Stability indicating method development and validation for simultaneous estimation of atorvastatin calcium and celecoxib in bulk and niosomal formulation by RP-HPLC

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The present work describes development and validation of a specific, sensitive, precise and stability-indicating high-performance liquid chromatographic method of analysis of atorvastatin calcium and celecoxib, both as a bulk drug and in niosomal formulation. The analysis has been performed by using Cosmosil-C18 column (4.6 mm×250 mm, 5 μ) at 25 °C using acetonitrile: ammonium acetate buffer pH 5.0: methanol (50:25:25 v/v/v) as mobile phase. The detection was carried out at 277nm with a flow rate of 1.0mL/min. The retention times of Atorvastatin calcium and Celecoxib were 6.195 and 3.989min, respectively. The method was validated according to ICH guidelines, for specificity, precision, linearity, accuracy and robustness. Atorvastatin calcium and Celecoxib were subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal degradation. The degradation was observed in oxidation and acid hydrolysis. The linearity for atorvastatin calcium and celecoxib were in the range of 100-500 μg/mL. The recovery study of atorvastatin and celecoxib were found to be in the range of 98.96 – 99.92% and 98.90–100%, respectively. The proposed method was validated and successfully applied to the estimation of Atorvastatin calcium and Celecoxib in combined in-house niosomal formulation.

Uniterms: Atorvastatin calcium/determination. Celecoxib/determination. High performance liquid chromatography/quality control. Niosomal formulation/quality control. Pharmaceutical formulations/ quality control.

O presente trabalho descreve o desenvolvimento e a validação de método de análise por cromatografia de alta eficiência específico, sensível, preciso e indicador de estabilidade de atorvastatina cálcica e celecoxibe, ambos como fármaco e como formulação niosômica. A análise foi realizada utilizando coluna Cosmosil-C18 (4,6 mm×250 mm, 5 μ) a 25 °C, e acetonitrila: tampão acetato de amônio pH 5,0: metanol (50:25:25 v/v/v) como fase móvel. A detecção foi realizada a 277 nm, com fluxo de 1,0 mL/min. Os tempos de retenção de atorvastatina cálcica e de celecoxibe foram 6,195 e 3,989 min, respectivamente. O método foi validado de acordo com as regras da ICH para especificidade, precisão, exatidão e robustez. A atorvastatina cálcica e o celecoxibe foram submetidos a condições de estresse por hidrólise, oxidação, fotólise e degradação térmica. A degradação foi observada por oxidação e hidrólise ácida. Observou-se a linearidade da atorvastatina cálcica e do celecoxibe na faixa de 100-500 μg/mL. A recuperação da atorvastatina e do celecoxibe foi observada na faixa de 98,96-99,92% e 98,90-100%, respectivamente. O método proposto foi validado e aplicado com sucesso para a determinação de atorvastatina cálcica e celecoxibe em formulação niosômica caseira combinada.

Unitermos: Atorvastatina cálcica/determinação. Celecoxibe/determinação. Cromatografia líquida de alta eficiência/controle de qualidade Formulação de niosomal/controle de qualidade. Formulações farmacêuticas/controle de qualidade.

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INTRODUCTION

Atorvastatin calcium is [R-(R*,R*)]-2-(4-fluorophenyl)-ß,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1*H*-pyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate [Figure1(a)] (IP, 2007). Atorvastatin is a selective, competitive inhibitor of HMG-CoA reductase, the rate-limiting enzyme that converts 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate, a precursor of sterols, including cholesterol. Atorvastatin lowers plasma cholesterol and lipoprotein levels by inhibiting HMG-CoA reductase and cholesterol synthesis in the liver and by increasing the number of hepatic LDL receptors; Atorvastatin also reduces LDL production and the number of LDL particles (Narwal, *et al.*, 2010; Moffat, c2004).

