Chromatographic resolution of drug analogues: 3-hydroxy-3-methylglutarylcoenzyme A reductase inhibitors (statins)

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Abstract: A high performance liquid chromatographic method for the simultaneous determination both qualitative and quantitative of cholesterol lowering statin drugs in pharmaceutical formulations has been developed. The most important advantage of developed method is that all seven statin drugs can be determined on a single chromatographic system without modification in detection wavelength. An organic modifier addition (25% v/v methanol) in the presence of buffer (20mM ammonium acetate; pH 4.0 adjusted with dilute acetic acid) played a key role in the resolution of statin drugs in gradient elution with acetonitrile. The drugs were separated on a Purospher Star 4.6mm × 25cm, 5µm, C_{18} column maintained at 25°C with 1mLmin^{-1} flow rate using ultra violet detection at 240nm. Good separation (Rs > 2.5) was achieved in a short analysis allowing simultaneous determination of all seven statins. The effect of variation in flow rate, detection wavelength and column oven temperature was also studied. The proposed method was statistically validated in terms of precision, accuracy, linearity, specificity and robustness. The newly developed method proved to be specific, robust and accurate for the quantification of seven statins in commercial pharmaceutical formulations.

Keywords: Hyperlipidemia, HPLC, 3-hydroxy-3-methylglutarylcoenzyme A reductase inhibitors (HMG-CoA).

INTRODUCTION

Analogue term, derived from Greek and Latin analogia, is frequently used to portray functional and structural similarities. This definition when extended to drug means that analogue of existing drug shares pharmacological and structural similarities with the innovative compound. In the light of this definition three different classes of drug analogues can be established: functional analogues are chemically dissimilar compounds displaying alike pharmacological properties; structural analogues possess structural similarities only and direct analogues possess pharmacological and chemical similarities. Drugs named statins (direct analogues) are among the frequently prescribed agents for mortality and morbidity reduction related to cardiovascular diseases (fig. 1). Atherogenic lipoproteins circulation reduction as a result of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibition is the major therapeutic action of these drugs (Taggart and Jones, 2008). This key enzyme catalyses the production of mevalonate from HMG-CoA, which is a key intermediate in biosynthesis of cholesterol (Endo *et al*., 1976). Clinical studies have discovered that these statins notably reduce heart attack and death risks in patients with established coronary artery disease, besides reducing cardiac events in patients with elevated cholesterol levels (Balk *et al*., 2004). In addition to lowering lipids, statins prevent formation of thrombus, improve endothelial function and maintain plaque stability. There is also an augmented curiosity regarding non-lipid activities of statins such as anti-inflammatory action (Griffin *et al*., 2011).

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In middle as well as in high income countries ischemic heart disease is the foremost reason of death killing over 7 million people each year. Cardiovascular disease is not limited to any specific gender, socio-economic or geographic boundaries and will stay the foremost basis of death globally in future. Therefore it is of significance importance to develop novel analytical methods for these statin drugs. Analytical methods in fact are engaged right throughout the drug life cycle, from drug design and manufacture, clinical trials, elucidating biotransformation means, quality control, fine-tuning of dosage scheme, it's prologue into the market place and pharmacovigilance to drug recycling and dumping with stress on environmental fortification.

All statins may exist in solution in lactone or free acid form. They may also exist as an equilibrium mixture of both lactone and free acid forms in a pH dependant manner (Alberts *et al*., 1980). For samples of lactone and hydroxy acids forms maintaining the solution pH in the region of 4-5 minimizes inter conversion. In order to recover them in high yields massive care must be exercised when handling these compounds and the analytical methods should be designed for the instantaneous quantification of two analytes that can potentially endure inter conversion at some point in analysis.

Literature survey revealed that analytical methods for the estimation of statin drugs have been reported individually, binary or in the form of a combination with some other drugs in pharmaceutical dosage forms as well as in biological samples by spectrophotometry (Tuljarani *et al*., 2010; Ashour *et al*., 2011; Sharma and Bhandari, 2012;

