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Original Article

SIMULTANEOUS DETERMINATION OF ATORVASTATIN AND ATENOLOL IN RABBIT PLASMA BY RP-HPLC METHOD AND ITS APPLICATION IN PHARMACOKINETIC STUDY

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ABSTRACT

Objective: The objective of the present study is to establish a simple, sensitive, and specific RP-HPLC technique to determine atorvastatin calcium (ATV) and atenolol (ATL) in rabbit plasma.

Methods: Both the drugs like atorvastatin calcium and atenolol have been extracted by simple liquid-liquid extraction with acetonitrile.

Results: The chromatographic separation has performed on a reversed-phase C_{18} column with a mobile phase of phosphate buffer (pH 4.0): acetonitrile: methanol (40:40:20, v/v). The techniques have been validated over the concentration range of 22.08-4778.37 ng ml^{-1} for ATV and 18.35-3969.20 ng ml^{-1} for ATL in rabbit plasma. The acceptability range was satisfied for all validation criteria. These methods have been profitably applied to the pharmacokinetic study of a bilayer tablet of ATV and ATL after an oral administration to a rabbit. A significant recovery of the drug from plasma resulted from acetonitrile as extracting solvent compared to other organic solvents.

Conclusion: The results obtained proved that the RP-HPLC method developed was accurate and reproducible, and the drug was stable in rabbit plasma.

Keywords: Atorvastatin, Atenolol, RP-HPLC, Pharmacokinetic study, Rabbit, Bilayer tablet

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INTRODUCTION

ATV, [R-(R,R*)]-2-(4-fluorophenyl)-β,δ-dihydroxy-5(1-methyethyl)-3-phenyl-4-[phenyl amino carboxyl]-1H-pyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate (fig. 1a), is a synthetic lower blood cholesterol substances. It's a particular competitive inhibitor of HMG-CoA reductase, an enzyme that catalyzes the conversion of HMG-CoA to mevalonate, a key rate-limiting step in cholesterol biosynthesis [1, 2]. It has been used for the treatment of hyperlipidemia. Several methods for its estimation using HPLC [3, 4] and HPTLC [5] were reported.

ATL, 4-[2-hydroxy-3-[(1-methyethyl)amino]propoxy]-benzene acetamide, (fig. 1b) is a cardioselective β1-adrenergic receptor blocking agent which is used for the treatment of hypertension, angina pectoris, and cardiac arrhythmias [6]. Several HPLC methods were reported in the literature for the quantitative determination of atenolol in biological samples [7-14], with fluorescence detection [15-19] and mass spectrometry [20-23]. Most of the analytical techniques for atenolol described in the literature are based upon the chromatographic determination of this drug in the pharmaceutical formulations with another active drug substance [24-28].

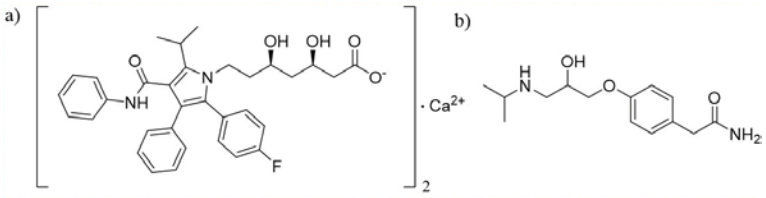

Fig. 1: Chemical scaffolds of a) ATV and b) ATL

Hypertension and hypercholesterolemia often coincide and may require to involve related treatment. The combination therapy of ATV and ATL may be valuable and effective in some situations, particularly in cardiovascular severe adverse effects such as severe hypertension, congestive heart failure, and exacerbation of angina may occur along with increased cholesterol level in the blood. In contrast, the fixed-dose combinations (FDCs) remain the preferable choice to the patient as compared to taking the single products two times. However, to the best of our knowledge, an RP-HPLC method for the simultaneous determination of ATV and ATL in co-formulated preparations has not been reported. Hereafter, the key objective of this work was to develop a simple, sensitive, rapid, and reliable RP-HPLC method for the simultaneous quantifications of ATV and ATL in rabbit plasma.

