Journal of Chromatographic Science 2013;51:222–228
doi:10.1093/chromsci/bms131 Advance Access publication August 3, 2012

Article

Development and Validation of Stability-Indicating Assay Method by UPLC for a Fixed Dose Combination of Atorvastatin and Ezetimibe

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Received 8 February 2012; revised 2 July 2012

A stability-indicating ultra-performance liquid chromatography method was developed and validated for the simultaneous determination of a fixed dose combination of atorvastatin and ezetimibe in bulk drugs. The developed method was successfully applied to the simultaneous quantitative analysis of the combination drugs in tablet. The chromatographic separation was performed on a Kromasil Eternity C18 UHPLC column (2.5 μm, 2.1 × 50 mm) using a gradient elution of acetonitrile and ammonium acetate buffer (pH 6.70; 0.01M) as the mobile phase at a flow rate of 0.2 mL/min with column oven temperature of 40°C. Ultraviolet detection was performed at 245 nm. Total run time was 5 min, within which the primary compounds and their degradation products were separated. The method was validated for accuracy, repeatability, reproducibility and robustness. Linearity, limit of detection and limit of quantitation were established for atorvastatin and ezetimibe.

Introduction

Atorvastatin (ATO) calcium, chemically, ([R-(R*, R*)]-2-(4-fluorophenyl)-b,d-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1H-pyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate), is a synthetic lipid-lowering agent. ATO is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis. ATO calcium is a white to off-white crystalline powder that is insoluble in aqueous solutions of pH 4 and below. ATO calcium is very slightly soluble in distilled water, pH 7.4 phosphate buffer and acetonitrile; slightly soluble in ethanol; and freely soluble in methanol (1). The lactone form of ATO (A-LCT) is the major degradation product of ATO (4). The chemical structure of ATO calcium is shown in Figure 1A.

Ezetimibe (EZE), chemically 1-(4-fluorophenyl)-3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidinone, is a white crystalline powder that is freely to very soluble in ethanol, methanol and acetone and practically insoluble in water. It melts at approximately 163°C and is reported to be stable at ambient temperature. It is one of the first new classes of lipid-lowering compounds that selectively inhibits the intestinal absorption of cholesterol and related phytosterols (2). The chemical structure of EZE is given in Figure 1B.

Clinical studies have shown that co-administration of EZE with statins could provide an additional reduction in LDL cholesterol in addition to total cholesterol. The combined use of these agents offers a satisfactorily tolerated lipid management strategy for patients with mixed hyperlipidemia (3).

ATO and its pharmaceutical formulation with EZE is not yet official in any pharmacopoeia. Several analytical methods, such as ultra-performance liquid chromatography (UPLC) (4–6), high-performance liquid chromatography (HPLC) (7–13), spectrophotometry (14–16), high-performance thin layer chromatography (HPTLC) (17, 18) and gas chromatography–mass spectroscopy (GC–MS) (19) have been reported for the determination of ATO, both individually and in combination with other drugs. Literature indicates spectrophotometry (20–23), HPLC (24–28) and HPTLC (29) methods for the determination of EZE in pharmaceutical formulations, drug substances and biological matrices. HPLC (30, 32), HPTLC (33, 34) and derivative ratio spectrophotometry (35) methods have been reported for the simultaneous determination of ATO and EZE. ATO and EZE should be monitored together with their degradation compounds, preferably in a single chromatographic run.

UPLC is a recent technique that enables significant reductions in separation time and solvent consumption. Due to very narrow and sharp peaks, more peaks may appear in less time, which may facilitate the analysis of complex mixtures, thus offering more information regarding the sample to be analyzed (4).

The purpose of this study was to develop a stability-indicating method for the simultaneous determination of ATO and EZE in bulk drugs and to apply the developed method for the quantitative determination of these drugs from tablets. The UPLC technique was chosen because of its previously mentioned advantages. The proposed method was able to separate the compounds of interest and their degradation products within 5 min. Thereafter, this method was validated as per International Conference on Harmonization (ICH) guidelines (36). A literature survey has shown that a stability-indicating UPLC method for the simultaneous determination of ATO and EZE has not been developed. The previously developed methods (30–35) have been able to separate both the drugs during a minimum run time, but they were not stability-indicating, i.e., the separation of various degradation products, employing ICH prescribed stress conditions, was not achieved.