Ashour, 2013; Lotfy and Hegazy, 2013; Naveed, 2014; Ramadani *et al*., 2014), high performance thin layer chromatography (Kumar and Baghyalakshmi, 2007), high performance liquid chromatography (Lily *et al*., 2000; Al-Rawithi *et al.*, 2003; Bahrami *et al.*, 2005; Mehta *et al.*, 2005; Kumar *et al.*, 2006; Pasha *et al*., 2006; Madan *et al.*, 2007; Petkovska *et al.*, 2008; Hameed *et al*., 2009; Gomes *et al*., 2009; Sultana *et al.*, 2010; Shah *et al*., 2011; Sultana *et al.*, 2011a; Sultana *et al.*, 2011b; Kublin *et al*., 2012; Al-Akkam *et al*., 2013; Kumar *et al*., 2013; Fagundes *et al*., 2014), capillary electrophoresis (Guihen *et al*., 2006), ultra performance liquid chromatography (Novakova *et al.*, 2009; Fukiwake *et al*., 2014) and liquid chromatography mass spectrometry (Miao *et al.*, 2003; Hermann *et al.*, 2005; Nirogi *et al.*, 2007; Ghosh *et al*., 2011; Vlckova *et al*., 2011; Macwan *et al*., 2011; Martin *et al.*, 2011; Macwan *et al*., 2012; Polagani *et al*., 2012; Ravi *et al*., 2012; Dhiman *et al*., 2015) but have not come across any method for simultaneous determination of all seven statins by high performance liquid chromatography. The present study was aimed to develop a simple chromatographic method, which will allow the separation, identification and determination of all seven statins in pharmaceutical formulations.

The advantage of developed method for simultaneous quantification of all seven statins is that only one sample is prepared and single chromatographic run is required to provide information on the identity, content uniformity, dissolution and purity of active pharmaceutical ingredients (APIs). Therefore, this method can be handy in daily sample handling in routine, when many samples of statins are analyzed in drug testing laboratories. A method of this kind would allow quantification of available statins in the market without the need of developing a new and separate method for individual statin and can be employed for simultaneous analysis of pharmaceutical dosage forms of statins in routine analysis.

MATERIALS AND METHODS

Instrumentation and reagents

Pravastatin sodium, rosuvastatin calcium, pitavastatin calcium, atorvastatin calcium, fluvastatin sodium, lovastatin and simvastatin used as standards were procured from AbMole Bio Science. Tablets Lipostat (pravastatin) by Bristol-Myers Squibb, Aurora (rosuvastatin) by Ferozsons Laboratories, Pitalo (pitavastatin) by Genix Pharmaceuticals, Lipitor (atorvastatin) by Pfizer, Lescol (fluvastatin) by Novartis, Mevacor (lovastatin) by MSD and Simva (simvastatin) by Nabiqasim were purchased from market. All reagents used in this particular study were of HPLC grade from Merck (Darmstadt, Germany). Acetate buffer of pH 4.0 was prepared from ammonium acetate ($1.54gL^{-1}$) and dilute acetic acid. Standard stock solution of pure drugs was made with diluent (prepared by mixing water, methanol and acetonitrile in 1:2:2 ratio v/v). Chromatography was conducted with a liquid chromatograph from Shimadzu (LC-20AT Series) Japan. Compounds were separated on a Purospher Star 4.6mm × 25cm, 5µm, C_{18} column from Merck (Darmstadt, Germany) maintained at 25°C. Injection volume was 10µL. Mobile phase flow rate employed was $1mLmin^{-1}$; the preferential detection wavelength was 240nm. The run time was 20 minutes for gradient elution. The elaboration of the chromatographic data was carried out from LC Solution software (Version 5.54 SP 5).

Method

Accurately weighed powder equivalent to about 8mg of pitavastatin, 15mg of fluvastatin and 20mg each of pravastatin, rosuvastatin, atorvastatin, lovastatin and simvastatin was transferred to a 100mL volumetric flask, added about 80% of the volume of diluent, dissolved by sonication for 15 minutes with occasional shaking and diluted to volume with diluent to get the desired concentration of $80\mu gmL^{-1}$ of pitavastatin, $150\mu gmL^{-1}$ of fluvastatin, $200\mu gmL^{-1}$ each of pravastatin, rosuvastatin, atorvastatin, lovastatin and simvastatin and filtered through a 0.22µm porosity membrane filter. Proposed method was successful when applied to determine statins in their dosage forms. HPLC chromatogram of sample is shown in (fig. 2).

The results summarized in (table 1) obtained for pravastatin, rosuvastatin, pitavastatin, atorvastatin, fluvastatin, lovastatin and simvastatin were comparable with the corresponding labeled amounts.

RESULTS

Validation results are summarized in (tables 3-9).

DISCUSSION

Method development

The foremost objective of current work was to develop and optimize a single HPLC method capable of simultaneous quantification of statins (pravastatin, rosuvastatin, pitavastatin, atorvastatin, fluvastatin, lovastatin and simvastatin) in pharmaceutical formulations.