Celecoxib is *p*-[5-*p*-methylphenyl-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzene sulfonamide [Figure 1(b)] (Moffat, c2004), it is a selective cycloxygenase-2 (PTGS2/ COX2) inhibitor used for treatment of osteoarthritis and rheumatoid arthritis. It acts by reducing prostaglandin synthesis through inhibition of COX-2. Selective COX-2 inhibitors appear to provide comparable anti-inflammatory effects to conventional non-steroidal anti-inflammatory agents (NSAIDs), while avoiding serious adverse reactions, in particular, gastrointestinal toxicity observed with chronic use of NSAIDs due to COX-1(PTGS1) inhibition (Konstantinopoulos, Lehmann, 2005).
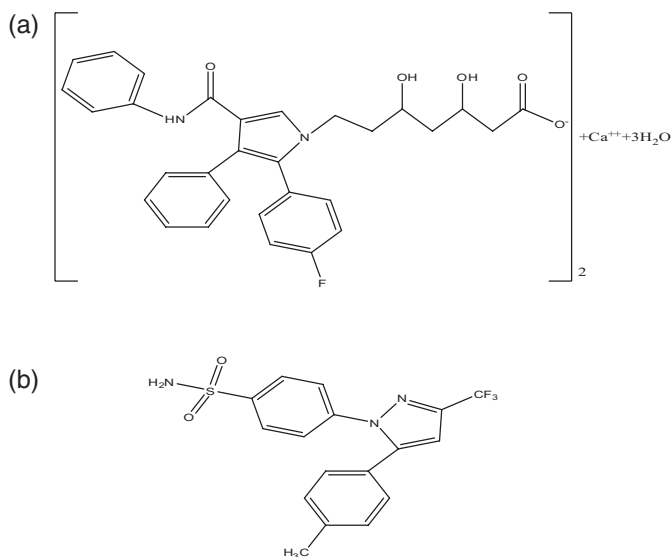

FIGURE 1 - Chemical structures of ATV Ca (a) and CXB (b).

Recent studies have indicated that in addition to the cholesterol-lowering effect, statins have pleiotropic activities that modulate other biological processes, such as cell proliferation and apoptosis. Hence, Atorvastatin acts as an anticancer agent. Celecoxib also induces the anticancer activity, the mechanism of action being that COX-2-derived PGE2 potentiates androgen biosynthesis by the cytochrome P450 enzyme aromatase and promotes angiogenesis, tumor maintenance/growth, and metastasis. The simultaneous inhibition of aromatase and COX-2 activity may therefore be responsible for the anticancer activity of celecoxib. Combinations of low doses of these anticancer agents i.e. Atorvastatin and celecoxib that have different mechanisms of action may together have significantly improved efficacy for inhibiting tumor formation and tumor growth when compared with individual anticancer substances alone (Xi, Xiao-Xing, Gina, 2007). However, both these drugs belongs to BCS class II and suffer from bioavalability problem (ATV Ca 14% and CXB 40%). Niosomes are microscopic lamellar structures formed on admixture of a nonionic surfactant, cholesterol and chloroform with subsequent hydration in aqueous media. They behave *in vivo* like liposomes prolonging the circulation of entrapped drug and altering its organ distribution. As drug delivery vesicles, niosomes have been shown to enhance absorption of some drugs across cell membranes, to localize in targeted organs and tissues and to elude the reticuloendothelial system. Niosomes can accommodate drug molecules with a wide range of solubility and thus can show better anti-angiogenic activity as compared to drugs as such (Abhinav *et al.,* 2011; Keservani *et al.*, 2011).

