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RESEARCH PAPER

STABILITY INDICATING REVERSE PHASE HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF CHLORAMPHENICOL AND FLURBIPROFEN SODIUM IN PHARMACEUTICAL DOSAGE FORM

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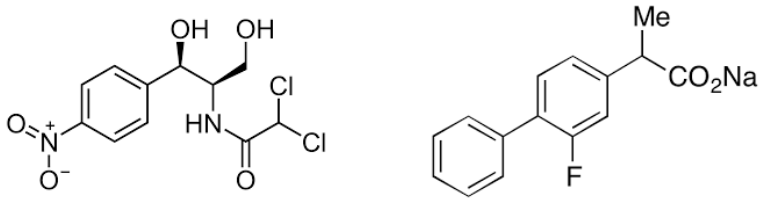
A simple, precise and rapid stability indicating RP-HPLC method is developed for the estimation of Chloramphenicol and Flurbiprofen sodium in formulation of Flubichlor eye drops in presence of degradation products. The separation was achieved using mobile phase Acetonitrile: Water: Glacial Acetic Acid (50:49:1 v/v/v) at flow rate of 1.0 ml/min. The effluent was monitored at 247 nm. The retention time of Chloramphenicol and Flurbiprofen sodium was found to be 1.980 and 7.727 min respectively. The total run time was 10.0 min within which two active compounds and their degradation products were separated. Acid, alkali, peroxide, thermal and photo degradation was carried out. The method was found to be specific enough to separate degradation products from main analytes. The described method was validated with respect to system suitability, specificity, linearity, accuracy, precision and robustness. Result of each parameter was met with its acceptance criteria. Method was found to be fast and is suitable for high-throughput analysis of the two drugs in presence of degradation products.

Key words: RP-HPLC, Chloramphenicol, Flurbiprofen sodium, Stability Indicating method.

INTRODUCTION

Chloramphenicol (CHLO) is a broad-spectrum antibiotic which acts by inhibiting protein synthesis. It is primarily bacteriostatic, having nitrobenzene substitution and is active against gram-positive and negative bacteria. It is used in the treatment of bacterial conjunctivitis and preferred drug for endophthalmitis caused by sensitive organisms. Flurbiprofen (FLUR), a non-steroidal anti-inflammatory drug, is a propionic acid derivative which acts by inhibiting the bodily synthesis of prostaglandins. It is used topically prior to ocular surgery to prevent or reduce intraoperative miosis. Combination of Chloramphenicol and Flurbiprofen is used in postoperative condition to reduce pain, swelling and eye infection, anti-infective and anti-septic, in bacterial conjunctivitis and is available as eye drops. The structures of Chloramphenicol and Flurbiprofen sodium are given in **Figure 1**.

Literature survey reveals that RP-HPLC (Koup *et al* 1978; Li *et al* 2002; Wang *et al* 2001; Rimawi and Kharoaf, 2011; Adewuyi *et al* 2011; Hossain *et al* 2011) and LC-MS (Tamosiunas *et al* 2006; Pan *et al* 2006) stability indicating method (Katakam and Sireesh, 2012; Katakam *et al* 2012) for estimation of chloramphenicol alone or in combination with other drugs from pharmaceutical formulation have been developed as well as spectrophotometric



(a) (b)

Fig. 1. Structures of (a) Chloramphenicol and (b) Flurbiprofen sodium

(Sanjeev and Jadhav, 2002; Chauhan *et al* 2007; Patel *et al* 2013), TLC (Jagathi *et al* 2011), RP-HPLC (Akhlaq *et al* 2011; Rajani *et al* 2014), high performance thin layer chromatography (Dhavse *et al* 1997), stability indicating method (Modi and Chaudhary, 2017) for estimation of Chloramphenicol and Flurbiprofen sodium. The aim of the present work is to develop and validate a new simple, rapid, selective, cost-effective and stability indicating RP-HPLC method for simultaneous determination of Chloramphenicol and Flurbiprofen sodium in a pharmaceutical formulation.

MATERIALS AND METHODS

Chemicals and Reagents

Analytical pure samples of Chloramphenicol (Swiss Parenteral Pvt. Limited, Gujarat, India) and Flurbiprofen Sodium (Sun Pharmaceuticals Industries Limited, Ankleshwar, Gujarat, India) were used in the study. The pharmaceutical dosage form used in the study was Flubichlor (Entod Pharmaceuticals Limited, Mumbai, India) procured from the local market and labeled to contain 0.5% *w/v* Chloramphenicol and 0.03% *w/v* Flurbiprofen sodium. All solvents and reagents used in the study were of AR grade.