MATERIALS AND METHODS

Chemicals

ATV and ATL were obtained from Cipla Ltd., Mumbai, India,as gift samples. Diltiazem was used as an internal standard (I. S.) and obtained from Cipla Ltd., Mumbai, India. The HPLC grade acetonitrile, methanol, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, and *o*-phosphoric acid were purchased from Merck, Mumbai, India. All reagents have been used analytical grade except acetonitrile and methanol, HPLC grade. Water was glass-double distilled and further purified from the Milli Q water purification system.

Instrumentation

HPLC analysis was performed with Waters, USA. HPLC system equipped with quaternary pump (Water™ 600), 7725i rheodyne

manual injector, and UV-Visible detector of module 2998. Data acquisition was performed with empower-II software.

Chromatographic conditions

The chromatographic separation was executed using a Hypersil BDS C_{18}column, with 250 mm × 4.6 mm and a 5 µm particle size (Thermo Technologies, USA). The mobile phase has been used for the separation of the analytes was phosphate buffer (pH 5.8): acetonitrile: methanol (40:40:20, v/v) with the flow rate of 1.0 ml min^{-1} was employed. The detector wavelength was set at 248 nm. The injection volume was 20 µl and the total run time was 15 min. The column was maintained at ambient temperature (24 °C).

Standard solutions

Separately, stock solutions were prepared by accurately transferring 10 mg of ATV and ATL into a 10 ml volumetric flask, dissolved in 2 ml of methanol, and the final volume was made to yield a concentration of 1 mg ml^{-1}. The I. S. working solution of 50 ng ml^{-1} was prepared by dilution with methanol. The stock solution of ATV and ATL were serially diluted with methanol to provide nine standard working solutions of desired concentrations of individuals. All the solutions were stored at 4 °C and bought to room temperature before use.

Calibration curves

A nine-point standard calibration solution of ATV and ATL were prepared by spiking blank plasma with appropriate amounts of analytes in rabbit plasma to yield final concentrations of 22.08, 50.97, 101.94, 203.88, 407.76, 815.91, 1529.08, 2867.04, and 4778.37 ng ml^{-1} for ATV and 18.35, 42.34, 84.68, 169.35, 338.71, 677.41, 1270.14, 2381.52, and 3969.20 ng ml^{-1} for ATL. Three quality control (QC) samples were prepared at three concentration levels of 101.94, 407.75, and 1529.08 ng ml^{-1} for ATV and 84.68, 338.71, and 1270.14 ng ml^{-1} for ATL. Calibration curves were plotted with a peak area ratio of drug and I. S. on the *Y*-axis and concentration on the *X*-axis.

Sample preparation and extraction

The liquid-liquid extraction procedure was used to extract the drug from the plasma. Blood samples were collected in disposable glass tubes (100 × 16 mm) and centrifuged at 4500 × g for 5 min. The plasma samples were kept at-80 °C until further analysis. A hundred microliter of plasma sample was taken in a 2 ml glass centrifuge tube; 10 µl of I. S. (50 µg/ml) was added, and the mixture was vortex for 10 sec using a multi-pulse vortexer (Glas-COL, USA). The above solutions were treated with 1.5 ml of acetonitrile. After vortex-mixed for 10 min in a sphinx vortexer (M37610-33, Barnstead International, USA) and centrifugation (Biofuge Fresco centrifuge, Heraeus, Germany) at 4 °C for 5 min at 10,000 rpm, the organic layer was removed and passes to a second tube using disposable Pasteur pipette. The collected organic layer was kept for evaporation to dryness under nitrogen gas flow using a nitrogen gas evaporator (Glas-COL, USA) at 25 psi at 40 °C. The residue was redeveloped in 100 µl of phosphate buffer (pH 5.8): acetonitrile: methanol (40:40:20, v/v), the solution was vortexes for 1 min using multi-pulse vortexer (Glas-COL, USA). Then 20 µl of aliquot was injected into the HPLC column for analysis.