Literature survey reveals spectroscopic methods for determination of ATV as single drug or in combination with other drugs (Prajapati, Bhandari, 2011; Dey, *et al.*, 2012). In addition, some HPLC, HPTLC and stability indicating methods have been reported for estimation of ATV in pharmaceutical dosage forms (Surekha, Swamy, Kumar, 2012; Krishnan *et al.*, 2011; Kumar, Ghosh, Chaudhary, 2012; Mhaske, *et al.,* 2012). CXB has been determined by UV-spectrophotometric, by HPLC and stability indicating methods (Revathi, *et al.,* 2011; Jadhav, Gowekar, Gowekar, 2012; Baboota, *et al.*, 2007). Plasma determination of CXB has also been reported (Emami, Fallah, Ajami, 2008). Various spectrophotometric and HPLC methods are reported for determination of atorvastatin and CXB alone and in combination with otherdrugs (Ahmed, Manohara, Ravi, 2012; Vijayamirtharaj *et al.*, 2010). However, no Reverse phase (RP)-HPLC method has been reported so far for simultaneous estimation of ATV Ca and CXB. The aim of the present work was to develop simple, precise, accurate and reproducible RP-HPLC method for simultaneous determination of this binary drug

formulation. The proposed method was optimized and validated in accordance with International Conference on Harmonization (ICH) guidelines (ICH, 2005).

EXPERIMENTAL

Chemicals and reagents

Pure samples of ATV Ca (Batch No. 121704002) and CXB (Batch No. 121983002) were provided as a gift sample by Lupin Research Park, Pune, India. Niosomes prepared by using span 60, cholesterol and cholroform. All the chemicals and reagents used were of HPLC grade and purchased from Loba chemie Pvt. Ltd., Mumbai, India.

Instrument

The development and validation of the method was performed on a Shimadzu (LC 2010 CHT, Kyoto, Japan) having quaternary system with automatic injection facility. The detector consists of a UV-Vis model and was operated at 277 nm. The software used was 'LC Solution'. The column used was Cosmosil C18 column (4.6 mm×250 mm, 5μ). Shimadzu (UV-1800, Japan) double beam spectrophotometer with two identical quartz cells was used for absorbance measurement.

Methods

Preparation of In house niosomes

Niosomal Formulation was optimized acc. to type of surfactant and method of preparation. The best surfactant was found to be span 60 among Span 20, Span 60, Span 80 and similarly better result were obtained with ether injectiom method over Hand shaking and sonication methods. Further optimization was done w.r.t concentration of span 60. The optimized formulation prepared by ether injection method containing Span 60:cholesterol: drug in 1:1:1(w/w/w) concentation (i.e 50 mg Span 60, 50 mg cholesterol, 25 mg ATV Ca+25mg CXB) was used for chromatographic evaluation.

Chromatographic conditions

Chromatographic separation was achieved at 250 °C and the detection was carried at 277nm at a flow rate of 1mL/min. Run time was kept at 10 min. Prior to the injection of drug solution, column was equilibrated for 60min with the mobile phase flowing through the system. The injection volume was 20 μL for assay level. The analysis was performed by using Cosmosil C18 (4.6 mm×250 mm, packed with 5 μm particle size) and the mobile phase consisting of acetonitrile: ammonium acetate buffer pH 5.0: methanol (55: 20:25v/v/v).

