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# Simultaneous Determination of Atorvastatin Calcium and Ezetimib by Ratio Spectra Derivative Spectrophotometry and Reverse Phase-High Performance Liquid Chromatography

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Rapid, accurate and precise ratio spectra derivative spectrophotometry and high-performance liquid chromatographic (HPLC) methods were described for the simultaneous determination of atorvastatin calcium and ezetimibe in combined pharmaceutical dosage forms. For ratio spectra derivative spectrophotometry, the signals for in the first derivative of the ratio spectra were measured at 266.6 nm for atorvastatin calcium and at 262.2 nm for ezetimibe in the mixture. The high-performance liquid chromatography (HPLC) method utilized hypersil ODS RP-18 column (250 mm × 4.6 mm) and methanol: water (70: 30 v/v) as a mobile phase at a flow rate of 1 mL $min^{-1}$. Detection was carried out using a UV detector at 242.0 nm. The retention time for atorvastatin calcium and ezetimib were found to be 2.491 and 8.057 min, respectively. Both the methods showed good linearity in the range of 3-15 µg/mL for both the drugs and the methods were successfully utilized for the commercial pharmaceutical preparation (Zatistat-10®, Torrent, Indrad) containing both the drugs in different proportions.

**Key Words: Atorvastatin calcium, Ezetimibe, RP High-performance liquid chromatography, Ratio spectra derivative spectrophotometry, Pharmaceutical formulations.**

## INTRODUCTION

Atorvastatin calcium[1] (ATVC), (βR,δR)-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-l*H*-pyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate; is a synthetic cholesterol-lowering agent, called HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitor. Ezetimibe[2] (EZTB), (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone is a class of lipid-lowering compound that selectively inhibits the intestinal absorption of cholesterol and related phytosterols. Combination therapy of ATVC and EZTB is used for the treatment of primary (heterozygous familial and non-familial) hypercholesterolemia. Analytical methods have been developed by one of the authors for estimation of ATVC with other

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antihypertensive agent[3,4]. Various analytical methods like spectrophotometric[5], HPLC[6,7], HPTLC[8] have been reported for the determination of ATVC and EZTB in binary mixture. The aim of this work is to develop a simple, rapid, sensitive and reliable spectrophotometric and RP-high performance liquid chromatography (RP-HPLC) method for the quality control of ATVC and EZTB in combined dosage forms with comparatively more sensitivity and less retention time with simple mobile phase composition than the reported one.

## EXPERIMENTAL

Spectrophotometric analysis was carried out on a Shimadzu 1601 double beam spectrophotometer with a fixed slit width (2 nm). The system software of the instrument was used for obtaining the ratio spectra and tracing the first derivative of the ratio spectra. The RP-HPLC system consisted of a Perkin Elmer, Series 200, USA model quaternary isocratic system pump with a rhenodyne valve injector with 20 µL fixed loop, equipped with a Diode array detector (UV-visible). The detector was set at 242.0 nm and peak areas were integrated automatically by computer using Borwin software programme. Pure drug sample of ATVC and EZTB was kindly gifted by M/s. Zydus Cadila Ltd., Ahmedabad, India and M/s. Sun Pharmaceuticals Ltd., Salvassa, India, respectively. Methanol analytical reagent grade (Allied Chemical Corporation, Vadodara, India) was used as solvent for spectrophotometric analysis, double distilled water and methanol HPLC grade (Spectrochem, Mumbai, India) were used for the RP-HPLC. All weighings were performed on an analytical balalance (BP211D) and sonicator (TEC-4) was used for mixing the solvent. A commercial pharmaceutical preparation (Zatistat-10®, Torrent, Indrad) was procured from commercial source.

### Ratio spectra derivative spectrophotometry

**Preparation of standard stock solutions of ATVC and EZTB:** Atorvastatin calcium and EZTB, 50 mg each, were accurately weighed and dissolved separately in 50 mL of methanol. Five mL of the above solution was separately diluted to 50 mL with methanol to produce 100 µg/mL each of ATVC and EZTB in methanol.