Method validation

The following features of the proposed technique were evaluated during validation: selectivity, specificity, linearity, precision, accuracy, and analyte stability. These validation experiments followed the FDA Guidance for Bioanalytical Method Validation.

Linearity and LLOQ

The linearity of the method was determined by analysis of five standard plots associated with a nine-point standard calibration curve. The ratio of area response for the analyte to I. S. was used for regression analysis. For calibration curve was analyzed individually using least square weighted ($1/x^2$) linear regression. The calculation was based on the peak area ratio of the analyte vs. the area of I. S. The concentration of the analyte was calculated from the calibration curve (y = MX+c, where y is the peak area ratio) using linear regression analysis with reciprocating of the drug concentration as a weighing factor ($1/x^2$). The lowest standard on the calibration curve was accepted as the LLOQ if the analyte response was at least five times more than that of drug-free (blank) extracted plasma. The deviation of standards other than LLOQ from the nominal concentration should not be more than±15%, and for LLOQ, it should not be more than±20%.

Specificity

The ability of an analytical technique to distinguish and quantify the analyte in the presence of other components in the sample is known as specificity. The specificity of the process was evaluated by the analysis of six rabbit blank plasma samples from different volunteers. These samples were pretreated according to the sample preparation procedure except for the addition of the I. S.

Accuracy and precision

The assay's inter-day precision and accuracy were evaluated by running three validation batches on three separate days. Each batch consisted of six replicates of quality control (QC) samples at low, medium, and high concentrations. The intra-day precision and accuracy consisted of six quality control (QC) samples at low, medium, high concentrations. A comparison was made between the obtained values and the experimental values. Precision was expressed as a percentage of relative standard deviation (% R. SD). The mean accuracy value should be within 15% of the actual value. The precision determined at each concentration level should not exceed 15% of R. SD

Extraction recovery

The analytes' extraction recovery from the plasma was evaluated by comparing the peak areas of QC samples at low, medium, and high concentrations with peak areas of corresponding standard solutions of the same concentration dissolved in the supernatant of the processed blank rabbit plasma.

Stability studies

The analyte stability in rabbit plasma was assessed by analyzing three low, medium, and high QC samples under different temperature and time conditions. Freeze-thaw strength was performed by subjecting unextracted QC samples to three freezes (-20 °C)-thaw (room temperature) cycles. QC samples were stored at-20 °C for 30 d and ambient temperature for 24 h to determine long-term and short-term stability, respectively. All stability testing QC samples were determined by using a calibration curve of freshly prepared standards. The concentrations obtained were compared with the actual values of the QC samples.

Pharmacokinetic studies

The pharmacokinetic studies were conducted under the Institute Animal Ethics Committee of the Indian Institute of Chemical Biology, Kolkata, India. Healthy rabbits (New Zeland albino) of either sex weighing 2.5-3.0 Kg were involved in the experiment. After a 12 h fast, all rabbits were divided into three groups, and each group contained six rabbits. The conventional ATV marketed tablet (Tonact 10 mg), sustained-release ATL marketed tablet (Aten 50 mg), and bilayer tablet prepared in our laboratory contained 10 mg ATV as a fast-release layer and 50 mg of ATL as sustained-release layer were administered in three groups. Blood sampling was carried out at suitable time intervals up to 36 h. The total area under the plasma concentration-time curve ($AUC_{0-\infty}$), the maximum plasma concentration (C_{max}), and the time to reach the maximum plasma concentration (T_{max}) were chosen as parameters for pharmacokinetic evaluation. The C_{max} and T_{max} were obtained directly from the plasma concentration vs. Time [21]. $AUC_{0-\infty}$ was obtained by adding the $AUC_{0-36\ h}$, which was calculated by the linear trapezoidal method and the area from the last experimental time point to infinite time ($AUC_{36\ h-\infty}$) using the technique of Shargel and Yu [22]. The student's t-test was used for statistical analysis of the data, and a probability value of $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Chromatography