Standard preparation

The standard stock solutions: 1000 μg/mL of ATV Ca and CXB were prepared separately by dissolving working standards in methanol and diluting with the same solvent. Standard calibration solutions of ATV Ca and CXB having concentrations in the range of 100-500 μg/mL were prepared separately by diluting stock solutions with methanol. A representative chromatogram of standard preparation is shown in Figure 2.
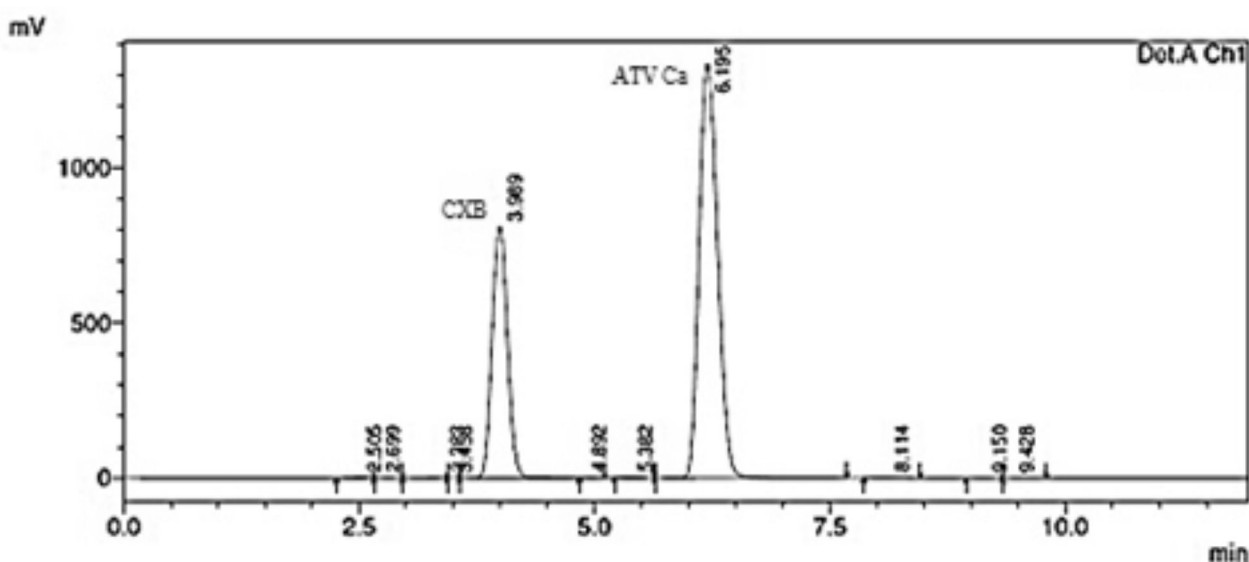

FIGURE 2 - Chromatogram of standard preparation of ATV Ca and CXB.

Sample solution

In house niosomal formulation of 50 mLwas prepared containing 25 mg ATV Ca and 25 mg CXB in combination. Sample of niosomal formulation equivalent to 5 mg of ATV Ca and CXB was accurately weighed and transferred into 10 mL volumetric flask and then diluted with methanol. The contents were sonicated for 15 minutes with intermittent shaking. This solution was filtered through 0.45 **μm nylon f**ilter, discarding first three mL of filtrate. Representative chromatogram of sample preparation is shown in Figure 3.

Analytical method validation

System suitability parameters

Mixed standard solution of ATV Ca and CXB was injected into the system for five times and system suitability parameters (tailing factor, retention time and theoretical plates) were checked.

Specificity

To assess the method specificity, blank and placebo solutions were injected into the HPLC system using the test conditions as specified above. The chromatogram was recorded and the responses of the peaks if any measured.

Linearity

The linearity of method was obtained by preparation of the calibration curve in 6 replicates. The calibration curve for ATV Ca and CXB was obtained by plotting separately the peak area of ATV Ca and CXB versus concentration of ATV Ca and CXB over the range of 100-500 μg/mL.

Accuracy

The recovery experiments were performed by adding a known quantity of pure standard drug into the solution of the tablet powder. The sample was then spiked with standard at levels 80%, 100% and 120% of test concentration. The resulting spiked sample solutions were assayed in triplicate.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Repeatability of standard application (System precision) and inter day precision study of ATV Ca and CXB were carried out by estimating the corresponding responses on the same day and on next day for 3 different concentrations of ATV Ca and CXB (200, 300, 400 μg/mL) in triplicate. Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Robustness

Robustness of the method was determined by analyzing standard solutions at normal operating conditions and by changing some operating analytical conditions such as flow rate (0.8, 1.0 and 1.2 mL/min), mobile phase ratio (52:23:25, 50:25:25 and 48:27:25 v/v/v), and detection wavelength (275, 277 and 279 nm).