**Preparation of calibration curve:** Suitable aliquots of the standard stock solution were diluted with methanol to produce standard solution of 10 µg/mL of ATVC and 20 µg/mL of EZTB for obtaining the divisor spectra. Different binary mixture solution containing ATVC and EZTB in 1:1 ratio (very near to clinical dose ratio 10 mg: 10 mg of ATVC and EZTB) were prepared by diluting different aliquots of the stock solution with methanol. The absorption spectra of 10 µg/mL of ATVC and 20 µg/mL of EZTB were recorded in the range of 200-400 nm and stored in the memory of the instrument as divisor spectra. The absorption spectra of the binary mixture solutions of ATVC and EZTB were recorded in the range of 200-400 nm and were stored in the memory of the instrument. The stored spectra of the binary mixture solutions were divided by a previously stored divisor spectrum of 20 µg/mL of EZTB to get

the ratio spectra of ATVC (Fig. 1a). The first derivative of the ratio spectra of ATVC were traced with $\Delta\lambda = 6$ interval (Fig. 1b), multiplication factor = 5 and the amplitudes at 266.6 nm were plotted against the respective concentrations of ATVC. Similarly, the absorption spectra of binary mixtures of ATVC and EZTB were divided by a previously stored standard divisor spectrum of 10 µg/mL of ATVC to get the ratio spectra of EZTB (Fig. 2a) and the first derivative of the ratio spectra were traced with $\Delta\lambda = 3$ interval (Fig. 2b), multiplication factor = 5 and the amplitude at 262 nm were then plotted against the respective concentrations of EZTB. The method was validated for linearity and range, interday and intraday precision, reproducibility and accuracy and the recovery data are shown in Table-1.

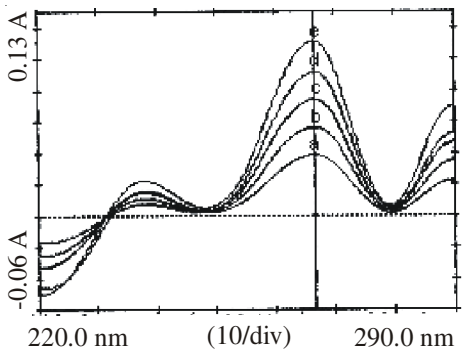


Fig. 1a. Ratio spectra of ATVC of (a) 3.0 µg/mL (b) 6.0 µg/mL (c) 9.0 µg/mL (d) 12.0 µg/mL (e) 15.0 µg/mL when 20 µg/mL EZTB used as divisor in methanol: water (70:30 v/v) ($\Delta\lambda = 6$ nm)

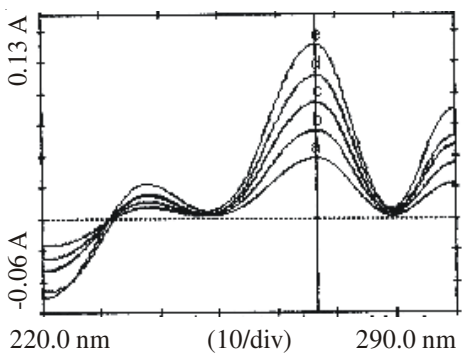


Fig. 1b. First derivative of ratiospectra of ATVC 6.0 µg/mL of (a) 3.0 µg/mL (b) (c) 9.0 µg/mL (d) 12.0 µg/mL (e) 15.0 µg/mL when 20 µg/mL EZTB used as divisor in methanol:water (70:30 v/v) ($\Delta\lambda = 6$ nm)

TABLE-1
RECOVERY STUDY DATA

| Level of standard addition (%) | % Recovery ± SD* | | | |
|---|---|---|---|---|
| | Method 1 | | Method 2 | |
| | ATVC | EZTB | ATVC | EZTB |
| 80 | 102.80 ± 0.902 | 102.88 ± 1.02 | 100.72 ± 0.325 | 98.90 ± 0.436 |
| 100 | 100.93 ± 0.348 | 98.46 ± 1.028 | 102.38 ± 0.768 | 100.52 ± 0.396 |
| 120 | 99.68 ± 0.516 | 101.28 ± 0.80 | 101.45 ± 0.527 | 98.40 ± 0.613 |

Mehtod 1 is the ratio spectra derivative spectrophotometry while Method 2 is RP-HPLC, *Values for recovery are mean for three determinations, SD is the standard deviation.