Experimental

Materials

Reference standards of ATO calcium and EZE were gifted by Ranbaxy Research Laboratory (Gurgaon, India) with declared purity of 99.5 and 99.0% respectively. Acetonitrile (ACN) for

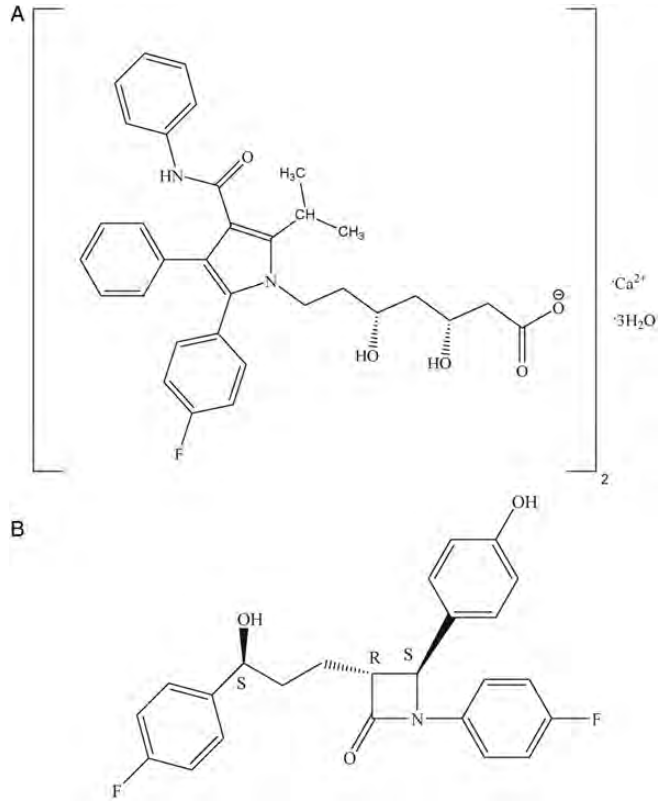

Figure 1. Chemical structures of: ATO (A); EZE (B).

HPLC was obtained from Spectrochem (Mumbai, India) and ammonium acetate was supplied by Qualigens Fine Chemicals (Mumbai, India). The 0.2-μm filter used to filter standard and sample preparation was a mdi SY13VF (PVDF), which was manufactured by Advanced Microdevices (Ambala, India). The tablet formulation (Aztor EZ) containing 10 mg each of ATO and EZE was procured from a local market in Delhi, India. Milli-Q water, obtained from Millipore (Bedford, MA), was used in making solutions.

Buffer preparation

A solution of ammonium acetate (0.01M) was prepared by dissolving approximately 0.77 g of ammonium acetate in one liter of water. The solution was sonicated in an ultrasonic bath for 3 min and finally filtered through a 0.2-μm filter.

Diluent preparation

Approximately 500 mL of ACN was transferred into a one-liter volumetric flask and 500 mL water was added to the flask. The solution was sonicated for 3 min and then filtered.

Chromatographic system

Analysis was performed on an Acquity UPLC system (Waters, Milford, MA), consisting of a binary solvent manager, sample manager and a photodiode array (PDA) detector. System control, data collection and data processing were accomplished using Waters Empower chromatography data software. The analytical column was a Kromasil Eternity C18 UHPLC column (2.5 μm, 2.1 × 50 mm). The separation of ATO and EZE was achieved by gradient elution using acetonitrile and ammonium acetate buffer (pH 6.70; 0.01 M). The final selected and optimized conditions were as follows: injection volume 1 μL, gradient elution (Table I), at a flow rate of 0.2 mL/min and column oven temperature of 40°C, detection wavelength 245 nm. Under these conditions, the backpressure in the system was 1,158 bar (equivalent to 16,791 psi).