The reversed-phase HPLC method was developed to provide a specific procedure suitable for the FDCs' rapid quality control analysis of ATV and ATL. Chromatographic investigations revealed that a mixture of ATV and ATL could be resolved from the co-formulated excipients using the C_{18} stationary phase. Various mobile phase system was prepared for chromatographic separation. Still, the proposed mobile phase comprising phosphate buffer adjusted pH to 4.0: acetonitrile: methanol (40:40:20 v/v) gave better resolution and sensitivity of ATV and ATL. The mobile phase composition was optimized. Under the described conditions, the analyte peaks were well defined, resolved, and free from tailing.

The chromatographic system described above allows good resolution of ATV, ATL, and I. S. peaks. At a flow rate of 1.0 ml min^{-1}, ATL, I. S., and ATV retention times were 8.7, 10.9, and 12.1 min, respectively. The optimum wavelength for detection was 248 nm,at which a good detector response was obtained. Representative chromatograms obtained from blank plasma and plasma spiked with ATV and ATL are presented. As shown in these fig. 2a and 2b, ATV, ATL, and I. S. were eluted, forming symmetrical peaks and well separated from the solvent front. No endogenous components from plasma were found to interfere with the elution of the analytes or I. S.

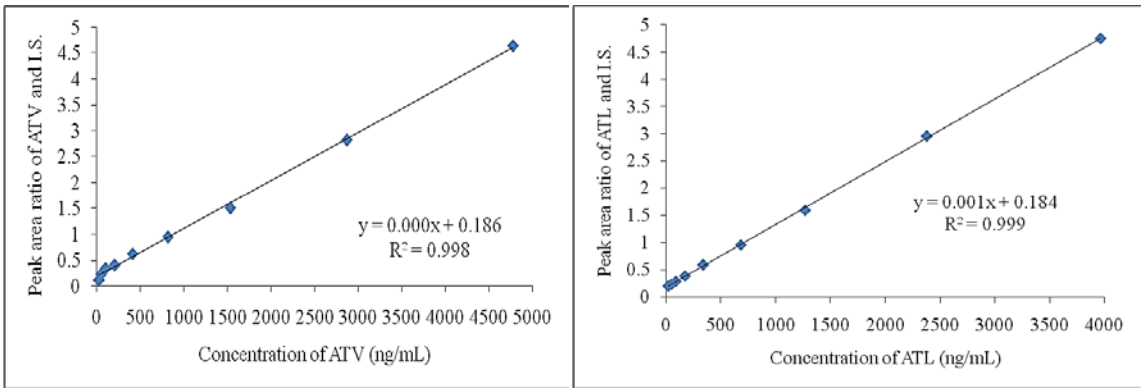

Fig. 2: Calibration plot of a) atorvastatin and b) atenolol by proposed HPLC method (n = 5)