**Analysis of tablets:** Twenty tablets were accurately weighed and powdered in a mortar. An amount equivalent to one tablet (containing 10 mg of ATVC and 10 mg of EZTB) was taken and dissolved in 10 mL of methanol by magnetically stirring it for five minutes. About 10 mL of methanol was added and stirred for further 5 min. The mixture was transferred to two centrifuge tubes and centrifuged at 1000 rpm

for 5 min. The supernatant was transferred to a 100 mL volumetric flask through a Whatman no 40 filter paper. The residue was washed thrice with methanol and the combined filtrate and washings were made up to the mark with methanol. The sample solution thus prepared was diluted with methanol to get the solutions containing ATVC and EZTB in 10:10 µg/mL proportions. The above solution was analyzed for the content of ATVC and EZTB using the method described above and the results are shown in Table-2.

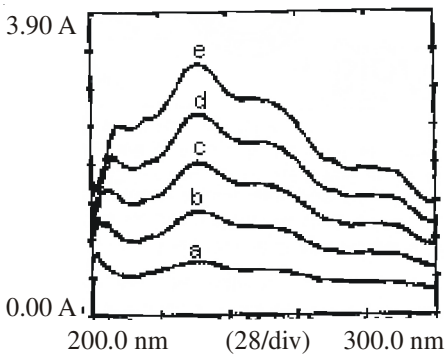


Fig. 2a. Ratio spectra of EZTB of (a) 3.0 µg/mL (b) 6.0 µg/mL (c) 9.0 µg/mL (d) 12.0 µg/mL (e) 15.0 µg/mL when 10 µg/mL ATVC used as divisor in methanol:water (70:30 v/v) ($\Delta\lambda$ = 3 nm)

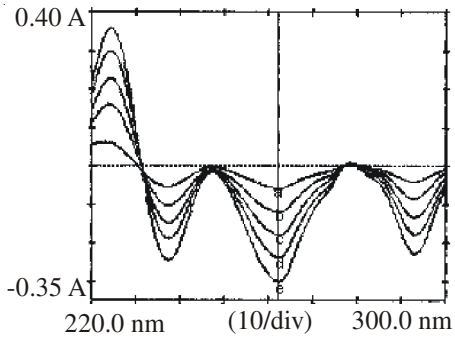


Fig. 2b. First derivative of ratio spectra of EZTB of (a) 3.0 µg/mL (b) 6.0 µg/mL (c) 9.0 µg/mL (d) 12.0 µg/mL (e) 15.0 µg/mL when 10 µg/mL ATVC used as divisor in methanol: water (70:30 v/v) ($\Delta\lambda$ = 3 nm)

TABLE-2
ANALYSIS OF COMMERCIAL TABLET FORMULATIONS

| Formulation | Label claim (mg/tablet) | | Method 1 | | | | Method 2 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Conc. found (mg) | | % Recovery* ± SD | | Conc. found (mg) | | % Recovery* ± SD | |
| | ATVC | EZTB | ATVC | EZTB | ATVC | EZTB | ATVC | EZTB | ATVC | EZTB |
| Zatistat-10® | 10 | 10 | 9.867 | 9.975 | 98.67 ± 0.831 | 99.75 ± 0.319 | 9.892 | 9.921 | 98.92 ± 0.438 | 99.21 ± 0.368 |

Method 1 is the ratio spectra derivative spectrophotometry while Method 2 is RP-HPLC,
*Values for recovery are mean for three determinations, SD is the standard deviation.

## RP-HPLC Method

**Chromatographic conditions:** Routine analysis was carried out isocratically on a hypersil ODS RP-18 column (250 mm × 4.6 mm i.d.) using methanol: water (70:30 v/v) as a mobile phase at a flow rate of 1 mL/min. All solvents were filtered through 0.45 µm millipore filter and degassed in an ultrasonic bath.