For analysis, the combination of ammonium acetate buffer with methanol and acetonitrile was tried as the mobile phase. The effect of change in buffer pH, buffer concentration and acetonitrile percentage was studied. The decisive selection on composition of mobile phase and its flow rate was made on the basis of the shape of the peaks (area, height and asymmetry); drift of baseline, analysis time (run time) and to some extent cost of solvent. Several mobile phase compositions were tried to

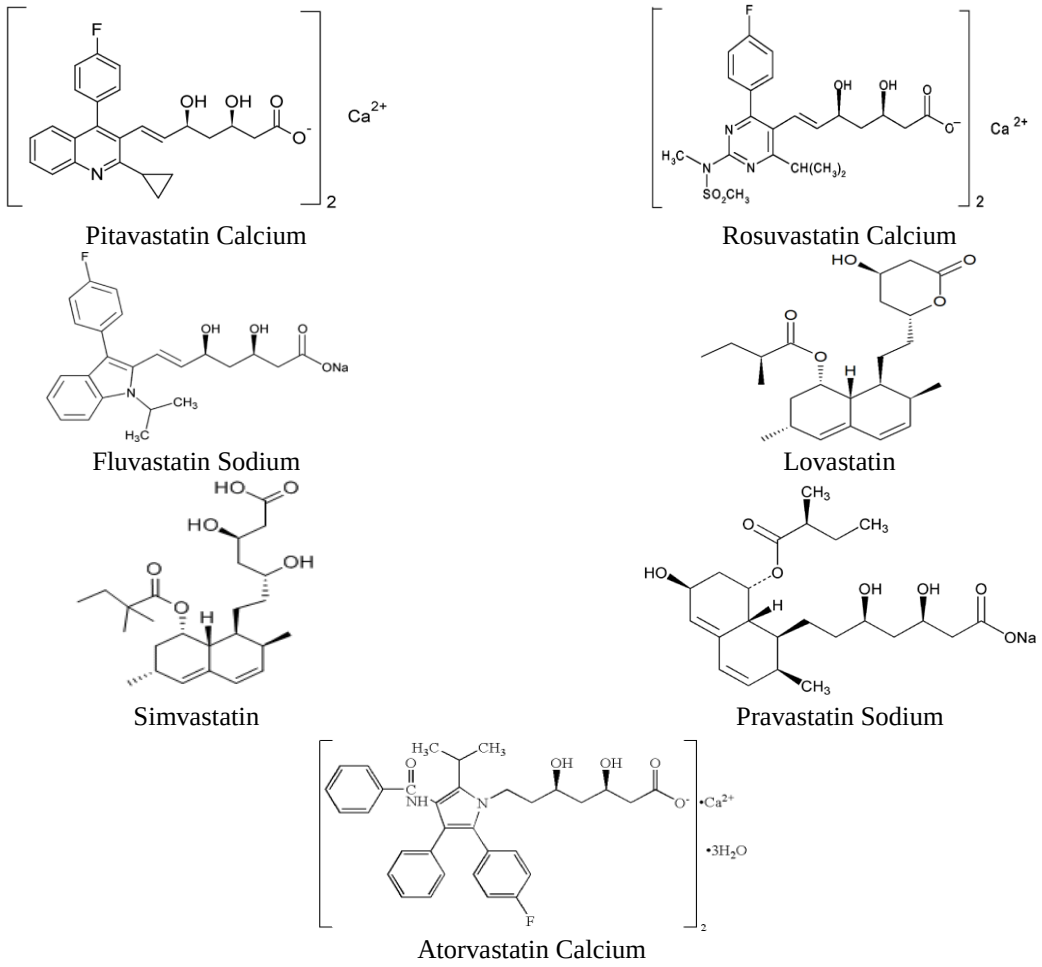

Fig. 1: Chemical structures of the examined statins

optimize LC parameters. From a number of solvents and their combinations mixtures investigated, gradient elution time programme described in (table 2) furnished sharp, well defined peaks with desired resolution and good peak asymmetry. For detection 240nm wavelength was selected because all of the statin drugs have an adequate absorption at this wavelength and extremely low analyte quantities can be correctly detected. Furthermore, at 240nm the calibration curves obtained showed excellent linearity with selected concentration of each statin. With regard to chromatographic procedure, different HPLC columns with C_8 and C_{18} stationary phases and of different dimensions and particle size from 3 to 10µm were evaluated but Purospher Star column with C_{18} stationary phase, 5µm particle size and dimensions i.e. 4.6mm × 25cm from Merck (Darmstadt, Germany) was column of choice to improve peak asymmetry, theoretical plates and resolution with reduced column back pressure at selected flow rate. The temperature of column oven was varied from 25 to 40°C but at 25°C the analysis was preferred as it ended in desired resolution and at the same time with improved peak asymmetry and theoretical plates. The injection volume varied between 5µL to 20µL, at 10µL peak asymmetry was good with good plate count and resolution. On the basis of solubility of all statins as well as stability, a mixture of water, methanol and acetonitrile (1:2:2) v/v was selected as diluent. The run time per sample is 20 minutes. The formulation excipients did not interfere in the precise and accurate estimation of statins drugs in pharmaceutical formulations.

Method validation

ICH guidelines were followed to validate method with respect to specificity, robustness, linearity, accuracy, and precision. Validation results are summarized in (tables 3-9).

Specificity

The method specificity was established by observing lack of interference encountered form the diluent (blank) and excipients of the tablets and capsules (placebo) peaks with the main (principal) peaks. The placebo of finished products was analyzed in triplicate along with standard solution and test solution. The method was specific since there was not any peak at retention times of pravastatin, rosuvastatin, pitavastatin, atorvastatin, fluvastatin, lovastatin and simvastatin. System suitability results are shown in (table 3).