Instrumentation

A HPLC system consists of Agilent 1260 ALS Autosampler (Agilent, United States) with microlitre syringe (20 μL) on symmetry C18 column (150 mm × 4.6 mm with 5 μm thickness, waters, Germany). Agilent PDA 1260 detector (Agilent, United States) with EZ chrome software and Shimadzu Aux 120 (Gottingen, Germany) analytical balance were used in study.

Preparation of standard solutions

Preparation of standard solution of Chloramphenicol

Chloramphenicol (50 mg) was dissolved in 50 mL of diluent to obtain a concentration of 1000 μg/mL. From, the standard stock solution, 1 mL was suitably diluted to 10 mL with diluent to obtain the working standard solutions of 100 μg/mL.

Preparation of standard solution of Flurbiprofen sodium

Flurbiprofen sodium (50 mg) was dissolved in 50 mL of diluents to obtain a concentration of 1000 μg/mL. From, the standard stock solution, 1 mL was suitably diluted to 10 mL with diluent to obtain the working standard solutions of 100 μg/mL.

Preparation of sample solution

One ml from eye drops (Flubichlor, labeled to contain 0.5% *w/v* Chloramphenicol and 0.03% *w/v* Flurbiprofen sodium) was transferred to a specified 10 ml of volumetric flask and dissolved in 5 mL of diluent, sonicated for 10 min and diluted up to mark. Then 2.0 mL of the solution was diluted to 10 mL to obtain the final concentration of 100 μg/mL Chloramphenicol and 6 μg/mL Flurbiprofen sodium.

Optimized chromatographic conditions

About 20 μL of the blank solution or standard solution was injected into symmetry, C18 (150 mm × 4.6 mm × 5.0 μ) column maintained at a temperature of 25±2 °C. The components were separated by using mobile phase of acetonitrile:water:glacial acetic acid in the ratio (50:49:1 *v/v/v*) at a flow rate 1.0 mL/min under an isocratic mode. The components were detected at a wavelength 247 nm using photodiode array (PDA) detector and all measurements were operated by EZchrom software.

Method validation

The method was validated in compliance with ICH guidelines (ICH, 1996).

System suitability studies

System suitability was established by injecting six replicates of a standard solution of Chloramphenicol and Flurbiprofen sodium and

the % RSD of retention time, asymmetry and theoretical plates were determined.

Linearity
A linear relationship between peak area and concentration of the drugs was evaluated over the concentration range expressed in µg/mL by making five replicate measurements in the concentrations range of 50–300 µg/mL for chloramphenicol and 6-18 µg/mL for flurbiprofen sodium, respectively.

Precision
The repeatability of the method was checked by analyzing (n=6) solutions of Chloramphenicol (200 μg/mL each) and Flurbiprofen sodium (12 μg/mL) the response were recorded. The intra-day (3 times on the same day) and inter-day (3 different days over a period of 1 week) precisions of the proposed method were checked by measuring the responses for 3 different concentration of 100, 150, 200 μg/mL for chloramphenicol and 6.0, 9.0, 12.0 μg/mL for Flurbiprofen sodium .The solution was injected and peak area was obtained. The % assay values were calculated. The % RSD was reported.

Recovery studies
Recovery studies were carried out by spiking three different known amounts of the standard substances to the drug product (standard addition method). Hence, 50, 100 and 150 μg/mL of Chloramphenicol and 3, 6, 9 μg/mL of Flurbiprofen sodium were spiked to the dosage form that contained 100 μg/mL of Chloramphenicol and 6 μg/mL of Flurbiprofen sodium, respectively, after sample dilution.

Limit of detection (LOD) and limit of quantitation (LOQ)
The limit of detection (LOD) and limit of quantitation (LOQ) was calculated by the equation:
LOD = 3.3 × (SD/Slope)
LOQ = 10 × (SD/Slope)
where:
SD = Standard deviation of the Y-intercepts of the calibration curve
Slope = Mean slope of the calibration curve

Robustness
The effect of deliberate variations in method parameters like the composition of the mobile phase, flow rate of mobile phase on chromatogram was evaluated in this study. The effect of these changes on peak areas was evaluated by calculating the relative standard deviations (RSD) for each parameter.

Analysis of marketed formulation
The eye drops sample solutions were prepared as per section 2.4. Suitable working sample solutions (20 μL) containing Chloramphenicol and Flurbiprofen sodium in the concentration ratio of 1:16.66 (100 μg/mL and 6 μg/mL of Chloramphenicol and Flurbiprofen sodium, respectively) were prepared, applied on HPLC column and analyzed under the optimized chromatographic conditions.