Table I
Gradient Program for Elution of ATO and EZE

Time (min)	Flow rate (mL/min)	A (%), acetate buffer (pH 6.70; 0.01M)	B (%), acetonitrile
Initial	0.200	42.0	58.0
1.00	0.200	40.0	60.0
2.00	0.200	20.0	80.0
3.00	0.200	20.0	80.0
4.00	0.200	42.0	58.0
5.00	0.200	42.0	58.0

Assay standard solution preparation

Approximately 100 mg each of standard substances were added to a 100-mL volumetric flask. Approximately 10 mL of methanol was added to this flask and sonicated for 2 min. This solution was then diluted to the mark with diluent and sonicated for 5 min. The solution was then filtered through a 0.2-μm filter and the filtrate was collected after discarding first few milliliters. A volume of 1 mL of the filtrate was transferred to a 100-mL volumetric flask, diluted to volume with diluent and then sonicated for 5 min, resulting in a solution containing 10 μg/mL each of ATO and EZE.

Sample preparation

Twenty tablets were crushed to fine powder. An accurately weighed portion of the powder equivalent to 10 mg each of ATO and EZE was placed in a 100-mL volumetric flask. Approximately 10 mL of methanol was added to this flask and sonicated for 3 min. This solution was then diluted to the mark with diluent and sonicated for 5 min. The solution was then filtered through a 0.2-μm filter and the filtrate was collected, after discarding first few milliliters. Five milliliters of the filtrate was transferred to a 50-mL volumetric flask, diluted to volume with diluent and then sonicated for 5 min, resulting in a solution containing 10 μg/mL each of ATO and EZE.

Method validation

System suitability

System suitability parameters were measured to verify the system performance. System precision was determined on six replicate injections of standard preparations. All important characteristics were measured, including peak resolution, tailing and theoretical plate number.

Specificity

As per ICH guideline Q1AR and ICH's Common Technical Document, forced degradation studies were performed on the drug substance (except photostability testing on the drug product) to establish its inherent stability characteristics to demonstrate the selectivity and stability-indicating capability of the proposed method (37). The standard substances were exposed to acidic (1N HCl, room temperature, 5 min), alkaline (0.01N NaOH, room temperature, 5 min), neutral (water, 80°C, 4 h), strong oxidizing (30% H_2O_2, 80°C, 1 h) and thermal degradation conditions (80°C, 1 day), and a photolytic stress study was conducted on a tablet sample (254 nm, 1 day). The blank solutions were also subjected to stress in the same manner as the drug solution. All of the exposed tablet samples, standards and blank solutions were then analyzed by the proposed method.

Linearity

Linearity was demonstrated from 50 to 150% of standard concentration using a minimum six calibration levels (50, 60, 80, 100, 120 and 150%) for both of the drugs. The method of linear regression was used for data evaluation. The peak area of standard substances was plotted against respective concentrations. Linearity was described by an equation and correlation coefficient was determined.

Precision

Precision was investigated by using the sample preparation procedure for six real samples of a commercial brand (Aztor EZ) of tablets and analyzing by the proposed method. Intermediate precision was studied using different equipment and columns and performing the analysis on a different day.

Accuracy

To confirm the accuracy of the proposed method, recovery experiments were conducted by the standard addition technique. Three different levels (80, 100 and 120%) of standards were added to pre-analyzed tablet samples in triplicate. The percentage recoveries of ATO and EZE at each level and each replicate were determined. The mean of percentage recoveries ($n = 9$) and the relative standard deviation (RSD) was calculated.

Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) of ATO and EZE were determined by using the signal-to-noise approach, as defined in ICH guideline (36). Increasingly dilute solutions of each drug were injected into the chromatograph and the signal-to-noise (S/N) ratio was calculated at each concentration.

Robustness

The robustness, as a measure of method capacity to remain unaffected by small but deliberate changes in chromatographic conditions, was studied by testing the influence of small changes in the pH of buffer (±0.2 units), column temperature (±5%), organic content of mobile phase (±2%, absolute) and flow rate (±5%).

Stability of sample preparation

The stability of the sample solution was established by storage of the sample solution at ambient temperature for 24 h. The sample solution was re-analyzed after 24 h and the assay was determined and compared against a fresh sample.