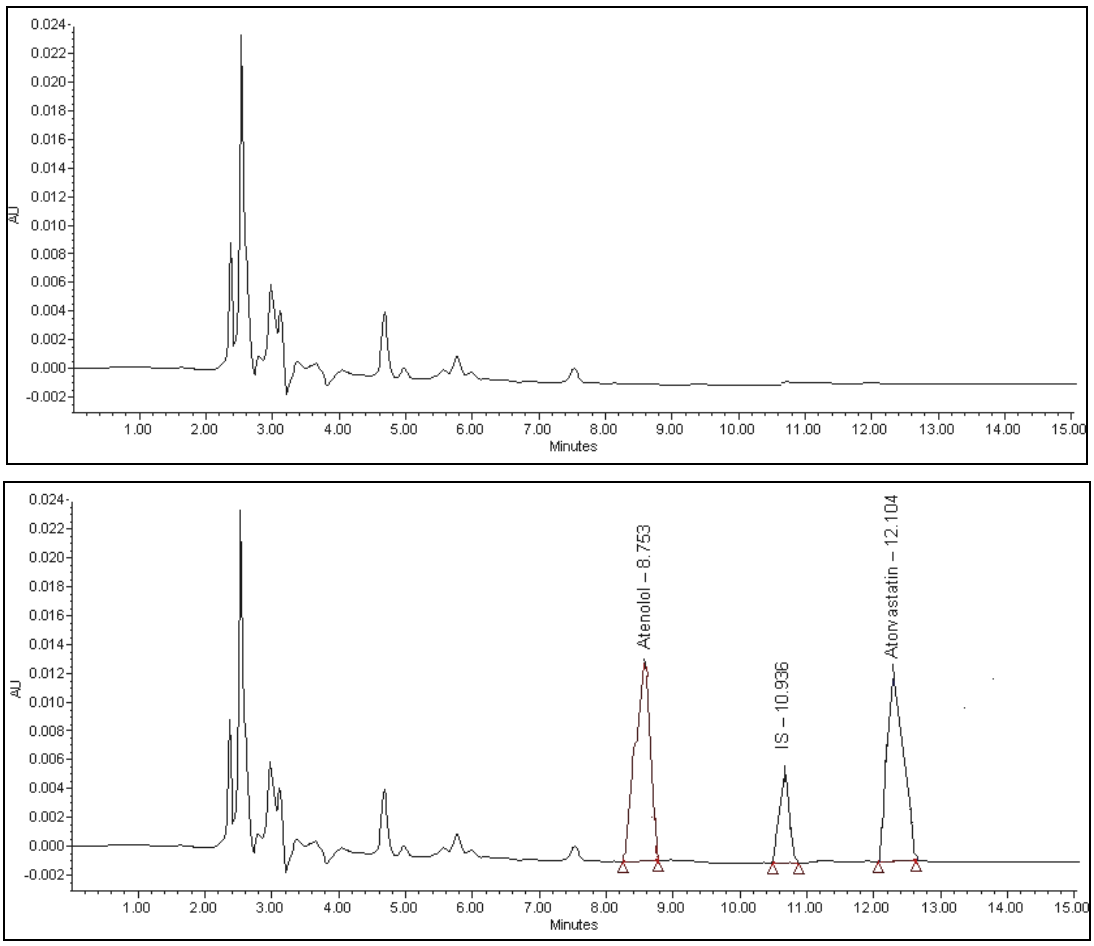

Fig. 3: Chromatogram of a) blank (ATV, ATL, and I. S. free rabbit plasma) b) plasma spiked with ATV, ATL, and I. S

Validation of the method

The analytical HPLC method was validated, calculating linearity, specificity, inter-day, and intra-day precision and accuracy, extraction recovery, and stability.

Linearity

A 1/concentration^2 weighting was used to fit a linear least-squares calibration curve to the response versus concentration data. Good linearity in the range of 22.09-4778.37 ng ml^{-1}and 18.35-3969.20 ng ml^{-1}were achieved for ATV and ATL, respectively. The regression equations(n = 6) were y = 0.0009x+0.1862 and y = 0.0012x+0.1858 for ATV and ATL, respectively, where y is the peak area ratio of the ATV or ATL to the I. S. and x is the ATV or ATL concentration (ng ml^{-1}). The typical correlation coefficient (r^2) was achieved between 0.9935 and 0.9986 for ATV and 0.9994 and 0.9998 for ATL. The calibration plot of ATV and ATL are shown in fig. 3a and 3b. The LLOQ was found to be 22.09 ng ml^{-1} and 18.35 ng ml^{-1} for ATV and ATL, respectively. In LLOQ, the mean peak area for the analyte peak was ~ 10-fold greater than the mean peak area for the blank rabbit plasma samples at the analyte retention time.