Stability studies
To evaluate the stability indicating properties of the developed HPLC method, forced degradation studies were carried out in accordance with the ICH guidelines. The standard drugs were subjected to acid, base, oxidation, thermal degradation and photo-degradation studies.

Stability stock solution
Accurately weighed 10 mg Chloramphenicol and 6 mg of Flurbiprofen sodium was diluted to 100 mL with diluent to obtain 100 μg/mL of Chloramphenicol and 60 μg/mL Flurbiprofen sodium.

Acid-induced degradation study
For acid degradation study, HCl (0.01 M, 1 mL) was added separately to 1 mL stability stock solution of Chloramphenicol and of Flurbiprofen sodium in 10 mL volumetric flasks. The mixtures were refluxed at 60 °C for 6 h and the volume was made up with diluent (100 μg/mL of Chloramphenicol and 6 μg/mL Flurbiprofen sodium). The forced degradation was performed in the dark to exclude the possible degradation effect of light. The resulting solution was applied to HPLC column and the chromatograms were run as described above.

Base-induced degradation study
For the base degradation study, NaOH (0.01 M, 1 mL) was added separately to 1 mL stability stock solution of Chloramphenicol and of Flurbiprofen sodium in 10 mL volumetric flasks. The mixtures were refluxed at 60 °C for 24 h and the volume was made up with diluent (100 μg/mL of Chloramphenicol and 6 μg/mL Flurbiprofen sodium). The samples were then applied and analyzed as described in the acid-induced degradation study.

Hydrogen peroxide-induced (oxidation) degradation study

H_2O_2 (3% *w/v*, 1 mL) was added separately to 1 mL stability stock solution of Chloramphenicol and of Flurbiprofen sodium in 10 mL volumetric flasks. The mixtures were refluxed at 60 °C for 4 h and the volume was made up with diluent (100 μg/mL of Chloramphenicol and 6 μg/mL Flurbiprofen sodium).The samples were then applied and analyzed as described in acid-induced degradation study.

Thermal degradation study

For dry heat degradation study, the standard powder drugs were placed in an oven at 60 °C for 6 h. Appropriate dilutions were prepared in methanol and then analyzed under the optimized chromatographic conditions.

Photo-degradation study

For the photo-degradation study, the standard powder drugs were exposed to UV light in a photo-stability chamber for 4 h. appropriate dilutions were prepared in methanol and then analyzed under the optimized chromatographic conditions.

RESULTS AND DISCUSSION

HPLC method optimization

For the selection of appropriate mobile phase for the effective separation of Chloramphenicol and Flurbiprofen sodium, several runs were made by using mobile phases containing solvents of varying polarity, at different concentration levels. Different mobile phase systems like at different concentration levels were tried. Among the different mobile phase combinations employed, the mobile phase consisting of acetonitrile: water: glacial acetic acid in the ratio of (50:49:1) *v/v/v* gave the best resolution with sharp well-defined peaks with R_t values of 1.980 min±0.02 and 7.727 min±0.02 for Chloramphenicol and Flurbiprofen sodium, respectively.

For the selection of analytical wavelength for the quantification of the drugs, their overlain spectra were obtained on the HPLC instrument. Both Chloramphenicol and Flurbiprofen sodium exhibited strong absorbance at about 247 nm which was selected as the analytical wavelength for further analysis.

Method validation

System-suitability parameters

The results of system-suitability test parameters were listed in **Table 1**. % RSD for all parameter was found to be less than 2 % which indicates system is suitable.

Table 1. Results of system suitability test parameters (n=6)

No.	Retention time (min)	Asymmetry	Theoretical plates	Retention time (min)	Asymmetry	Theoretical plates
	CHLO			FLUR		
1	1.98	1.61	4413	7.72	1.56	5213
2	1.97	1.62	4453	7.71	1.54	5221
3	1.95	1.59	4408	7.81	1.56	5261
4	1.95	1.60	4465	7.73	1.55	5289
5	1.99	1.61	4467	7.73	1.53	5289
6	1.98	1.62	4468	7.74	1.52	5278
Mean	1.97	1.60	4445.66	7.75	1.54	5260
SD	0.01	0.011	27.81	0.04	0.01	35.60
% RSD	0.94	0.68	0.62	0.57	0.64	0.69

Linearity

Linearity is the property of a mathematical relationship or function which means that it can be graphically represented as a straight line. Calibration graphs were constructed in the concentration range of 50–250 μg/mL for Chloramphenicol and 3-18 μg/mL for Flurbiprofen sodium. The correlation coefficients, y-intercepts, and slopes of the regression lines of the two drugs were calculated and are presented in **Table 2**.