LOD & LOQ

Six sets of Calibration curve were prepared and LOD and LOQ were calculated by using the formula:
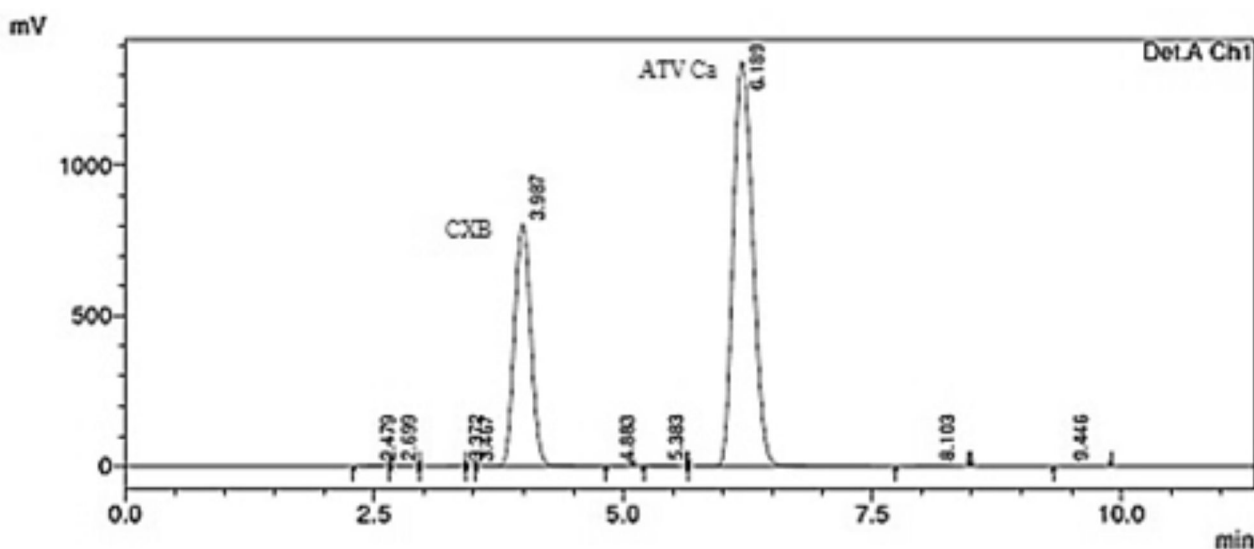

FIGURE 3 - Chromatogram of sample preparation of ATV Ca and CXB.

$$LOD = 3.3 \frac{SD}{S} \qquad LOQ = 10 \frac{SD}{S}$$

where SD is standard deviation of y-intercept of the calibration curves; S is mean slope of six calibration curves.

Forced Degradation Studies

ATV Ca and CXB standard samples were degraded under different stress conditions like acidic, alkali, oxidative hydrolysis and dry heat conditions. For acid and base degradation, samples were refluxed with 1M HCl and 1M NaOH at 70 °C for 3 h. For oxidation, 30% v/v H_2O_2 was refluxed at 70 °C for 3 h. For thermal degradation, sample was exposed to heat in oven at 100 °C for 6 h and for photo degradation; drug was exposed to direct sunlight for 6 h. Acid and base treated samples were neutralized and all the samples were then diluted to a concentration of 500 μg/mL with methanol. The samples were withdrawn and subjected to RP-HPLC analysis under optimized conditions.

RESULTS AND DISCUSSION

Method

For HPLC method optimization, different ratios of acetonitrile and ammonium acetate buffer pH5 were tried but resolution of peak was not achieved. So, methanol was used as third mobile phase. Different ratios of acetonitrile, Ammonium acetate buffer pH5 and methanol were tried i.e. 60:20:20 v/v/v, 65:15:20 v/v/v, 55:25:20v/v/v. It was found that acetonitrile: ammonium acetate buffer: methanol in the ratio 50:25:25 v/v/v as mobile phase at a flow rate of 1.0 mL/min gave good peaks. Thus, Cosmosil-C18 (4.6 mm×250 mm, 5 μm) column, 1 mL/min flow rate, 20 μL injection volume, 25 °C column oven temperature, 277 nm wavelength were the conditions, which were found to be suitable for the separation of peaks. These chromatographic conditions gave a retention time of 6.195 and 3.989 min for ATV Ca and CXB, respectively.