Specificity

The specificity of the method has been analysed by comparing chromatograms of six different sources of rabbit plasma. No significant peaks were observed at ATV, ATL, and I. S. retention times in blank rabbit plasma. Representative chromatograms of rabbit blank plasma and plasma spiked with the ATV, ATL, and I. S. are shown in fig. 2a and b, respectively.

Accuracy and precision

Results of the intra-day and inter-day validation assays for ATV and ATL are presented in table 1. The inter-and intra-day precisions were≤5.86 and ≤4.80, whereas the inter-and intra-day accuracies were in the range of 93.90-102.57% and 96.16-98.78%, respectively, for ATV. Results of the inter-and intra-day precisions were≤5.07 and ≤3.95, whereas the inter-and intra-day accuracies were in the range of 96.35-102.09% and 96.56-98.32%, respectively, for ATL. Both precision and accuracy were within the acceptable ranges for bioanalytical purposes. The assay method demonstrated a high degree of accuracy and precision.

Table 1: Inter-and intra-day precision and accuracy for ATV and ATL (mean±SD; *n* = 6)

Analyte	QC sample (ng ml^{-1})	Inter-day variation			Intra-day variation		
		mean±SD	R. SD%*	Accuracy %	mean±SD	R. SD%*	Accuracy %
	101.94	99.54±1.63	1.64	97.65	100.70±4.78	4.75	98.78
ATV	407.95	418.23±24.51	5.86	102.57	392.10±11.37	2.90	96.16
	1529.08	1435.83±81.41	5.67	93.90	1471.08±70.61	4.80	96.21
	84.68	83.11±1.79	2.16	96.35	81.16±2.93	3.61	97.19
ATL	338.71	342.06±17.34	5.07	102.09	332.89±9.29	2.79	96.56
	1270.14	1259.25±61.33	4.87	95.12	1251.58±49.44	3.95	98.32

*% Relative standard deviation (R. SD) = (standard deviation/mean) × 100

Extraction recovery

Recovery of the ATV and ATL from the liquid-liquid extraction procedure was examined by comparing the detector response obtained from the extracted sample and the detector response obtained for direct injection of standard solution. A recovery experiment was performed with six replicates at three concentration levels (low, medium, and high). Recovery results presented that maximum recovery was achieved with 92.37% for ATV and 92.53% for ATL, whereas extraction recovery for I. S. ranged from 75.16% to 86.25% (table 2). The extraction recovery was satisfactory as it was consistent, precise, and reproducible. Thus, the single-step liquid-liquid extraction procedure used in this method proved efficient and straightforward enough to extract three drugs (including I. S.) simultaneously from rabbit plasma.

Table 2: Extraction recovery of ATV, ATL, and I. S. (*n* = 6)

Analyte	QC sample (ng ml^{-1})	Relative standard deviation %*	Extraction recovery %
	101.94	2.08	89.82
ATV	407.95	3.91	92.37
	1529.08	3.17	91.44
	84.68	3.22	92.53
ATL	338.71	2.51	90.28
	1270.14	1.08	91.48
I. S.	500	2.57	86.25

*% Relative standard deviation (R. SD) = (standard deviation/mean) × 100

Table 3: Stability studies of ATV and ATL (mean±SD; *n* = 6)

Stability	ATV				ATL			
	QC sample (ng ml^{-1})	mean±SD	R. SD%	Accuracy %	QC sample (ng ml^{-1})	mean±SD	R. SD%	Accuracy %
Freeze-thaw	101.94	95.29±7.56	7.93	93.47	84.68	87.62±5.70	6.50	95.27
	407.75	424.06±29.26	6.90	104.01	338.71	328.57±26.91	8.19	102.54
	1529.08	1515.15±146.36	9.66	99.09	1270.14	1238.14±98.80	7.98	98.69
Short term	101.94	101.29±10.79	10.65	99.37	84.68	82.11±7.37	8.98	98.53
(24 h)	407.75	413.74±44.48	10.75	101.47	338.71	339.45±34.83	10.26	102.82
	1529.08	1488.44±176.68	11.87	97.34	1270.14	1249.21±119.80	9.59	99.12
Long term	101.94	99.33±10.23	10.30	97.44	84.68	84.26±7.20	8.54	96.23
(30 d)	407.75	422.52±31.82	7.53	103.62	338.71	326.07±23.12	7.09	98.12
	1529.08	1491.50±28.19	1.89	97.54	1270.14	1269.33±133.91	10.55	95.94

