APPLICABILITY OF COMPREHENSIVE MOBILE PHASE IN VALIDATED BIO ANALYTICAL QUANTITATIVE ESTIMATION OF ROSUVASTATIN, EZETIMIBE, AMLODIPINE, VALSARTAN, LERCANIDIPINE AND FLUVASTATIN IN HUMAN PLASMA BY RP-HPLC METHOD

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ABSTRACT

A bio analytical method was developed with compatible mobile phase for estimation of drugs viz. rosuvastatin and ezetimibe, amlodipine and valsartan, lercanidipine and fluvastatin in human plasma. Liquid chromatographic system takes ample of time to stabilise and to attain equilibrium on changeover of mobile phase. Compatible mobile phase was beneficial to separate and estimate many drugs simultaneously; and saved time and solvent consumption. The technique was developed and equipped with reverse phase high performance liquid chromatograph (RP-HPLC), using RP - C_{18} column with UV detector. For estimation of all these drugs, the mobile phase [acetonitrile: acetate buffer pH 4.0 (pH adjusted with acetic acid)] was pumped at a flow rate of 0.8 mL min^{-1} in the ratio of 60:40 V/V and the eluents were monitored at 245 nm. The method was studied and validated as per ICH guidelines and US FDA guidelines for selectivity, specificity, accuracy and precision and stability study. All these drugs shown linear response between concentration and response as a peak area and chromatograms with well resolved peaks were sign of methods efficiency. Statistical data of calibration graph to study linearity of these drugs in biological matrix was found within prescribed limit. Prepared quality control samples were estimated, accuracy and precision results of rosuvastatin and ezetimibe were 97-100.93 as % nominal concentration and 0.8210-2.0367 as % CV for amlodipine and valsartan 98.43-99.81 as % nominal conc. and 0.9480-2.4753 as % CV and for lercanidipine and fluvastatin 97.18-103.5 as % nominal conc and 0.5258-3.8301 as % CV and were within prescribed limit. The bio analytical method is simple as it is free from solvent extraction and solid phase extraction, precise, accurate, and consumes less solvent due to compatible mobile phase. The method suits for quantification of these drugs in plasma, hence applied for bio equivalence, bioavailability study in real clinical samples.

INTRODUCTION

The risk of non-communicable diseases such as cardiovascular diseases, diabetes and cancer is rapidly replacing infectious diseases, causing increased morbidity and mortality in developing countries. Cardiovascular drugs play important role in controlling disorders like hypertension, hyperlipidaemia, angina and cardiac arrhythmia. Also, rational use and benefits of combination drug therapy of cardiovascular agents is proven. Concentration measurement of drug(s) and their metabolite(s) in biological matrices are an important aspect of drug development.

Rosuvastatin (RV), chemically 7-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-(methyl-methylsulfonyl-amino)-pyrimidin-5-yl] 3, 5-dihydroxy-hept-6-enoic acid, is one of the more recently introduced statins. Statins are HMG-CoA reductase inhibitors. The conversion of 3-hydroxy 3 methyl glutaryl (HMG)-COA to mevalonic acid is especially important, because it is a primary control site for cholesterol bio synthesis[1-4].

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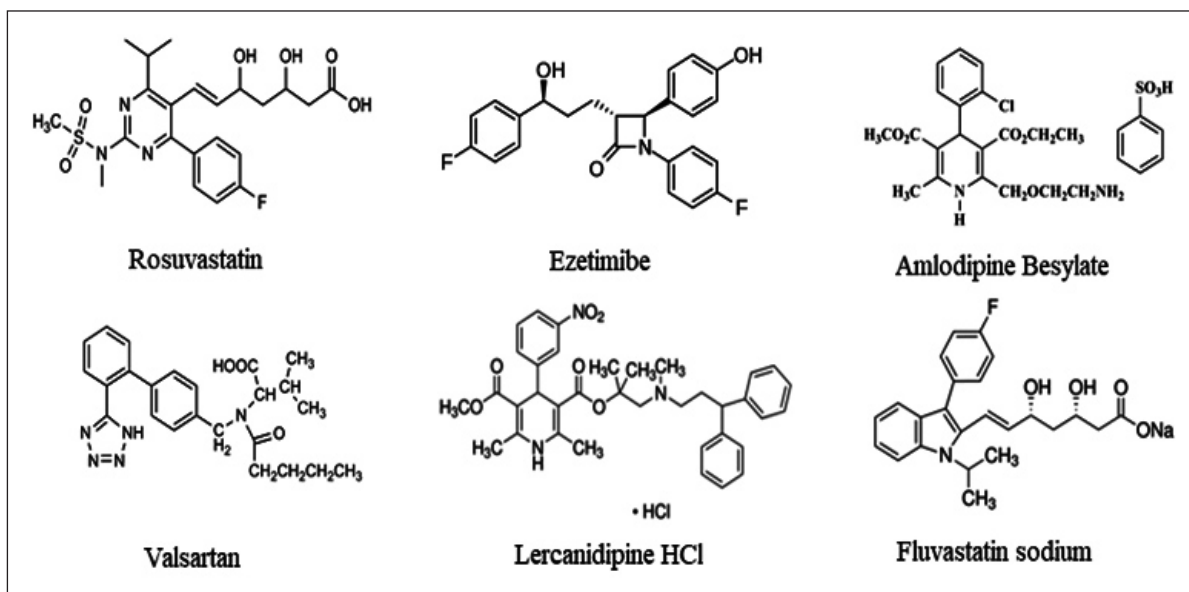*For Correspondence: E-mail: dyadegk@gmail.com

Fig. 1: Chemical structure of drug molecule

Methods such as bio analytical mass spectrometry[5] and RP-HPLC[6-9] have been reported for estimation of RV alone or in combined formulation with other drugs.