Results and Discussion

Method development and optimization

The primary criteria for development of the successful UPLC method for the determination of ATO and EZE were that the method should be able to determine both drugs in a single run, should be accurate, precise, reproducible, robust and free of interference from degradation products and can be applied for routine use in a quality control (QC) laboratory.

The retention of both ATO and EZE increased with an increase in column length, so a shorter column (50 × 2.1 mm) was selected to provide the shortest possible run time without compromising on the resolution. A lower particle size (2.5 μm) column was chosen to increase the resolution between both drugs.

To identify a suitable organic modifier, various compositions of acetonitrile and methanol were tested. Methanol produced a high retention time for ATO and high column pressures due to the high viscosity. ACN was found to display advantageous separations. The buffer pH of 6.70 was found to be the most appropriate for robust resolution of the components of interest in a minimum run time.

A change of percentage of ACN in the mobile phase strongly influenced the retention time of the two drugs. When ACN content was lower than 50%, retention time of ATO increased rapidly, and when the ACN content was higher than 80%, resolution between ATO and EZE was unsatisfactory. Thus, the final gradient run was chosen with regards to the peak resolution, tailing and analysis time. The gradient program is given in Table I.

The flow rate of 0.2 mL/min was optimized with regard to the backpressure and analysis time. The standard and sample concentration of 10 μg/mL and injection volume of 1 μL were also optimized with regard to peak response, resolution and tailing. At the wavelength of 245 nm, the response of both was satisfactory and thus, their assay determination was possible in a single run. ATO and EZE were well resolved in reasonable time of approximately 5 min with retention times (RTs) of 0.675 and 1.254 min, respectively. A typical chromatogram showing separation of both drugs is shown in Figure 2.

The developed method has a resolution of approximately 7.36 between the two drugs, which suggests no interference from the degradation products, making it superior to already developed methods (30–32). The earlier methods lacked stability-indicating capability and had resolutions of approximately 2.0–4.0 between ATO and EZE.

Analytical parameters and validation

After satisfactory development of method, it was subjected to method validation as per ICH guidelines (36). The method was validated by the standard procedure to evaluate adequate validation characteristics, to demonstrate its suitability for its intended purpose.

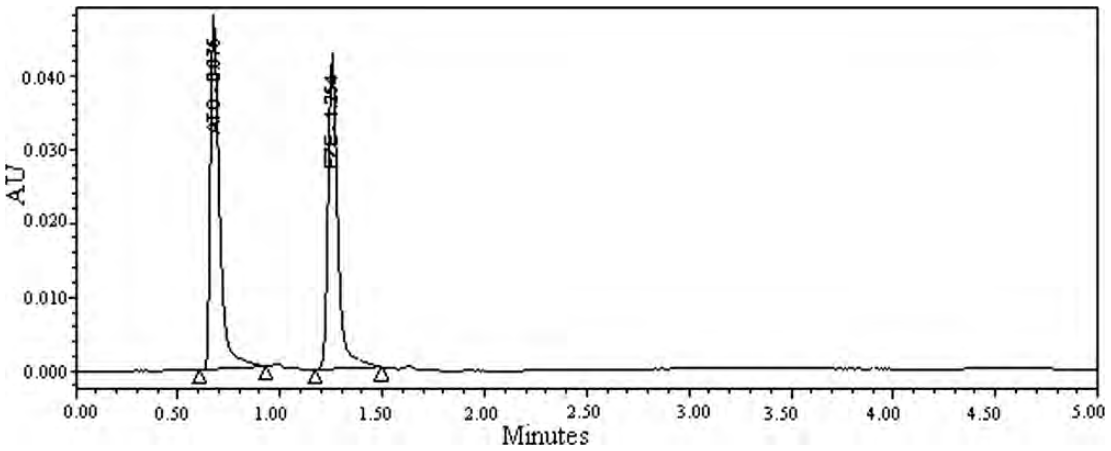

Figure 2. Chromatogram showing separation of ATO and EZE.