Specificity

Specificity of the proposed method was determined by injecting about 20 μL of the blank or working standard of Chloramphenicol and Flurbiprofen sodium or sample solution into the HPLC system and chromatograms were recorded under the optimized chromatographic conditions as shown in **Figure 2**, **3**. The chromatograms of standard Chloramphenicol and Flurbiprofen sodium and sample showed

that two peaks with 100% peak area at retention time 1.980 min and 7.727 min for Chloramphenicol and Flurbiprofen sodium respectively.

Table 2. Linear regression data for the calibration curve (n=5)

Parameters	CHLO	FLUR
Linearity range	50-300 μg/mL	6-18 μg/mL
Linearity regression equation	y = 33,884.330x - 396,374.467	y = 192,920.438x - 225,416.933
Slope ± SD	33,884.330	192,920.438
Intercept ± SD	396,374.467	225,416.933
Correlation coefficient (r^2)	0.999	0.998

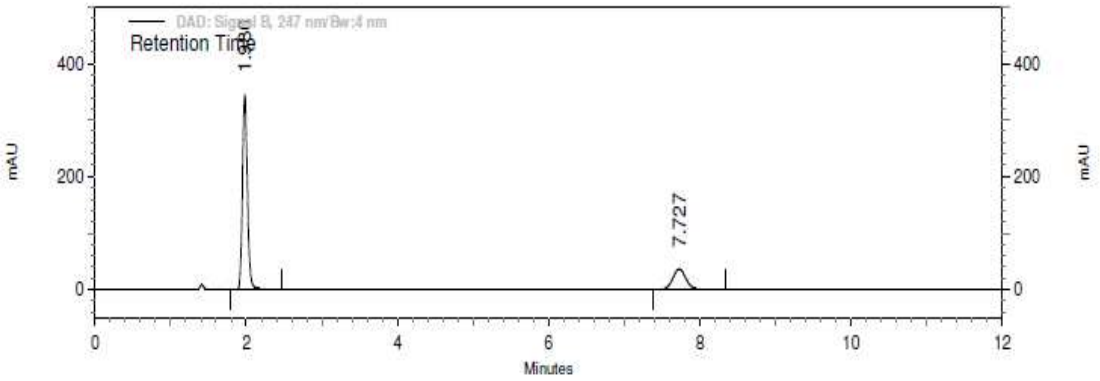


Fig. 2. RP-HPLC chromatogram of standard Chloramphenicol (100 μg/mL) and Flurbiprofen (6 μg/mL)

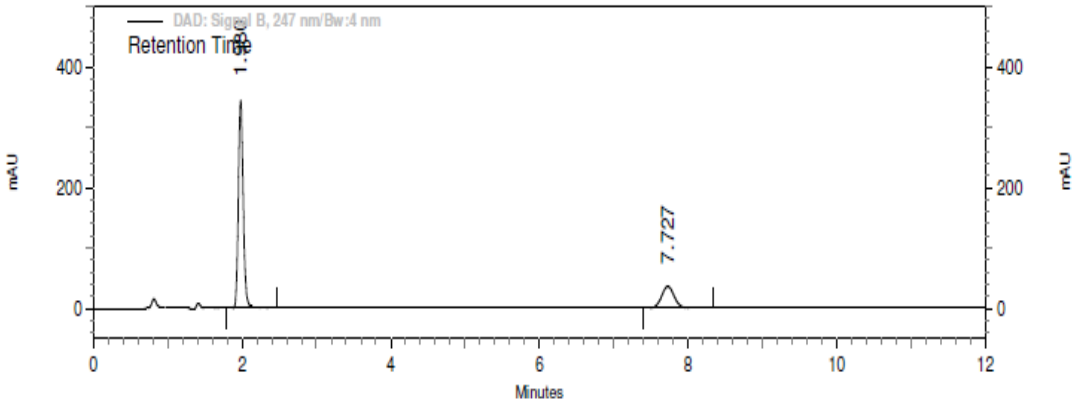


Fig. 3. RP-HPLC chromatogram of sample Chloramphenicol (100 μg/mL) and Flurbiprofen (6 μg/mL)

Precision

Repeatability and intermediate precision of the developed method were expressed in terms of relative standard deviation (RSD) of the peak area. The results showed that the repeatability solutions of Chloramphenicol (200 μg/mL each) and Flurbiprofen sodium (12 μg/mL) and inter-day variation of the results at a concentration of 100, 150, 200 μg/mL for Chloramphenicol and 6, 9, 12 μg/mL for Flurbiprofen sodium were within the acceptable range. The results of repeatability are as shown in **Table 3**. % RSD for repeatability of Chloramphenicol and Flurbiprofen sodium was 0.007 and 0.33 respectively. The results of intra-day precision and inter-day precision are shown in **Table 4**. The % RSD for intra-day precision and inter-day precision were found to be less than two, which indicated the method precision.