Ezetimibe (EZE), chemically (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone[1], is an antihyperlipidemic agent that has usefulness in lowering cholesterol levels[2]. It acts by decreasing cholesterol absorption in the intestine by blocking the absorption of the sterol at the brush boarder. Specifically, the β-lactam binds to the Niemann-pick C1 like 1(NPC1L1) protein on the gastrointestinal tract that is responsible for cholesterol absorption[3]. Although it may be used alone, it is marketed as a combination product with simvastatin.

Various analytical techniques have been reported in the literature for the estimation of EZE alone or in combination with other anti-hypertensive agents or antihyperlipidemic drugs in pharmaceutical dosage forms. These include RP-HPLC[10-11], degradation study[12], UV spectroscopy[13-15] and bio analytical method[16].

Amlodipine besylate (AD), chemically, 2 - [(2 - amino ethoxy) - methyl] - 4 - (2 - chloro phenyl) -1, 4 -dihydro - 6 - methyl - 3, 5 - pyridine dicarboxylic acid 3 - ethyl - 5 - methyl ester, benzene sulfonate, is a potent dihydro calcium channel blocker[1-4]. Although it is usually used alone, amlodipine is also marketed as a combined formulation with valsartan.

Various analytical techniques have been described in the available literature for the estimation of AD alone

or in combination with other angiotensin inhibiting drug in pharmaceutical formulations. These include UPLC-MS/MS bio analytical[17-19] and UV spectroscopy[20].

Valsartan (VAL), chemically, N - (1 - oxopentyl) - N - [(2' - (1H - tetrazol - 5 - yl) (1, 1' - biphenyl) - 4 - yl) methyl] - L - valine, is a potent angiotensin receptor blocker[1-4]. Methods such as bio analytical[21] and RP-HPLC[22] have been reported for detection of VAL alone or in combined formulation along with other drug.

Lercanidipine (LER), chemically 1,4 dihydro-2,6 dimethyl-4(3-nitrophenyl)-3-5-pyridinedicarboxylic acid-3-[2[(3,3 diphenyl propyl)methyl amino]-1,1 dimethyl ethyl] 5 methyl ester, is a dihydropyridine calcium channel blocker with actions similar to those of nifedipine. It is used in the treatment of hypertension. Although it may be used alone, lercanidipine is also marketed as a combined product with atenolol[1,3].

In the reviewed literature, various analytical techniques have been reported for the estimation of LER alone or in combination with other anti - hypertensive agents or antihyperlipidemic drugs in pharmaceutical formulations. These include UPLC-MS/MS bio analytical method[23], LC-MS/MS bio analytical method[24], planar chromatography[25] and HPLC[26, 27].

Fluvastatin (FV), chemically sodium (3R*5S*6E)-7-[3-(4-fluoro-phenyl)-1-(propan-2-yl)-1H-indol-2-yl]-3,5-dihydroxy-6 heptanoate, is 3-hydroxy 3-methyl glutaryl coenzyme a (HMG-COA) reductase inhibitor and is a lipid regulating drug with the actions on plasma lipids

Table I: Results of calibration study of drugs

Drug Parameter	RV	EZE	AD	VAL	LER	FV
Regression equation	Y=0.0354X - 0.226	Y=0.0292X + 0.1275	Y=0.0188X - 0.3294	Y=0.0223X + 0.149	Y=0.04X + 0.0891	Y=0.0169X – 0.0047
r^2	0.9999	0.9999	0.9987	0.9996	0.9999	0.9999
Wavelength	245 nm	245 nm	245 nm	245 nm	245 nm	245 nm
Conc range	320-3200 ng mL^{-1}	320-3200 ng mL^{-1}	320-2560 ng mL^{-1}	320-5120 ng mL^{-1}	80-480 ng mL^{-1}	320-1000 ng mL^{-1}

r^2 - Correlation coefficient

analogous to those of simvastatin. It is used to lower LDL cholesterol, total cholesterol, apo lipoprotein B and triglycerides and to upsurge HDL-cholesterol in the treatment of hyperlipidaemias[1-4].

Methods such as UV spectrophotometric methods[28, 29] are reported for the estimation of FV alone or in combination with other drugs.

Amlodipine, valsartan, rosuvastatin and fluvastatin are official in BP[30] while rosuvastatin, ezetimibe, amlodipine, valsartan and fluvastatin are official in IP[31].

The chemical structures of RV, EZE, AD, VAL, LER and FV are shown in Fig. 1.

Literature survey reported that few bio analytical techniques have been described for the estimation of these drugs individually as well as in combination with other cardiovascular drugs[5,6]. A LC-MS/MS bio analytical method have been reported to quantify some of these drugs in human plasma[5,24], so we tried to develop bio analytical method for quantification of drug employing protein precipitation to separate plasma. In the present work, an attempt has been made to develop a bio analytical method for simultaneous detection of these selected drugs in human plasma. HPLC methods are extensively applied for routine quality control estimation of drugs because of their accuracy, repeatability, selectively, sensitivity and specificity. Validation of bio analytical method is essential before use in accordance with ICH guidelines[32] and US FDA guidelines[33], so the proposed RP-HPLC method was validated by evaluating its selectivity, accuracy, precision and sensitivity.