Table 1: Results of commercial formulations.

Sample	Labeled amount (mg)	Amount taken for assay (µg/ml)	Amount found* (µg/ml)	Label claim %
Pravastatin	40	200	198.74 ± 0.32	99.37
Rosuvastatin	10	200	199.66 ± 0.50	99.83
Pitavastatin	4	80	80.29 ± 0.16	100.37
Atorvastatin	10	200	199.24 ± 0.40	99.62
Fluvastatin	20	150	149.70 ± 0.33	99.80
Lovastatin	20	200	198.70 ± 0.22	99.35
Simvastatin	20	200	198.70 ± 0.32	99.35

*Each value is a mean of six observations.

Table 2: Gradient elution time programme.

Time (minutes)	Acetate buffer pH 4.0: Methanol (3:1) % v/v	Acetonitrile HPLC % v/v	Comments
0 – 3	55	45	Isocratic
3 – 6	55 → 30	45 → 70	Linear gradient
6 – 10	30 → 10	70 → 90	Linear gradient
10 – 16	10 → 55	90 → 45	Return to initial composition
16 – 20	55	45	Isocratic re-equilibration

Table 3: System suitability results.

Active Ingredient	Asymmetry	No. of Theoretical Plates (USP)	Resolution (Rs)	Capacity Factor (k')	Separation Factor
Pravastatin	1.267	7340	-	0.496	-
Rosuvastatin	1.194	9871	7.256	1.042	2.139
Pitavastatin	1.091	21679	18.877	2.789	2.652
Atorvastatin	1.163	41954	3.446	3.104	1.114
Fluvastatin	1.211	56653	2.780	3.317	1.067
Lovastatin	1.237	111575	20.351	4.756	1.444
Simvastatin	1.218	123437	5.303	5.124	1.078

Table 4: Precision and intermediate precision results.

Active Ingredient	Precision (Repeatability)		Precision (Reproducibility)	
	System*	Method*	Intra-day*	Inter-day*
Pravastatin	0.16	99.37 ± 0.30	99.37 ± 0.30	99.87 ± 0.60
Rosuvastatin	0.25	99.83 ± 0.30	99.83 ± 0.30	100.44 ± 0.35
Pitavastatin	0.21	100.37 ± 0.58	100.37 ± 0.58	98.63 ± 0.50
Atorvastatin	0.20	99.62 ± 0.31	99.62 ± 0.31	99.79 ± 0.64
Fluvastatin	0.16	99.80 ± 0.22	99.80 ± 0.22	100.33 ± 0.65
Lovastatin	0.11	99.35 ± 0.25	99.35 ± 0.25	99.78 ± 0.76
Simvastatin	0.16	99.35 ± 0.16	99.35 ± 0.16	99.71 ± 0.59

*Each value is a mean of six observations.

Precision (repeatability)

Single analyst demonstrated the precision of the test method in single day. Analyst prepared one standard solution and six sample preparations each of Lipostat 40mg tablets, Aurora 10mg tablets, Pitalo 8mg tablets, Lipitor 10mg tablets, Lescol 20mg capsules, Mevacor 20mg tablets and Simva 20mg tablets and performed the assay as per the developed method. Both system and method precisions were determined by measurement of six replicates of standard and sample respectively. The mean, standard deviation (SD), relative standard deviation (% RSD) and confidence interval of responses were calculated. The % RSD values summarized in (table 4) indicate that proposed method is repeatable.

