastatin were prepared using diluent and further diluted to yield solutions in the concentration range of 75-1500 µg/ml, 50-1000 µg/ml, 5-100 µg/ml, 10-200 µg/ml of aspirin, metoprolol, 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 20 µl in HPLC. Linearity data for ENL, HCT, ASP and ATR are given in the table-2. Linearity plots were shown in figues-III to VI.

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD is defined as the smallest level of analyte that gives a measurable response. LOD is based on S/N ratio (signal/noise) typically for HPLC methods. Six replicates of the analyte were measured.

The LOQ is the lowest concentration that can be quantified reliably with a specified level of accuracy and precision. It is the lowest concentration at which the precision expressed by relative standard deviation (RSD) is less than 2% and accuracy expressed by relative difference in the measured value and true value is also less than 2%. In other words, the analyte response is 10 times greater than the noise response. Six replicates of the analyte were analyzed and quantified.

The LOD and LOQ of aspirin, hydrochlorothiazide, enalapril and atorvastatin were 1.474µg/ml and 4.466µg/ml; 0.737µg/ml and 2.233µg/ml; 0.545µg/ml and 1.653µg/ml and 1.641µg/ml and 4.972µg/ml respectively.

Accuracy

The accuracy of the method was determined by spiking a know mixture of the drugs corresponds to 40 %, 50 % and 60 % of aspirin (200 µg/ml, 250 µg/ml, 300 µg/ml), hydrochlorothiazide (100 µg/ml, 125 µg/ml, 150 µg/ml), enalapril (100µg/ml, 125µg/ml, 150µg/ml) and atorvastatin (400µg/ml, 500 µg/ml, 600 µg/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 table-3.

Precision

The intra- and inter-day precision were determined by analyzing 250 µg/ml aspirin, 125 µg/ml hydrochlorothiazide , 125 µg/ml enalapril and 300 µg/ml atorvastatin on same day and consecutive days, respectively. The intermediate precision was determined by changing column brand also whole experiment was conducted by different analyst on different instrument. The results intraday and interday precisions are depicted in table-4.

Robustness

The robustness of the method was determined as per USP guidelines under a variety of conditions such as change in flow rate and pH of buffer used. The results obtained by deliberate variation in method parameters and data are summarized in table-5.

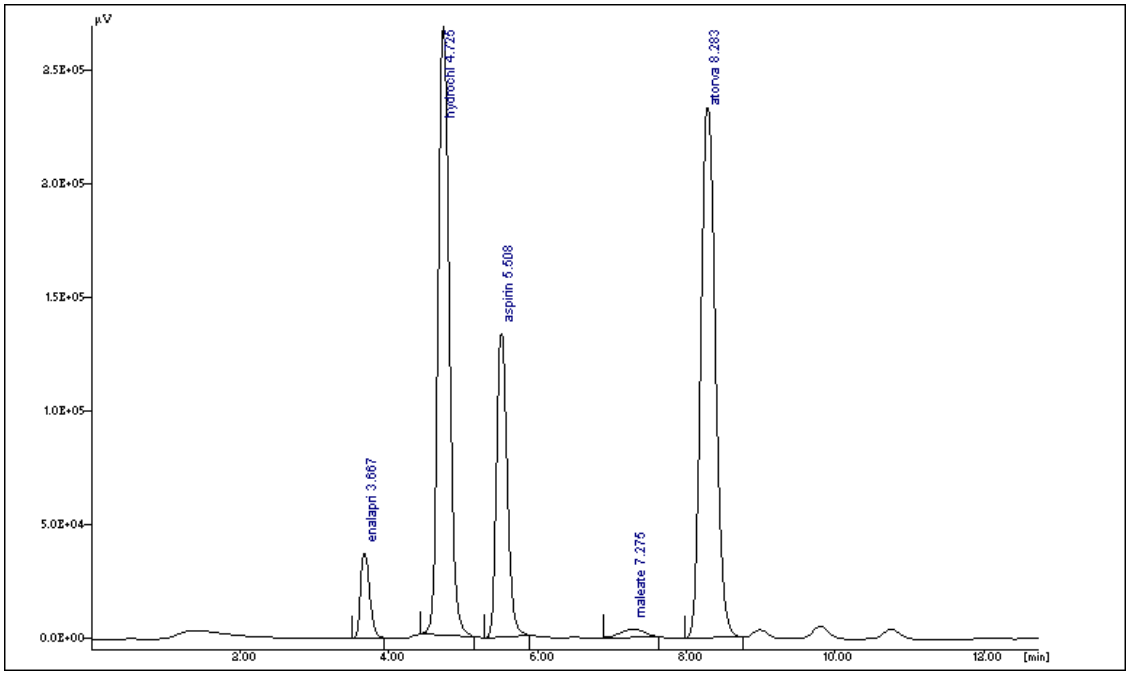


Fig 5 Chromatogram of standard solution of aspirin, enalapril, hydrochlorothiazide and atorvastatin

RESULTS AND DISCUSSION

To optimize the mobile phase, various proportions of buffer with methanol and acetonitrile were tested. The use of acetonitrile, methanol and buffer in the ratio of 50:25:25v/v resulted in peaks with good shape and resolution. A flow rate of 0.6 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 enalapril, hydrochlorothiazide, aspirin and atorvastatin were found to be 3.667, 4.725, 5.508 and 8.283 min respectively. Quantitative linearity was obeyed in the concentration range of 25-500, 12.5-250, 12.5-250 and 50-1000 µg/ml of enalapril, hydrochlorthiazide, aspirin and atorvastatin respectively. The regression equations of concentration over their peak areas were found to be y=20912x+45828 (R^2=0.999), y= 20912x+22914 (R^2=0.999), y= 18084x-19048(R^2=0.999), y= 24000x+21765(R^2=0.999) respectively where y is the peak area and x is concentration of enalapril, hydrochlorthiazide, aspirin and atorvastatin (µg/ml). The number of theoretical plates obtained were 9708.70, 5997.14, 5362.77 and 10276.21 respectively which indicates the efficient performance of the column. The limit of detection and limit of quantitation were found to be 1.474 and 4.466, 0.737 and 2.233, 0.545 and 1.653, and 1.641 and 4.972 µg/ml respectively, which indicates the sensitivity of the method. The high percentage recovery indicates that the proposed method is highly accurate.

CONCLUSION

A simple and efficient HPLC method has been developed, optimized and validated for the isocratic separation and simultaneous determination of ENL, HCT, ASP and ATR in their simulated dosage form. The method, suitable for routine quality control, has been successfully applied to the determination of four analytes in commercial dosage forms.

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