MATERIALS AND METHODS

Instrumentation

Analysis was performed with a Shimadzu (Japan) Prominence chromatograph equipped with an LC-20 AT solvent delivery system, a universal loop injector (Rheodyne 7725) of injection capacity 20 µL, and an SPD - 20 A UV–Visible detector set at 245 nm. The equipment was controlled by a PC work station with clarity software. Compounds were separated on Phenomenex Luna C$_{18}$ column (250 mm × 4.6 mm i.d., 5-µm particle size) under reversed phase partition conditions. For estimation of these drugs, the optimized mobile phase was 60:40 (V/V) mixture of acetonitrile: acetate buffer pH 4.0 ± 0.2 (adjusted with glacial acetic acid). The flow rate and the run time were kept at 0.8 mL min^{-1} and 10-12 min, respectively. For weighing of the drugs, weighing balance Dhona 100 DS model no 11526 and Afcoset ER 200A accuracy ±0.1 mg make (The Bombay Burmah Trading Corp. Ltd Mumbai. Sr No 0412097) was utilised. For plasma separation from blood- programmable, microcentrifuge (Bioera Life Sciences Pvt. Ltd.) Sr No LQK 236 A was utilised. For drug plasma matrix mixing, vortex mixer (Yorco Sales Pvt. Ltd.) was utilised.

In the bio analytical method, both the mobile phase and sample solutions were degassed by the use of a sonicator (Lab Man Scientific Instruments Chennai, 1.8 L, serial No. L 6732) and these were filtered through a 0.22 µm filter (Pall Corporation, Mumbai). The identities of the analytes were established by comparing the retention time of analytes in the mixed solution with those in standard solutions. Chromatography was performed at room temperature, and temperature of flow cell was kept at 40 ± 1 °C. The UV spectrum of all these drugs for selecting the working wavelength of detection was taken using a Shimadzu-1700 a double beam UV - Visible spectrophotometer (Shimadzu, Kyoto, Japan).

Reagents and chemicals

A pharmaceutically pure sample of VAL (USP grade with 99.1 % w/w purity) was procured from FDC Ltd., Mumbai, AD (IP grade with 99.3 % w/w) was procured from Smruthi Organics Ltd., and RV (Eur Ph grade with 99.7 % w/w), EZE (IP grade 100.2 % w/w), LER (99.5 % w/w purity) and FV (99.3 % w/w purity) were procured
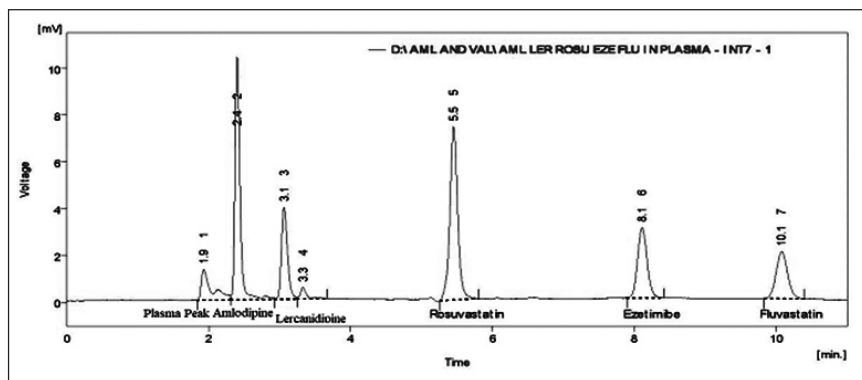
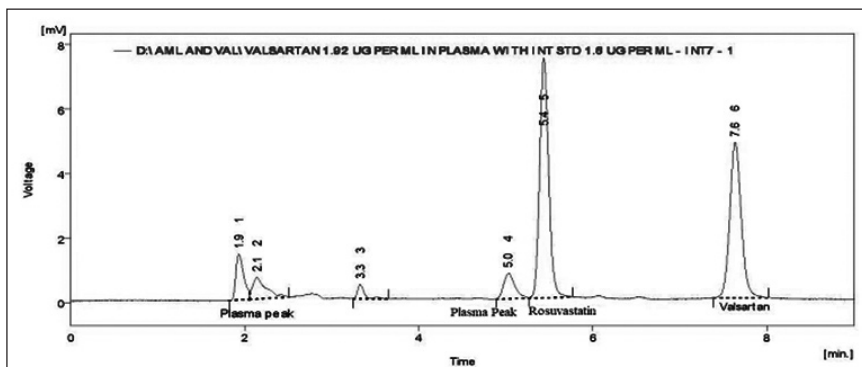**Fig. 2: Optimized chromatograph of mixed drug in comprehensive mobile phase**

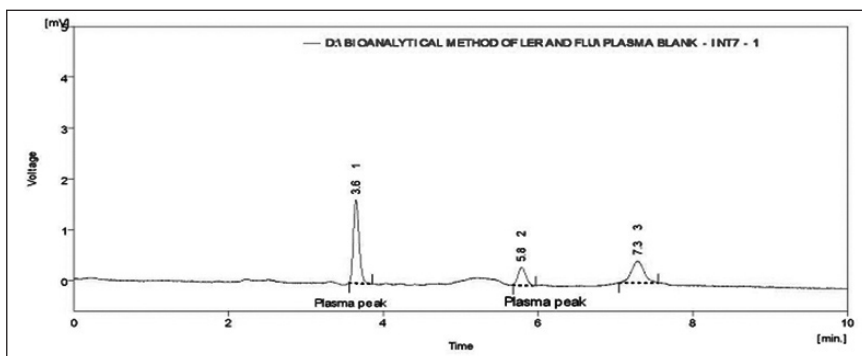**Fig. 3: Optimized chromatograph of mixed drug in comprehensive mobile phase**

Fig. 4: Chromatograph of blank plasma in optimized mobile phase

and obtained from Swapnroop Drugs and Pharma, Aurangabad. HPLC grade acetonitrile and water were procured from Merck Life Sciences Pvt. Ltd., Mumbai and acetic acid (HPLC grade) was procured from Qualigens India Pvt. Ltd., Mumbai, India.

Collection of Plasma

For plasma collection, human blood sample was drawn. Blood was centrifuged (Micro Centrifuge Bio Era Life Sciences Pvt. Ltd.) at 4000 RPM for 10 min. The supernatant clear plasma was carefully recovered in clean and dry tubes, stored in defreezer till use in the bio analytical method.