Table 5: Accuracy results.

Active Ingredient	Amount Added (µg/mL)	Amount Found (µg/mL)	Mean Recovery (%)*	Average Recovery (%)**
Pravastatin	120	120.54	100.76	99.89
	200	199.84	100.22	
	280	275.54	98.70	
Rosuvastatin	120	120.53	100.64	100.96
	200	202.02	101.21	
	280	282.38	101.05	
Pitavastatin	48	47.73	100.68	99.64
	80	78.90	99.84	
	112	108.86	98.40	
Atorvastatin	120	120.58	100.65	100.58
	200	200.92	100.62	
	280	280.87	100.47	
Fluvastatin	90	91.52	101.37	101.12
	150	152.44	101.31	
	210	212.12	100.69	
Lovastatin	120	120.62	101.46	100.29
	200	199.07	100.48	
	280	274.44	98.94	
Simvastatin	120	120.65	101.05	99.83
	200	199.05	100.03	
	280	274.20	98.42	

* Mean of 3 readings for individual level; **Average recovery for all levels

Table 6: Linearity results.

Active Ingredient	Concentration Range (µg/mL)	Correlation Coefficient	% y-intercept	Slope
Pravastatin	120-280	1.0000	2.8	30875.2
Rosuvastatin	120-280	1.0000	0.2	26414.5
Pitavastatin	48-112	0.9999	3.7	53972.8
Atorvastatin	120-280	0.9999	1.0	24121.7
Fluvastatin	90-210	1.0000	1.3	47931.6
Lovastatin	120-280	1.0000	3.4	27432.5
Simvastatin	120-280	1.0000	3.5	29326.6

Table 7: Robustness results (cumulative % RSD of precision and robustness).

Active Ingredient	Change in Flow Rate (mL/min)		Change in Detection Wavelength (nm)		Change in Column Temperature (°C)	
	0.8	1.2	238	242	23	27
Pravastatin	0.28	0.32	0.29	0.29	0.28	0.30
Rosuvastatin	0.35	0.54	0.37	0.34	0.32	0.38
Pitavastatin	1.04	0.74	0.84	0.99	0.91	0.86
Atorvastatin	0.38	0.31	0.31	0.36	0.32	0.31
Fluvastatin	0.22	0.39	0.38	0.34	0.30	0.38
Lovastatin	0.30	0.41	0.44	0.49	0.48	0.42
Simvastatin	0.21	0.33	0.34	0.36	0.36	0.33

Intermediate precision (reproducibility)

Method precision procedure was repeated on a different day, by a different analyst, using a different HPLC system. Intra-day and inter-day precisions, both were calculated. The method is reproducible since differences between RSD (%) for intra-day and inter-day precision (table 4) were not significant.

Accuracy

Standard addition method was employed in recovery experiment. Placebo of finished products was spiked with pravastatin, rosuvastatin, pitavastatin, atorvastatin, fluvastatin, lovastatin and simvastatin API stock solution in triplicate at 60%, 100% and 140% concentration used in the method and the difference between the theoretical

Table 8: Limit of detection and limit of quantitation.

Active Ingredient	Limit of Detection (ppm)	DL Co-efficient	Limit of Quantitation (ppm)	QL Co-efficient
Pravastatin	1.224	3.3	3.711	10
Rosuvastatin	3.915		8.943	
Pitavastatin	8.730		26.45	
Atorvastatin	5.857		17.749	
Fluvastatin	2.219		6.726	
Lovastatin	2.366		7.171	
Simvastatin	3.133		9.495	

Table 9: Analytical solution stability results (cumulative % RSD of precision and solution stability).

Active Ingredient	Precision (% label claim*)	Stability 24 Hours (% label claim**)	Cumulative RSD (%)
Pravastatin	99.37	99.58	0.29
Rosuvastatin	99.83	100.78	0.46
Pitavastatin	100.37	100.95	0.59
Atorvastatin	99.62	99.54	0.29
Fluvastatin	99.80	99.77	0.20
Lovastatin	99.35	99.00	0.27
Simvastatin	99.35	99.13	0.17

*Each value is a mean of six observations; **Mean of three readings.