System suitability parameters

System suitability parameters (tailing factor, retention time and theoretical plates) were found within acceptance criteria. Relative standard deviation (RSD) for area was found satisfactory and meeting ICH specifications (% RSD≤2%). Summary of system suitability parameters is tabulated in Table I.

TABLE I - Summary of system suitability parameters

System suitability parameter	Observations		Acceptance criteria
	ATV Ca	CXB	
Tailing factor	1.209	1.089	≤ 2
Retention Time (t_R) (min)	6.195	3.989	≥ 2
Theoretical plates	4484.450	2729.27	≥ 2000
% RSD* of area	0.144	0.356	≤ 2

* n=5

Specificity

Specificity of the method was checked by injecting the placebo solution; no peaks were found at the retention time of ATV Ca and CXB.

Linearity

A good linear relationship as indicated by correlation coefficient value 0.997 and 0.999 were observed for ATV Ca and CXB, respectively over the range of 100-500μg/mL. The slope and intercept value for calibration curve was found as; y=16398x+18575 for ATV Ca and y=9235x+30017 for CXB. The results of linearity are tabulated in Table II.

TABLE II - Results of linearity

Parameter	ATV Ca	CXB
Linearity range (μg/mL)	100-500	100-500
r^2 ± SD*	0.997 ± 0.0005	0.999 ± 0.0057
Slope ± SD*	16206 ± 23.51	9099.8 ± 26.52
Intercept± SD*	360880 ± 5643.49	45259.33 ± 2738.38
LOD (μg/mL)	1.149176	0.993063
LOQ (μg/mL)	3.482351	3.009282

*n=6

Accuracy

The accuracy of the method was determined by recovery studies and the percentage recovery was calculated. The recoveries of ATV Ca and CXB were found to be in the range of 98.96 - 99.92% and 98.90 - 100%, respectively. The results were compared with the expected results and expressed as percentage as given in Table III.

Precision

The precision of the method is satisfactory as % RSD is not more than 2%. The results of the precision study are given in Table IV.
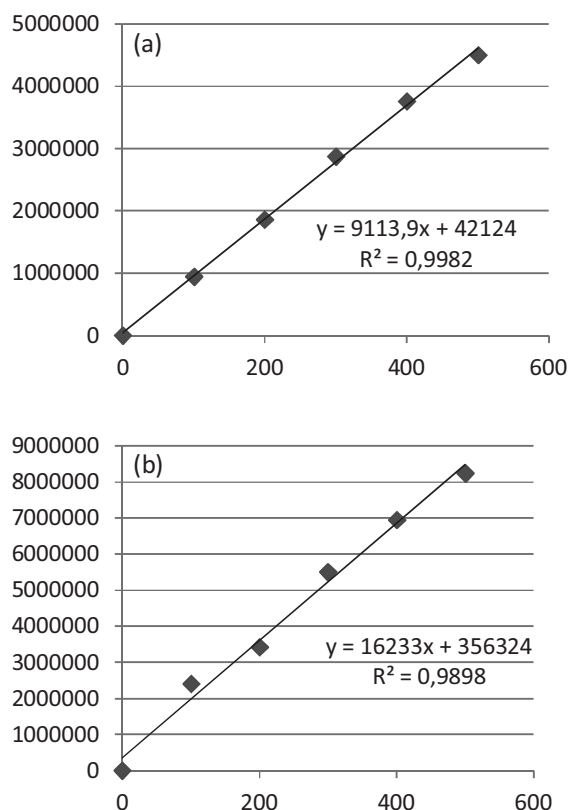
y = 9113,9x + 42124
R² = 0,9982

y = 16233x + 356324
R² = 0,9898

FIGURE 4 - **(a)** Linearity of CXB ; **(b)** Linearity of ATV Ca.

Robustness
    The results for robustness of the method are given in Table V; the results obtained were satisfactory as % RSD found to be less than 2%. The parameters like tailing factor and retention times showed adherence to the limits.

LOD and LOQ
    Results of LOD and LOQ are tabulated in Table II.