Preparation of standard solutions of RV, EZE, AD, VAL, LER and FV

About 4 mg of each drug was separately weighed accurately and transferred to separate 10 mL volumetric flasks. The pure drugs were solubilized in mobile phase and made the volume up to the mark i.e. 10 mL with mobile phase. Sample was sonicated for 10 min for degassing and the solution was marked as standard stock solution. Secondary working stock solution of each drug with concentartion 40 µg mL^{-1} was separately prepared from aliquots of primary stock solution.

Combined drug elution in optimized mobile phase

Mobile phase was prepared and pumped at a flow rate 0.8 mL min^{-1}. Also mixed standard solutions of all these drugs were prepared. Freeze dried plasma was thawed and 200 µL of plasma was transferred into 5 mL volumetric flask, 1 mL of mixed standard solution added, vortexed for 5 min; and volume was made to 5 mL with mobile phase and again homogenized on vortex shaker for 10 min. Further, drug-plasma admixture was centrifuged at 7000 rpm (5 min). Clear separated supernatant liquid was filtered through 0.22 µ syringe filter and aliquots of solution were further diluted, degassed and 20 µL injected into the column to obtain chromatograph.

Drugs in plasma matrix for plot of calibration curve

ICH guidelines[32] and US FDA guidelines[33] recommend calibration standards should be prepared in the same biological matrix as the study samples. The calibration range is defined by the LLOQ (Lower limit of quantification), which is the lowest calibration standard, and the ULOQ (Upper limit of quantification), which is the highest calibration standard. There should be one calibration curve for each analyte studied during method validation and for each analytical run. A calibration curve

Table II (a): Results of accuracy and precision for RV and EZE

Parameter	Concentration							
	LLOQ		LQC		MQC		HQC	
	320 ng mL^{-1}		960 ng mL^{-1}		1600 ng mL^{-1}		2560 ng mL^{-1}	
	RV	EZE	RV	EZE	RV	EZE	RV	EZE
Within batch n=5								
Mean	313.6	323	955.8	953.4	1552	1570	2520	2506
% CV	2.0367	1.7919	1.2909	0.8210	1.2393	1.7443	0.8875	1.7952
% Nominal	98.00	100.93	99.56	99.31	97.00	98.12	98.43	97.89

Table II (b): Results of accuracy and precision for AD and VAL

Parameter	Concentration							
	LLOQ		LQC		MQC ng mL^{-1}		HQC ng mL^{-1}	
	320 ng mL^{-1}		960 ng mL^{-1}		1280	1920	1920	3560
	AD	VAL	AD	VAL	AD	VAL	AD	VAL
Within batch n=5								
Mean	319.4	315	946.4	952.2	1239	1905.2	1905	3512
% CV	2.2628	2.4753	1.3241	1.2503	1.9511	0.9480	1.0495	1.5428
% Nominal	99.81	98.43	98.58	99.18	96.79	99.22	99.21	98.65

Table II (c): Results of accuracy and precision for LER and FV

Parameter	Concentration							
	LLOQ ng mL^{-1}		LQC ng mL^{-1}		MQC ng mL^{-1}		HQC ng mL^{-1}	
	80	320	240	640	320	720	400	800
	LER	FV	LER	FV	LER	FV	LER	FV
Within batch n=5								
Mean	82.8	324	238	639.6	311	714.2	404.6	798
% CV	0.5258	3.8301	3.0875	1.1125	3.1174	4.9871	1.4326	2.5282
% Nominal	103.5	101.25	99.16	99.93	97.18	99.19	101.15	99.75

should be generated with a blank sample, LLOQ and the ULOQ.

100 µL of freeze dried thawed plasma was transferred into 5 mL volumetric flask, 40 µL of RV of conc. 40 µg mL^{-1} added, vortexed for 5 min; and volume was made to 5 mL with mobile phase and again homogenized on vortex shaker for 10 min. Further, drug-plasma admixture was centrifuged at 7000 rpm (5 min). Clear separated supernatant liquid was filtered through 0.22 µ syringe filter and filtrate was stored in vial, labelled as RV of conc 320 ng mL^{-1}. Similar procedure was followed to prepare RV of conc. in the range 320-3200 ng mL^{-1}; this process was repeated without drug to obtain blank plasma. Similarly, calibration standards of EZE, AD, VAL, LER and FV in plasma were also prepared in defined conc. range. To know interference in detection, chromatograph of mixed standard solution was also obtained.

Preparation of quality control (QC) samples in plasma matrix

Quality control (QC) samples, a requisite for bio analytical method validation, were prepared in the laboratory as per ICH guidelines[32] and US FDA guidelines[33]. During method validation, the QCs should be prepared at a minimum of 4 concentration levels within the calibration curve range: the lowest quality control sample (LLOQ), within three times of the LLOQ (low QC i.e. LQC), around 30 - 50% of the calibration curve range (medium QC i.e. MQC) and at least 75 % of the ULOQ (high QC i.e. HQC).

Table III (a): Results of stability studies for RV and EZE

Parameter	Short term stability				Freeze thaw stability				Long term stability			
	LQC		HQC		LQC		HQC		LQC		HQC	
	960 ng mL^{-1}		2560 ng mL^{-1}		960 ng mL^{-1}		2560 ng mL^{-1}		960 ng mL^{-1}		2560 ng mL^{-1}	
	RV	EZE	RV	EZE	RV	EZE	RV	EZE	RV	EZE	RV	EZE
Mean	944.3	940	2516.6	2506.6	949	951.3	2550	2533.3	918.66	915.3	2431.6	2503.3
% CV	2.2979	2.2471	2.2981	2.3570	2.1952	1.4672	1.3305	2.1462	1.3778	1.4080	2.2067	1.0365
% Nominal	98.36	97.91	98.30	97.91	98.85	99.09	99.60	98.95	95.69	95.34	94.98	97.78