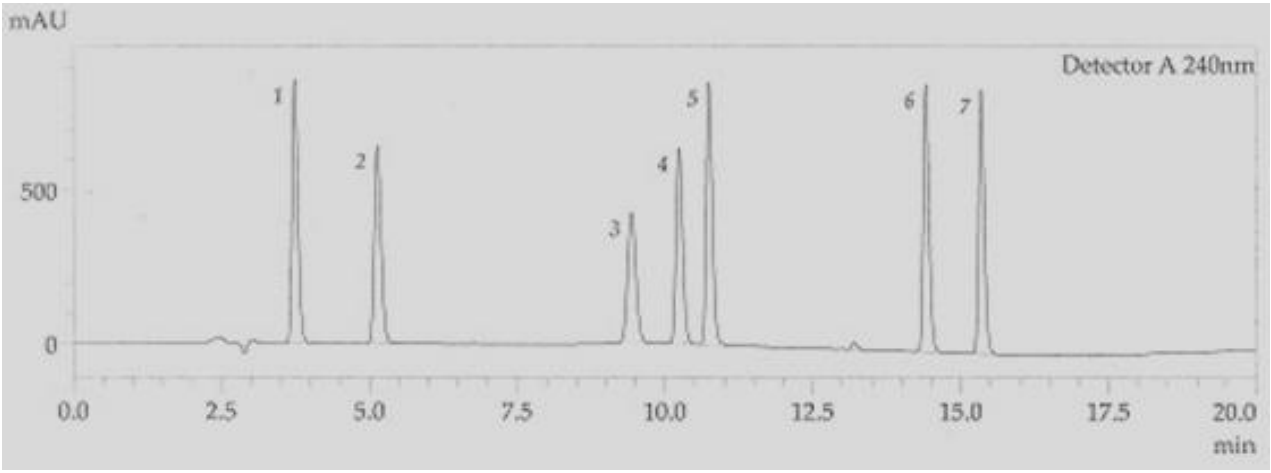

Fig. 2: HPLC chromatogram of sample solution – showing separation of statins like Pravastatin (1), Rosuvastatin (2), Pitavastatin (3), Atorvastatin (4), Fluvastatin (5), Lovastatin (6) and Simvastatin (7).

value and the actual value found in the analyses are reported (table 5).

Linearity and range

To demonstrate the linearity, five standard solutions ranging from 60%-140% of assay sample concentration of pravastatin, rosuvastatin, pitavastatin, atorvastatin, fluvastatin, lovastatin and simvastatin were assayed on HPLC as per the developed method. The responses of each solution were recorded and linearity results were calculated for slope of regression line, % y-intercept and correlation co-efficient (table 6). The concentration ranges were found to be 120-280µg/ml for pravastatin, rosuvastatin, atorvastatin, lovastatin and simvastatin, 48-112µg/ml for pitavastatin and 90-210µg/ml for fluvastatin over which Beer Lambert's law is obeyed by drugs.

Robustness

Method robustness was determined to evaluate the effect of deliberate variation of the chromatographic conditions, though small in magnitude on the quantification of statins. Robustness was dogged by changing; flow rate of mobile phase, analytical wavelength and column oven temperature. The difference between the results ascertained under varied parameters and normal conditions are insignificant. Hence the method is termed as robust (table 7).

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

Detection and quantitation limits were derived from the residual standard deviation of regression line constructed from standards (five levels) prepared for linearity studies. Detection and quantitation limit co-efficient were 3.3 and 10.0 respectively. The data (table 8) shows that proposed method is sensitive for detection and quantitation of statins.

Analytical solution stability

The drug solution stability during analysis was evaluated by repetitive analysis of sample solution prepared for precision study after storage of drug solution for 24 hours under laboratory bench conditions against freshly prepared reference solution to establish the solution stability period. The cumulative % RSD of content of initial analysis and solution stability were less than 2.0%, also no extraneous peak of impurities or degradants is observed in chromatogram of sample for chromatographic method proving the solution stability for the predetermined time interval (table 9).

CONCLUSION

The developed method enabled quantification of the statin drugs with reasonably good precision and accuracy in solid pharmaceutical dosage forms. In all cases recoveries obtained were good. The statins can be determined efficiently with satisfactory precision in oral dosage forms with the proposed method. This method is measured straightforward, consistent and discriminating providing results with adequate accuracy and precision with lower detection and quantification limits, thus making it further sensitive and specific. Furthermore, shorter analysis time for statins makes this analytical method apposite for routine analysis of pharmaceutical dosage forms.

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