**Preparation of mobile phase:** A mixture of 70 mL methanol and 30 mL double distilled water was sonicated for 2 min and then filtered through 0.45 µm membrane filters in a flask. This was used as a mobile phase.

**Preparation of standard stock solutions of atorvastatin calcium and ezetimibe:** Atorvastatin calcium and ezetimibe, 10 mg each, were accurately weighed and dissolved separately in 10 mL of mobile phase. One mL of the above solution was diluted to 10 mL with mobile phase to produce 100 µg/mL each of ATVC and EZTB in mobile phase.

**Preparation of calibration curve:** Different binary mixture solutions containing ATVC and EZTB in 1:1 ratio like 3:3, 6:6, 9:9, 12:12 and 15:15 µg/mL (very near to clinical dose ratio 10:10 of ATVC:EZTB) were prepared by mixing suitable aliquots of the standard stock solutions and diluting it with mobile phase. These binary mixture solutions were chromatographed and the peak area of these solutions were measured at 2.491 and 8.027 min corresponding to the retention time of ATVC and EZTB, respectively (Fig. 3). Calibration curves for both the drugs were plotted separately against the corresponding concentration to obtain the calibration graph. The method was validated for linearity and range, interday and intraday precision, reproducibility and accuracy and the recovery data are shown in Table-1.

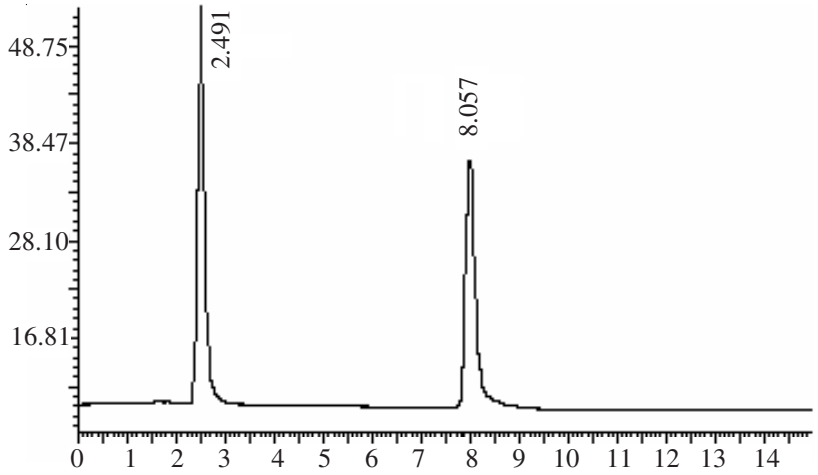


Fig. 3. Chromatogram of mixed standard solution containing 10 µg/mL ATVC and EZTB using mobile phase as methanol:water (70:30) at flow rate 1.0 mL $min^{-1}$

**Analysis of tablets:** Twenty tablets were accurately weighed and powdered in a mortar. An amount equivalent to one tablet (containing 10 mg of ATVC and 10 mg of EZTB) was taken and dissolved in 15 mL of mobile phase by ultrasonicating for 2 min. The mixture was transferred to two centrifuge tubes and centrifuged at 1000 rpm for 5 min. The supernatant was transferred to a flask through a 0.45 µm membrane filter and volume was adjusted to the mark with the same mobile phase. The sample solution thus prepared was diluted with mobile phase to get the solution containing ATVC and EZTB in 9:9 µg/mL. A total of 20 µL volume of final solution was injected into the chromatograph and analyzed for the content of ATVC and EZTB using the method described above and the results are shown in Table-2.

## RESULTS AND DISCUSSION

Ratio spectra derivative spectrophotometry and RP-HPLC methods for the simultaneous determination of ATVC and EZTB from their binary mixture were successfully developed. Both the methods showed good linearity in the range of 3-15 μg/mL for both the drugs with correlation coefficient of 0.9996 for ATVC and 0.9974 for EZTB in case of ratio spectra derivative spectrophotometry and 0.9992 for ATVC and 0.9986 for EZTB in case of RP-HPLC.