Table III (b): Results of stability studies for AD and VAL

Parameter	Short term stability				Freeze thaw stability				Long term stability			
	LQC		HQC ng mL^{-1}		LQC		HQC ng mL^{-1}		LQC		HQC ng mL^{-1}	
	960 ng mL^{-1}		1920	3560	960 ng mL^{-1}		1920	3560	960 ng mL^{-1}		1920	3560
	AD	VAL	AD	VAL	AD	VAL	AD	VAL	AD	VAL	AD	VAL
Mean	950	940	1906.6	3486.6	926.6	920	1906.6	3510	890	880	1856.6	3486.6
% CV	2.2979	2.2471	2.2981	2.3570	2.1952	1.4672	1.3305	2.1462	1.3778	1.4080	2.2067	1.0365
% Nominal	98.95	97.91	99.30	97.93	96.52	95.83	99.30	95.83	92.70	91.66	96.70	97.94

Table III (c): Results of stability studies for LER and FV

| Parameter	Short term stability				Freeze thaw stability				Long term stability			
	LQC ng mL^{-1}		HQC ng mL^{-1}		LQC ng mL^{-1}		HQC ng mL^{-1}		LQC ng mL^{-1}		HQC ng mL^{-1}	
	240	640	400	800	240	640	400	800	240	640	400	800
	LER	FV	LER	FV	LER	FV	LER	FV	LER	FV	LER	FV
Mean	236.6	633.66	393.3	786.66	236.66	626.6	405	740	220	621.66	368.3	776.6
% CV	2.6423	1.9146	3.1735	2.1624	2.6423	2.0941	3.0240	4.8014	3.7204	1.3684	1.6945	1.6061
% Nominal	98.58	99.7	98.32	98.33	98.60	97.91	101.2	92.5	91.66	97.13	92.07	97.03
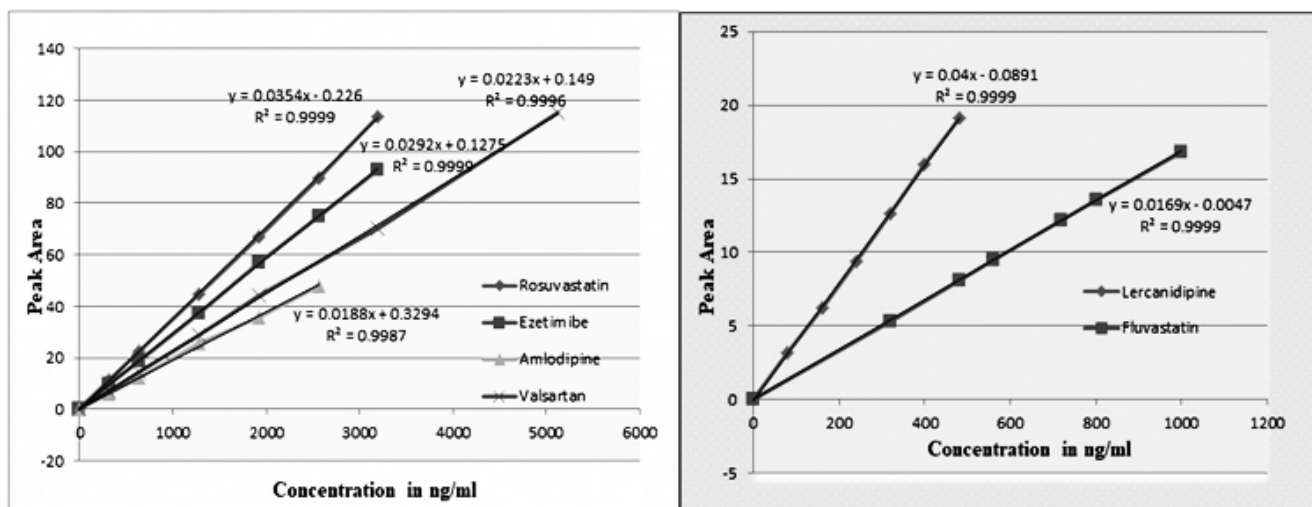
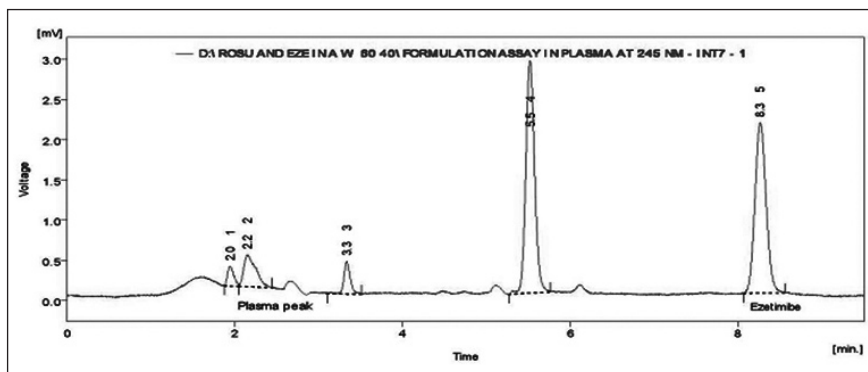Fig. 5: Calibration curve of all six drugs in plasma

Fig. 6: Accuracy and precision study of RV and EZE in plasma
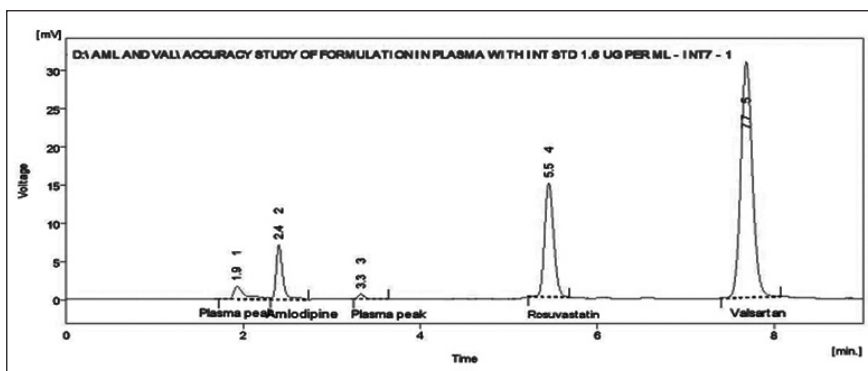