Stability studies

Table 3 summarizes the stability study results carried out under various conditions. The analytes were stable at ambient temperature (20-30 °C) for at least 24 h in rabbit plasma. The accuracy percentage obtained was more than 97.34% for ATV and 98.53% for ATL. The analytes remained unaffected at-20 °C for one month, and the rate of accuracy was found to be more than 97.44% for ATV and 95.94% for ATL. The freeze-thaw stability results showed that both the analytes were stable for at least three freeze-thaw cycles. Stability results indicated that rabbit plasma samples could be thawed and refrozen without compromising the integrity of the samples.

Table 4: Pharmacokinetic parameters of tonact 10 mg, Aten 50 mg, and bilayer tablets containing 10 mg of ATV and 50 mg of bilayer tablets after oral administration to healthy rabbit volunteers (*n* = 3)

Parameter*	Tablet			
	Tonact 10 mg	Aten 50 mg	Bilayer tablet	
			ATV	ATL
C_{max} (µg ml^{-1})	17.00±4.47	27.53±3.53	18.19±5.10	26.55±8.24
T_{max} (h)	0.5±0.0	3.0±0.0	0.5±0.0	3.0±0.0
$AUC_{0-\infty}$ (µg h ml^{-1})	81.04±22.29	342.65±62.82	106.47±31.48	347.12±74.67
$AUMC_{0-\infty}$ (µg h^2 ml^{-1})	334.89±79.35	2796.51±52.50	780.37±102.84	2847.31±96.49
MRT (h)	5.29±0.48	8.16±0.15	7.33±0.62	8.20±0.94
$t_{1/2}$ (h)	3.04±0.41	7.54±0.64	3.31±0.24	9.90±0.55
K_{el} (h^{-1})	0.23±0.01	0.10±0.01	0.21±0.03	0.07±0.01

*T_{max} = time to reach plasma concentration (C_{max}); AUC = Area under plasma concentration-time curve; AUMC = Area under the first momentum plasma concentration-time curve; MRT = Mean residence time; $t_{1/2}$ = plasma half-life of drug; K_{el} = Elimination rate constant.