In case of ratio spectra derivative spectrophotometry, the instrumental parameters like divisor spectra and smoothing factor ($\Delta\lambda$) of tracing the first derivative of ratio spectra were optimized for the reliable determination of subject components. Some divisor concentrations were tested selecting the standard solution as divisor at an appropriate concentration and it was observed that the standard solution of 10 μg/mL of ATVC was suitable for determination of EZTB and 20 μg/mL of EZTB was suitable for determination of ATVC. The smoothing factors $\Delta\lambda$ = 6 nm for ATVC and $\Delta\lambda$ = 3 nm for EZTB were found to be optimum for tracing the first derivatives of ratio spectra as linearity and sensitivity is concerned. The results are shown in Figs. 1a, 1b, 2a and 2b. The experiment was repeated five times in a day for intra-day and on five different days for inter-day precision. The method was found to be precise as % RSD for intra-day and inter-day precision were 0.701 and 0.804 for ATVC and 0.902 and 1.018 for EZTB, respectively. The reproducibility of the method was determined by using methanol from three different manufacturers (Allied Chemical Corporation, Vadodara, S.D. Fine Chemicals, Mumbai and Qualigens, Mumbai) for the preparation of stock solution of standard drugs. The average value of % RSD, 0.909 for ATVC and 1.133 for EZTB reveals the reproducibility of the method. The accuracy of the method was determined by performing recovery studies by standard addition method in which pre-analyzed samples were taken and standard drug was added at three different levels. As evident from the results shown in Table-1, the % recovery ± SD lies in the range of 99.68 ± 0.516 to 102.80 ± 0.902 for ATVC and 98.46 ± 1.028 to 102.88 ± 1.02 for EZTB. The analysis of commercially available tablet formulations reveal satisfactory results, 98.67 ± 0.831 for ATVC and 99.75 ± 0.319 for EZTB as evident from the results shown in Table-2.

A simple and rapid RP-HPLC method was also developed for the simultaneous estimation of ATVC and EZTB using hypersil ODS RP-18 column. In order to effect the simultaneous elution of the two component peaks under isocratic conditions, the mobile phase composition was optimized. A satisfactory separation was obtained with a mobile phase consisting of methanol:water (70:30 v/v) at a flow rate 1 mL $min^{-1}$. Under the described chromatographic conditions, the analyte peaks were well defined, resolved and almost free from tailing and the retention times for ATVC and EZTB were found to be 2.491 and 8.057 min, respectively (Fig. 3). The optimum wavelength for the detection was 242.0 nm at which much better detector responses for both drugs were obtained. There was good repeatability of the proposed method

as the precision of the method was less than 2 % for both the drugs. The coefficient of variance for ATVC and EZTB were found to be 0.86 and 0.54 %, respectively that shows the method is highly precise.

Linearity experiment was performed thrice for both the drugs and the response was found to be linear in the concentration range of 3-15 µg/mL for both the drugs. Regression lines were obtained at 95 % confidence interval using least square method. Correlation coefficient 'r' values for both the drugs were ≥ 0.999. Accuracy of the method was determined by recovery studies (n = 3). The concentration of the standard spiked to the sample were 80-120 %. Recovery data from the study are reported in Table-1 and the % recovery was found to be in the range of 100.72 ± 0.325 to 102.38 ± 0.768 for ATVC and 98.42 ± 0.613 to 100.52 ± 0.396 for EZTB. The content of drugs in the commercial dosage form was found to be 98.92 ± 0.438 for ATVC and 99.21 ± 0.368 for EZTB (Table-2). The estimated amount was within the acceptable limits of the labeled claim of the formulation.

The developed methods *viz*., ratio spectra derivative spectrophotometry and the RP-HPLC, provides convenient and efficient methods for the separation and estimation of ATVC and EZTB in their combined dosage form. There was no interference from the excipients used in the tablet formulations and hence the methods are suitable for analysis of tablets. The results of validation show that the proposed methods are simple, linear, precise, accurate and selective and can be employed in routine assay of ATVC and EZTB in tablets.

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