Fig. 7: Accuracy and precision study of AD and VAL in plasma

Quality control samples of RV and EZE

QC samples were prepared by dissolving drug powder equivalent to 4 mg each of RV and EZE in mobile phase into 10 mL volumetric flask. It was mixed well and sonicated for 10 min and secondary stock solution was obtained from main stock. 200 μL of plasma was transferred into 5 mL volumetric flask, 0.2 mL of mixed solution containing 40 μg mL^{-1} each of RV and EZE added, vortexed for 5 min, and volume was made to 5 mL with mobile phase and homogenized on vortex shaker (Vortex Mixer Yorco Sales Pvt. Ltd.) for 10 min. It was centrifuged and the supernatant liquid was filtered through 0.22 μ syringe filter and labelled as 1600 ng mL^{-1} (MQC) sample of RV and EZE. Similarly 320 ng mL^{-1} (LLOQ), 960 ng mL^{-1} (LQC) and 2560 ng mL^{-1} (HQC) were obtained in plasma by using aliquots of secondary stock solutions.

Quality control samples of AD and VAL

The QC samples containing AD and VAL each 320 ng mL^{-1} (LLOQ), AD and VAL each 960 ng mL^{-1} (low QC i.e. LQC), AD 1280 ng mL^{-1} and VAL 1920 ng mL^{-1} (medium QC i.e. MQC); and AD 1920 ng mL^{-1} and VAL 3560 ng mL^{-1} (high QC i.e. HQC) were prepared and obtained in the plasma using aliquots of AD and VAL from their secondary stock solutions.

Quality control samples of LER and FV

The QC samples containing LER 80 ng mL^{-1} and FV 320 ng mL^{-1} (LLOQ), LER 240 ng mL^{-1} and FV 640 ng mL^{-1} (LQC), LER 320 ng mL^{-1} and FV 720 ng mL^{-1} (MQC), and LER 400 ng mL^{-1} and FV 800 ng mL^{-1} (HQC) were prepared in the plasma using aliquots of secondary stock solutions of LER and FV.

Validation

ICH guidelines[32] stated the objective of the bio analytical method validation to be demonstrate that it is suitable for its intended purpose. Bio analytical method validation is essential to ensure the acceptability of method performance and the reliability of analytical results. The results of pivotal nonclinical toxicokinetic/pharmacokinetic studies and of clinical trials, including comparative bioavailability/bioequivalence studies, are utilised to make regulatory decisions regarding the safety and efficacy of drug products[34]. Efforts were given to develop well characterized, appropriately validated and documented bio analytical method which one generated reliable data to support regulatory guidelines. Validation of bio analytical method is described in ICH guidelines[32] and US FDA guidelines[33]. The method was validated for selectivity, specificity, calibration curve, accuracy and precision and stability.

Selectivity

Selectivity is nothing but the ability of an analytical method to differentiate and quantify the analyte along with or in the presence of other components in the blank biological matrix. Evidence was provided by injecting 20 μL blank plasma in column to obtain chromatograph.

Specificity

Specificity is the capability of a bio analytical method to detect and differentiate the analyte from other substances, for specificity study mixed solution of RV, EZE, AD, LER, FV and VAL was used; and injected to obtain chromatograph.

Calibration curve of drugs in plasma matrix and plot of calibration curve

Calibration curve of each drug was generated by preparing standard solutions in conc. range RV (320-3200 ng mL^{-1}), EZE (320-3200 ng mL^{-1}), AD (320-2560 ng mL^{-1}), VAL (320-5120 ng mL^{-1}), LER (80- 480 ng mL^{-1}) and FV (320-1000 ng mL^{-1}). All solutions were degassed and injected 20 µL into the column to obtain chromatograph. The obtained chromatograph was read and the peak areas were measured. Peak areas were then plotted on the ordinate against their respective conc. on abscissa. Standard regression curve analysis was obtained by use of Microsoft Office Excel software and correlation coefficient, slope and standard deviation were obtained.

Accuracy and precision

The accuracy was determined by replicate analysis of samples containing added known amount of the analyte. Accuracy and precision was studied by preparing and measuring five determinations per concentration, in each analytical run.

Analyte peak response should be identifiable, discrete, and reproducible, and the back calculated concentration should have the overall accuracy at each concentration level within ±15 % of the nominal concentration, except at the LLOQ, where it should be within ±20 %. The precision (% CV) of the concentrations determined at each level should be within 15 %, except at the LLOQ, where it should not exceed 20 % as stated in ICH guidelines[32] and US FDA guidelines[33].

Study for RV and EZE

The QC samples containing RV and EZE each 320 ng mL^{-1} (LLOQ), 960 ng mL^{-1} (low QC), 1600 ng mL^{-1} (medium QC); and 2560 ng mL^{-1} (high QC) were prepared. Before injecting the solution was sonicated for 10 min for degassing, and 20 µL solution was injected in the column using Hamilton syringe. Obtained chromatograph was interpreted.

Study for AD and VAL

The QC samples containing AD and VAL each 320 ng mL^{-1} (LLOQ), AD and VAL each 960 ng mL^{-1} (low QC), AD 1280 ng mL^{-1} and VAL 1920 ng mL^{-1} (medium QC); and AD 1920 ng mL^{-1} and VAL 3560 ng mL^{-1} (high QC) were prepared. Before injecting, the solution was degassed for 10 min in the sonicator, and 20 µL solutions injected in the column using Hamilton syringe. The

obtained chromatograph was read and the peak areas were measured to know the conc. of solution.