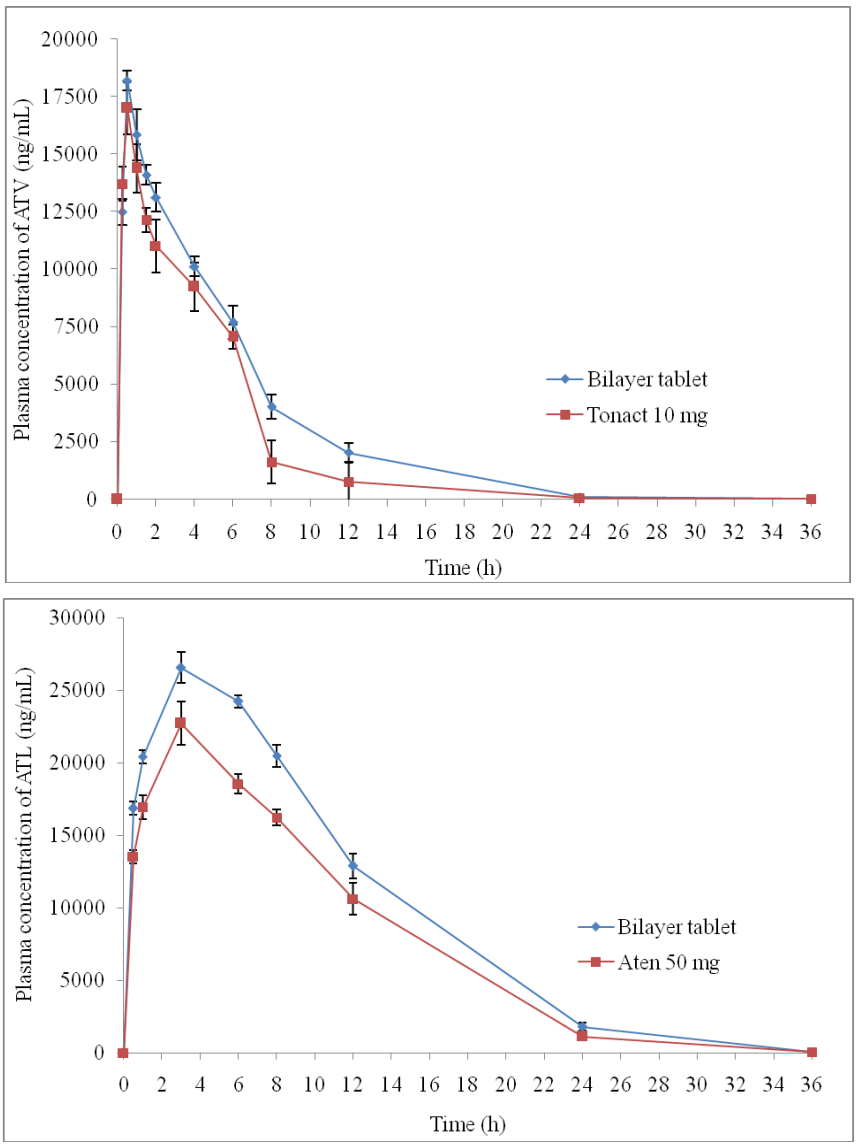

Fig. 4: Plasma concentration profiles of a) ATV and b) ATL following oral administration of marketed formulation (Tonact 10 mg or Aten 50 mg) and bilayer tablets in healthy rabbits (n = 6)

Pharmacokinetic studies

The method was successfully applied to detect ATV and ATL in rabbit plasma for 36 h after oral administration of Tonact 10 mg, Aten 50 mg, and bilayer tablet consisting of 10 mg of ATV and 50 mg of ATL. The rabbit plasma concentration of the ATV-time profile and the rabbit plasma concentration of the ATL-time profile are shown in fig. 4a and fig. 4b. The pharmacokinetic parameters (T_{max}, C_{max}, AUC_{0-t}, $AUC_{0-\infty}$, $t_{1/2}$, and K_{el}) are listed in table 4.

CONCLUSION

A novel, sensitive and straightforward RP-HPLC method has been developed and validated to estimate ATL and ATV in rabbit plasma using UV-detector simultaneously. The suitable resolution was obtained between ATL, ATV, and I. S. with ATL, I. S., and ATV retention times were 8.7, 10.9, and 12.1 min, respectively. There was no interference, i.e., peaks observed around ATL, ATV, and I. S retention time. A maximum recovery of drug from plasma resulted using acetonitrile as extracting solvent compared to other organic solvents. The results obtained proved that the RP-HPLC method development was accurate and reproducible, and the drug was stable in rabbit plasma. In summary, the validated chromatographic technique can be successfully applied for the pharmacokinetic studies of ATV and ATL bilayer tablets. This study may be extended to determine the pharmacokinetics of ATV and ATL in human plasma.

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Nil

AUTHORS CONTRIBUTIONS

Mr. Suraj Kumar Shah-Conceptualization and Methodology

Dr. Sanjay Dey and Dr. Sourav De-Investigation and Writing-Original draft preparation

CONFLICTS OF INTERESTS

The authors declare no conflict of interest.

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