Table II
Method Validation Results for the Assay of Individual Compounds

Parameter	ATO	EZE
System precision* (%RSD)	0.61	0.83
Specificity†	No interference	No interference
Linearity‡ (correlation coefficient)	0.9985	0.9983
Linearity‡ (equation)	$y = 15531x - 4041.6$	$y = 14345x - 5169$
Repeatability§ (%assay)	100.7	100.8
Repeatability** (%RSD)	0.7	0.8
Intermediate precision§ (%assay)	100.2	100.3
Intermediate precision** (%RSD)	0.6	0.7
Accuracy†† (%RSD)	0.5	0.5
Accuracy†† (%recovery)	100.1	100.4
LOD (μg/mL)	0.05	0.03
LOQ (μg/mL)	0.07	0.07
Stability−24 h (%)‡‡	99.9	99.9

*Determined by six replicate injections.
†Demonstrated by forced degradation and separation of degradation products.
‡Six levels of standard concentration, from 50 to 150%.
§Average of six determinations.
**Determined on six values.
††Determined at three levels (80, 100 and 120%) with triplicate determination at each level.
‡‡Correlated with freshly prepared sample.

Table III
Forced Degradation Data

Degradation condition	Assay of ATO (%)	Assay of EZE (%)
No degradation (control)	100.3	99.4
Acid hydrolysis (1N HCl, 25°C, 5 min)	80.9	98.6
Alkali hydrolysis (0.01N NaOH, 25°C, 5 min)	100.5	75.6
Neutral hydrolysis (water, 80°C, 4 h)	87.4	73.5
Oxidation (30% H_2O_2, 80°C, 1 h)	72.8	83.5
Thermal (80°C, 1 day)	99.5	98.7
Photolytic (UV at 254 nm, 1 day)	99.8	98.8

System suitability

The percentage RSD of area count of six replicate injections of the standard solution of individual drugs was below 2.0%, which indicates that the system is precise. The results of system precision are presented in Table II.

Specificity

The results of the forced degradation study are given in Table III. ATO was found to be sensitive to acid hydrolysis. Less than 10% degradation of ATO was found when using 0.1N HCl. Therefore, 1N HCl was selected for degradation studies, which yielded A-LCT as a major degradation product at RT 1.941 min with an area of 20.17% with respect to ATO. A chromatogram of the acid degraded standard solution is presented in Figure 3A.

EZE was found to be sensitive to alkali hydrolysis. The reaction in 0.1N NaOH at 80°C was so fast that the whole of the drug degraded in 0 min, and at room temperature, there was more than 30% degradation of the drug. Subsequently, studies were performed in 0.01N NaOH at room temperature (25°C). Drug degradation was associated with a rise in a major degradation product (DP1) at RT 1.636 and a very minor degradation product (DP2) at RT 2.352 min, with areas of 3.73 and 0.79%, respectively, as shown in Figure 3B. The degradation of EZE was found to be directly proportional to the strength of alkali; additionally, the drug gradually decreased with time on heating at 50°C in 0.01N NaOH.

Both ATO and EZE were stable to water at room temperature. Upon heating the solution at 80°C for 4 h, ATO yielded a major degradation product at RT 1.932 min with an area of 2.07%, whereas the degradation of EZE was associated with degradation products at RT 0.979 (DP1) and 1.626 (DP2) min, with areas of 1.10 and 5.77%, respectively (Figure 3C). The rate of hydrolysis of ATO was found to be slower than EZE.

Both drugs were stable to hydrogen peroxide (30%) at room temperature. Upon heating the solution in H_2O_2 at 80°C for 1 h, ATO yielded two minor degradation products at RT 2.292 (DP1) and 2.661 (DP2) min with areas of 1.24 and 1.38%, respectively, with respect to ATO, whereas the degradation of EZE was associated with a rise in a major degradation product at RT 1.609 min with an area of 2.32% with respect to EZE (Figure 3D).

Both ATO and EZE were found to be stable to the effect of temperature. When the mixture of drug powders was exposed to dry heat at 80°C for one day, no decomposition of the drugs was observed; additionally, both drugs were stable in light degradation.