Study for LER and FV

The QC samples containing LER 80 ng mL^{-1} (LLOQ), 240 ng mL^{-1} (low QC), 320 ng mL^{-1} (medium QC); and LER 400 ng mL^{-1} (high QC) were prepared. The QC samples containing FV 320 ng mL^{-1} (LLOQ), 640 ng mL^{-1} (low QC), 720 ng mL^{-1} (medium QC); and 800 ng mL^{-1} (high QC) were prepared. Before injecting, the solution was sonicated for 10 min for degassing; and 20 µL solution was injected in the column using Hamilton syringe. The obtained chromatograph was read.

Sensitivity

It is well defined as the lowest analyte concentration that can be measured with acceptable accuracy and precision i.e. analyte containing (LLOQ). Drugs solutions with conc. LLOQ were injected to obtain data and chromatographs.

Reproducibility

Reproducibility of the method is evaluated by replicate measurements of the QCs and is usually included in the assessment of precision and accuracy.

Stability

Stability of the analyte in the studied matrix is evaluated using low and high concentration stability QCs. Aliquots of the low and high stability QCs are analysed at time zero and after the applied storage conditions that are to be evaluated. A minimum of three stability QCs should be prepared and analysed per concentration level/storage condition/time point.

The chemical stability of an analyte in a given matrix under specific conditions; for given time interval was assessed by below mentioned ways.

For RV and EZE

The QC samples containing RV and EZE each 960 ng mL^{-1} (LQC), 2560 ng mL^{-1} (HQC) were prepared. The solution was degassed for 10 min in the sonicator, and 20 µL solutions injected in the column by Hamilton syringe. The obtained chromatograph was read and the peak areas were measured to know the conc. of QC sample solution.

For AD and VAL

The QC samples containing AD and VAL each 960 ng mL^{-1} (LQC), and AD 1920 ng mL^{-1} and VAL 3560 ng

mL^{-1} (high QC) were prepared. For degassing, the solution was sonicated for 10 min, and 20 μL solution injected in the column by Hamilton syringe. Retention time, Peak area were recorded from the obtained chromatographs.

For LER and FV

The QC samples containing LER 240 ng mL^{-1} (low QC), LER 400 ng mL^{-1} (high QC); and the QC samples containing FV 640 ng mL^{-1} (low QC), 800 ng mL^{-1} (high QC) were prepared. Before injecting, the solution was sonicated for degassing for 10 min; and 20 μL solutions injected in the column by Hamilton syringe. The obtained chromatograph was read and the peak areas were measured to know the conc. of solution.

As per guidelines[32,33] at least three replicate at each of the low and high concentrations should be assessed. Stability sample results should be within 15 % of nominal concentrations.

Freeze and thaw stability

Prepared QCS samples were frozen and thawed, and three freeze-thaw cycles were carried out to study, which mimics the intended sample handling conditions to be used during sample analysis.

Bench-top stability

At laboratory temperature stability of QCS was done, which mimics design of expt and cover the laboratory handling conditions that are expected for study samples.

Long-term stability

The QCS were stored, and storage time in a long term stability evaluation was equal to the time period between the first sample collection date and the last sample analysis date.

Stability testing should evaluate the stability of the analyte during sample collection and handling, after long term (frozen at the intended storage temperature) and short term (bench top room temp) storage, and after freeze and thaw cycles and the analytical process. The procedure should also include an evaluation of analyte stability in stock solution.

Carry-over

Carry-over is an alteration of a measured concentration due to residual analyte from a preceding sample that remains in the analytical instrument. Carry-over should be assessed and minimised during method development. During validation carry-over should be assessed by analysing blank samples after the calibration standard at the ULOQ.

RESULTS AND DISCUSSION

Bio analytical method development and mobile phase optimization

This research of commonly used cardiovascular drugs was focused on optimization of the conditions for a simple, rapid as well as a low cost and effective bio analytical method including a selection of the proper column and or comprehensive mobile phase to obtain satisfactory results.

To determine the appropriate wavelength for simultaneous estimation, these analytes in mobile phase were scanned in the range of 200–400 nm. From the overlaid UV spectra, suitable wavelength considered for monitoring the analyte was 245 nm. It was observed that all analytes absorbed well at 245 nm, and at this wavelength there was no interference from the mobile phase like baseline disturbance, and therefore, concluded that 245 nm was the most suitable wavelength for analysis of drugs with appropriate sensitivity.

To optimize mobile phase, chromatogram of drugs were obtained in mobile phase acetonitrile: water in the ratio 75: 25 V/V, the drugs eluted but resolution was not proper. Therefore, the proportion of water was increased; and mobile phase with composition acetonitrile: acetate buffer (60:40 V/V) was found most suitable.

The optimized mobile phase acetonitrile: acetate buffer in composition 60: 40 V/V of pH 4.0 (adjusted with acetic acid) was selected for bio analytical method and processed drug plasma matrix solutions were chromatographed in this mobile phase, shown in Figs. 2 and 3.

These changes resulted into elution of drugs with reasonable retention factor and symmetry in the peak. The chromatograph with well resolved peak was obtained for AD, LER, RV and EZE with retention times 2.42, 3.13, 5.534 and 8.334 minuets respectively. Further, efficiency of mobile phase for bio analytical method was ascertained by estimating all these drugs from human plasma.