Analysis of In-house niosomal formulation
    The proposed liquid chromatographic method was applied to the determination of ATV Ca and CXB in their combined dosage forms (In-house prepared niosomal formulation). The results for ATV Ca and CXB were comparable with the corresponding added/labeled amounts given in Table VI.

Forced degradation study
    The drugs were degraded under acidic and oxidative condition while they were stable in alkaline, photolytic and thermal conditions. The ATV Ca gets degraded to form the lactone compound while the degradation product of CXB is unknown (Shah, Kumar, Singh, 2008). The chromatogram of forced degradation studies is given in Figure 5 and the results of stability studies are given in Table VII.

TABLE III - Results of Accuracy study

Drug	Level	Theoretical Amount (µg/mL)	Mean % Recovery	% RSD*
ATV Ca	50%	150	98.75	0.34
	100%	300	98.25	0.39
	150%	450	99.81	0.57
CXB	50%	150	99.44	0.45
	100%	300	97.56	0.81
	150%	450	99.65	0.74

*n=3

TABLE IV - Results of precision study

Drug	Concentration (µg/mL)	Inter-day (%RSD*)	Repeatability (%RSD*)
ATV Ca	200	0.125	0.132
	300	0.178	0.183
	400	0.171	0.179
CXB	200	0.07	0.05
	300	0.04	0.08
	400	0.04	0.05

*n=3

TABLE V - Results of robustness studies

	Robustness study of ATV Ca Chromatographic changes			Robustness study of CXB Chromatographic changes			
Factor	Level	Retention time (t_R) (min)	Area	Factor	Level	Retention time (t_R) (min)	Area
Flow rate (mL/min)				Flow rate (mL/min)			
0.8	-0.2	6.172	13044484	0.8	-0.2	3.995	7350366
1.0	0	6.12	12666143	1.0	0	3.985	7196852
1.2	0.2	6.173	12933150	1.2	0.2	3.993	7255729
%RSD*		0.4925	1.5094	%RSD*		0.1325	1.0656
Ratio of Mobile phase (ACN: Ammonium acetate buffer pH 5.0: Methanol)				Ratio of Mobile phase (ACN: Ammonium acetate buffer pH 5.0: Methanol)			
52:23:25	-2	6.119	12670019	52:23:25	-2	3.982	7196852
50:25:25	0	6.12	12666143	50:25:25	0	3.985	7196852
48:27:25	2	6.166	12924476	48:27:25	2	3.994	7330361
%RSD*		0.4376	1.1607	%RSD*		0.1566	1.0644
Wavelength (nm)				Wavelength (nm)			
275	-2	6.08	12449856	275	-2	3.961	7217802
277	0	6.12	12666143	277	0	3.985	7196852
279	2	6.104	12758710	279	2	3.98	6983030
%RSD*		0.3299	1.2554	%RSD*		0.3185	1.8215

* n=3

TABLE VI - Result of analysis of In-house niosomal formulation by proposed RP-HPLC method

Parameters	ATV Ca	CXB
Label Claim (mg)	25	25
Actual content found* (mg)	24.78±0.78	24.82±0.08
% Recovery	99.12	99.28
% RSD*	0.53%	0.8%

*n=3

CONCLUSIONS

The HPLC method developed for the analysis of mixture of ATV Ca and CXB in their pharmaceutical preparations is stability indicating, precise, accurate and with a short run time. The method was fully validated showing satisfactory data for all the method validation parameters tested. The developed method can be conveniently applied for the routine estimation of ATV Ca and CXB.