In the preceding stress studies, purity angle was found to be less than the purity threshold for all analytes, suggesting that

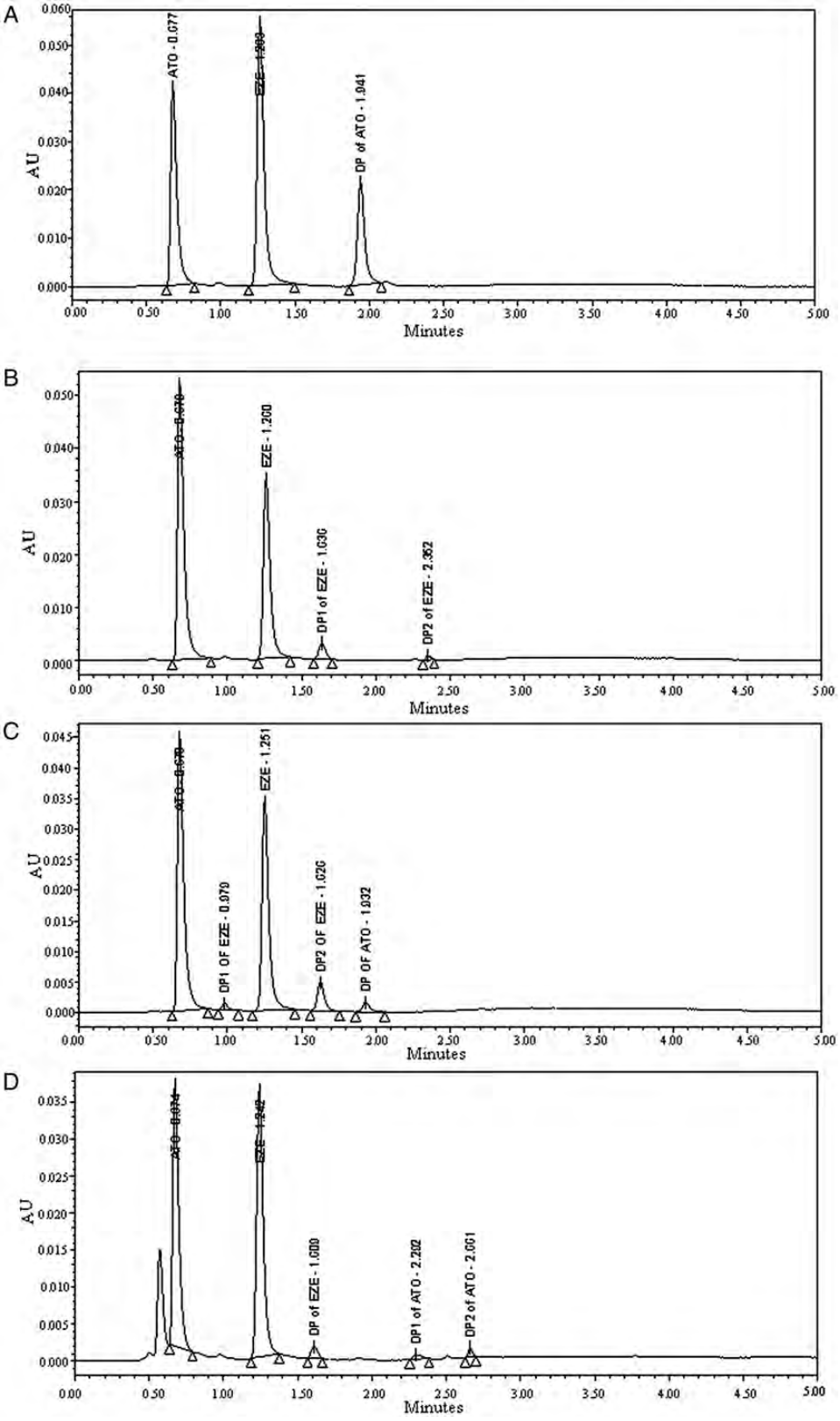

Figure 3. Chromatograms of: acid degraded standard solution (A); alkali degraded standard solution (B); neutral stress study (C); oxidative stress study (D).