METHOD VALIDATION

Selectivity for analyte

Evidence of the selectivity was provided by testing blank plasma in absence of analyte. The evaluation of
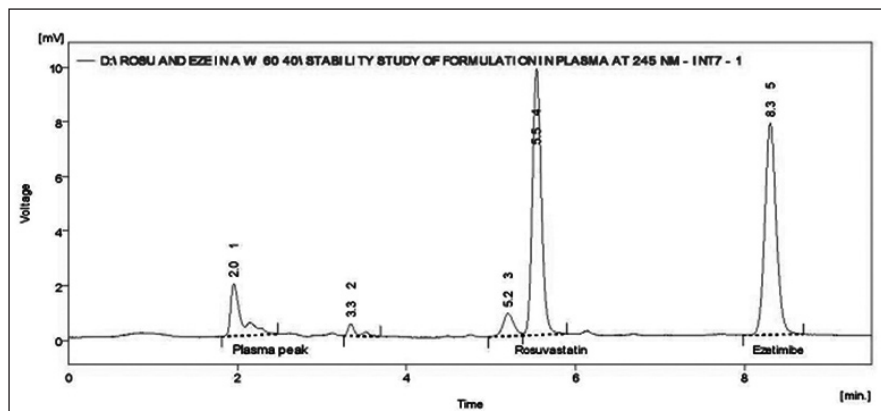
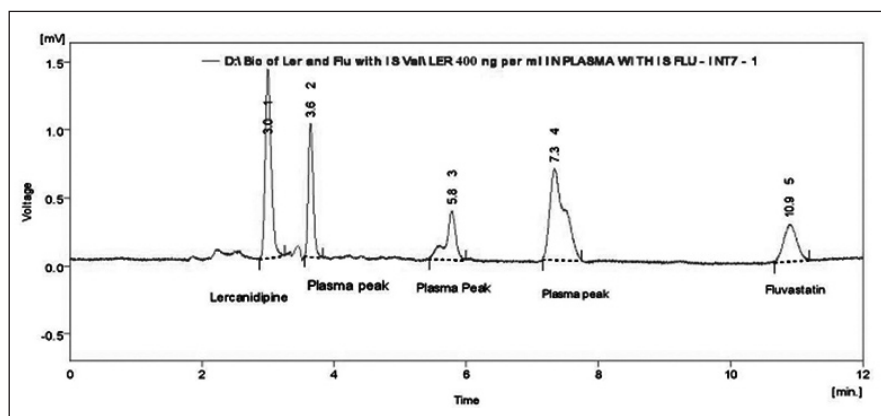Fig. 8: Freeze thaw stability study of RV and EZE

Fig. 9: Short term stability study of LER and FV

selectivity from the chromatograph demonstrated that no significant response attributable to interfering analytes or components was observed at the retention time(s) of the analyte in the blank samples shown in (Fig. 4).

Specificity

Specificity is the capability of a bio analytical method to detect and differentiate the analyte from other substances. For specificity study mixed solution of RV, EZE, AD, LER, FV and VAL was used; and injected to obtain chromatograph of LER, AD, EZE, RV and FV shown in the Fig. 2 and of RV, VAL shown in the Fig. 3.

Calibration curve of drugs in plasma matrix and plot of calibration curve

As per guidelines calibration curve for these drugs was established in conc. range RV (320-3200 ng mL^{-1}), EZE (320-3200 ng mL^{-1}), AD (320-2560 ng mL^{-1}), VAL (320-5120 ng mL^{-1}), LER (80- 480 ng mL^{-1}) and FV (320-1000 ng mL^{-1}) by linear regression analysis shown in Fig. 5. The regression equation obtained for standard curve

with coefficient of regression (r^2) and slope for these drugs are given in Table I.

Accuracy and precision

Accuracy and precision study of the method for RV and EZE is shown in Fig. 6 and for AD and VAL is shown in Fig. 7. The accuracy and precision within the batch was calculated and results are tabulated in Table II (a, b, c). Precision was measured as percentage of coefficient of variation (% CV) or relative standard deviation and accuracy was determined as percent of nominal conc (% nominal). The within batch % CV was found in the range (0.8875-2.0367) for RV and (0.8210-1.7952) for EZE, (1.0495-2.2628) for AD and (0.9480-2.4753) for VAL, (0.5258-3.0875) for LER and (1.1125-4.9871) for FV at all conc. levels. The data of % nominal conc. were varied between (97 to 99.86) for RV and (97.89 to 100.93) for EZE, (96.79-99.81) for AD and (98.43-99.22) for VAL and (97.18-103.5) for LER and (99.19-101.25) for FV at all conc. levels. All the obtained values were within acceptable limits as per guidelines.

Reproducibility

Reproducibility of the method was assessed by replicate measurements using the prepared quality controls samples.

Stability study

Obtained chromatograph of freeze thaw stability of RV and EZE is shown in Fig. 8 and short term stability of LER and FV is shown in Fig. 9. From the stability results, it was observed that no substantial degradation was observed in the samples stored at different conditions. It was concluded that sample was stable at 12 h (Short term stability), for three freeze-thaw cycles and for 10 days at -20° C (Long term stability), and results are shown in Table III (a, b, c).

Carry over

No residual analyte was found from a preceding sample that remains in the analytical instrument. The

blank plasma injected after calibration study and obtained chromatogram showed no presence of significant peak or response at retention time of analyte.

CONCLUSION

The selected analytes were estimated from the plasma; and any interference of the plasma matrix was not observed during the development of RP - HPLC bio analytical method. The method is simple as protein precipitation technique was employed, so free from separation techniques like solid phase extraction or liquid-liquid extraction. The method was validated for sensitivity, accuracy and precision, specificity, and stability as per ICH and USFDA guidelines, and obtained statistical data of validation were within prescribed range conforms the rigidity of method. Applicability of comprehensive mobile phase for quantitative estimation of these drugs saved time and solvent consumption. The run time was relatively short (10 and 12 mins), which enables rapid quantification of many samples, moreover, this bio analytical approach can be rationally applied for relative bioavailability and bioequivalence study of these drugs.

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