TABLE VII - Degradation studies of ATV Ca and CXB by HPLC

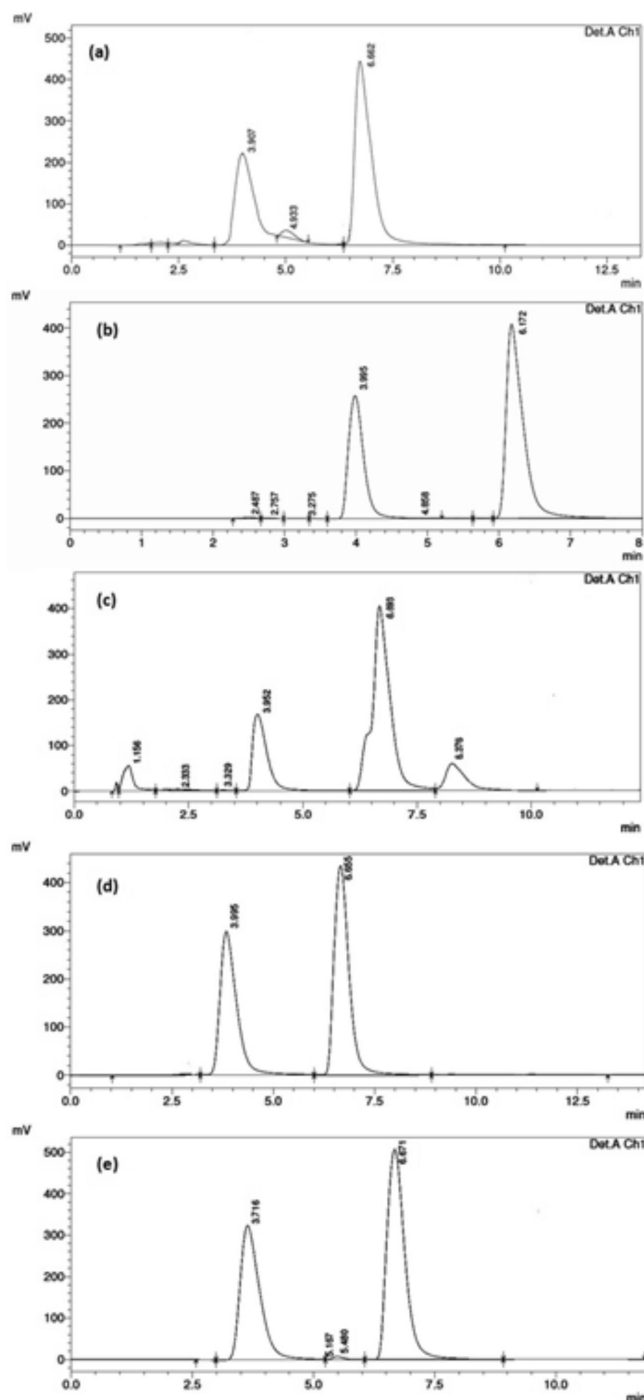| Stress condition	Amount of ATV Ca degraded (%)	Amount of ATV Ca recovered (%)	Amount of CXB degraded (%)	Amount of CXB recovered (%)	t_R of degraded products (min)
1 M HCl (3 h at 70 °C)	11.19	88.80	4.29	95.70	4.933
1 M NaOH (3 h at 70 °C)	0.039	99.96	0.12	99.87	-
H_2O_2 (3 h at 70 °C)	28.81	71.18	14.89	85.10	1.156, 8.376
Dry heat (6 h at 100 °C)	2.52	97.47	0.076	99.92	-
Sunlight(6 h)	2.18	97.81	0.094	99.90	-

t_R= Retention time, - = no degradation

FIGURE 5 - HPLC chromatogram of **(a)**acid (1M HCl, 3 h at 70 °C) treated ATV Ca; t_R 6.662 and CXB; t_R3.907, degradation product (D1); t_R 4.933. **(b)**Alkali (1 M NaOH, 3 h at 70 °C) treated ATV Ca; t_R 6.172 and CXB; t_R3.995 **(c)** H_2O_2 (30% H_2O_2, 3 h at 70 °C) treated ATV Ca; t_R 6.693 and CXB; t_R3.952, degradation products (D1); t_R1.156, (D2); t_R8.376 **(d)** Heat (6 h at 100 °C treated ATV Ca; t_R 6.665 and CXB; t_R3.995 **(e)** Sunlight (6 hr.) treated of ATV Ca; t_R6.671 and CXB; t_R 3.716.

ACKNOWLEDGEMENTS

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