Table IV
System Suitability Parameters and Robustness

System suitability parameter	Robustness parameter	ATO	EZE
Resolution	No change (repeatability)	—	7.36
	pH of buffer (+0.2 units)	—	7.12
	pH of buffer (−0.2 units)	—	7.13
	Column temperature (+5%)	—	6.82
	Column temperature (−5%)	—	7.15
	Flow rate (+5%)	—	7.13
	Flow rate (−5%)	—	7.16
	Organic content of mobile phase (+2%)	—	6.55
	Organic content of mobile phase (−2%)	—	7.81
USP tailing	No change (repeatability)	1.7	1.4
	pH of buffer (+0.2 units)	1.7	1.4
	pH of buffer (−0.2 units)	1.7	1.4
	Column temperature (+5%)	1.7	1.4
	Column temperature (−5%)	1.7	1.4
	Flow rate (+5%)	1.7	1.4
	Flow rate (−5%)	1.7	1.4
	Organic content of mobile phase (+2%)	1.7	1.5
	Organic content of mobile phase (−2%)	1.7	1.4
Column efficiency	No change (repeatability)	2,760	3,605
	pH of buffer (+0.2 units)	2,885	3,528
	pH of buffer (−0.2 units)	2,992	3,528
	Column temperature (+5%)	3,085	3,566
	Column temperature (−5%)	2,876	3,479
	Flow rate (+5%)	3,110	3,543
	Flow rate (−5%)	2,850	3,664
	Organic content of mobile phase (+2%)	2,890	3,258
	Organic content of mobile phase (−2%)	2,985	3,795

the peak of the analytes was pure and that the degradation products did not interfere with the analytes.

Linearity

The response was found to be linear from 50 to 150% of the standard concentration. For both drugs, the correlation coefficient was greater than 0.998. Correlation coefficients and linearity equations of the primary compounds are presented in Table II.

Precision

The results of the precision study are shown in Table II along with intermediate precision data. Low RSD values indicate that the method is precise.

Accuracy

The amount recovered was within $\pm 2\%$ of the amount added, which indicates that the method is accurate and no interference due to excipients is present in tablets. The results of the recoveries for the assay are shown in Table II.

LOD and LOQ

The concentration (in μg/mL) with S/N ratio of at least 3 was confirmed to be the LOD and a concentration with an S/N ratio of at least 10 was confirmed to be the LOQ, which meets the criteria defined by ICH guidelines. The LOD and LOQ results of ATO and EZE are presented in Table II.

Robustness

No significant effect was observed on system suitability parameters such as resolution, tailing and theoretical plates of the drugs when small but deliberate changes were made to the chromatographic conditions. The results are presented in Table IV, along with system suitability parameters of normal methodology. Thus, the method was found to be robust with respect to variability in the previously mentioned conditions.

Stability of sample solution

The sample solution did not show any appreciable change in assay value when stored at ambient temperature up to 24 h. The assay results are presented in Table II.

Conclusions

A novel UPLC method was successfully developed and validated for the simultaneous determination of ATO and EZE. The total run time was 5 min, within which both drugs and their degradation products were separated. Method validation results have proved the method to be selective, precise, accurate, robust and stability-indicating. This method can be successfully applied for routine analysis by the industry. It can also be utilized for the determination of content uniformity and dissolution profiling of this product, in which sample load is higher and high throughput is essential for faster delivery of results.

Acknowledgments

The authors are appreciative of Daiichi Sankyo Life Science Research Centre in India (DS-RCI), Gurgaon, Haryana for providing necessary facilities in their analytical laboratory. Ranbaxy Research Laboratory, Gurgaon, Haryana, India is kindly acknowledged for providing working standards of atorvastatin calcium and ezetimibe. The authors would also like to thank Dr. Gyan C. Yadav of Department of Chemistry, DS-RCI and Dr. Saranjit Singh of Department of Pharmaceutical Analysis, NIPER, Punjab, India for their kind support throughout the research project.

Declaration of Interest

The authors state no conflict of interest and have received no payment in the preparation of this manuscript.

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