Accuracy/recovery studies

The recovery studies were carried out at 50%,

100% and 150% of the test concentration. The percentage recovery of Chloramphenicol and Flurbiprofen sodium at all the three levels was found to be satisfactory (**Table 5**). For Chloramphenicol, the % recovery was found between 98.83% and 100.33% and for Flurbiprofen sodium between 99.92% and 100.37%, respectively.

Table 3. Results of repeatability (n=6)

Drug	Conc.	1	2	3	4	5	6	SD	%RSD
CHLO	200	6409454	6410500	6409648	6410325	6410216	6409545	450.5779	0.007029
FLUR	12	2096215	2089541	2095481	2088195	2098125	2079581	6902.4	0.33007

Table 4. Intraday and Interday precision of the method (n=3)

Drug	CHLO			FLUR		
Conc. (*μ*g/ml)	100	150	200	6	9	12
Intraday precision						
Mean peak area	2982720	4675089	6410500	944350	1566497	2096215
± S.D.	12801.580	15069.603	17844.531	2382.142	5198.589	4766.628
% R.S.D	0.42	0.32	0.27	0.29	0.34	0.22
Interday precision						
Mean peak area	2970268	4658494	6434258	946695.7	1512253	2086350
± S.D.	16318.930	28618.000	32525.44	4567.028	10301.580	7339.803
% R.S.D	0.54	0.61	0.50	0.48	0.68	0.44

Table 5. Recovery as accuracy studies of the proposed HPLC method (n=3)

Drug	Spike level %	Taken	Added	Found	Mean peak area	% Recovery
CHLO	50	100	50	149.25	4660976.7	99.06
	100	100	100	200.92	6411781.3	101.20
	150	100	150	247.79	7965981	98.70
FLUR	50	6	3	8.99	1510256.3	98.20
	100	6	6	12.00	2089635	99.15
	150	6	9	14.89	2647795.3	98.24

Limit of detection (LOD) and quantitation (LOQ)

The limits of detection and quantitation were found to be 1.05 and 3.18 *μ*g/mL for Chloramphenicol and 0.075 and 0.23 *μ*g/mL for Flurbiprofen sodium, respectively, indicating the sensitivity of the developed method.

Robustness of the method

The robustness of the method evaluated by assessing the effect of variations in method parameters on peak areas showed low RSD values (less than 2.0%) indicating the robustness of the method (**Table 6**).

Table 6. Robustness of the developed HPLC method (n=3)

Parameters	Drug	Mean area ± SD	RSD
Mobile phase composition (+2 mL)	CHLO	3021766 ± 19228.03	0.63
	FLUR	946076 ± 8418.73	0.88
Mobile phase composition (-2 mL)	CHLO	2970918 ± 23765.87	0.79
	FLUR	944176 ± 6722.63	0.71
Flow rate (1.2 mL)	CHLO	2972538 ± 25688.99	0.86
	FLUR	944146 ± 8266.74	0.87
Flow rate (0.8 mL)	CHLO	3035169 ± 30834.16	0.95
	FLUR	947552 ± 9005.28	0.86

Analysis of marketed formulation

The marketed formulation, Flubichlor was analyzed using the developed method. The chromatogram of eye drops sample showed only two peaks at R_t value of 1.980 min and 7.727 min for Chloramphenicol and Flurbiprofen sodium, respectively, indicating that there is no interference of the excipients present in the tablet formulation. The content of Chloramphenicol and Flurbiprofen sodium was

calculated by comparing peak areas of the sample with that of the standard (**Table 7**).

Table 7. Assay results of the pharmaceutical dosage form (n=5)

Drug	Amount present per 1 ml of solution	% Amount found	SD	RSD
CHLO	5 mg	101.49 %	0.00827	0.16
FLUR	0.3 mg	99.78 %	0.00032	0.10

Stability studies

The results of the forced degradation study of Chloramphenicol and Flurbiprofen sodium using acetonitrile: water: glacial acetic acid (50:49:1 *v/v/v*) as the mobile phase system are summarized in **Table 8**.

Table 8. Summary of forced degradation studies of Chloramphenicol and Flurbiprofen sodium

Exposure conditions	Drug	Time	% Recovery	Degradation products (%)	R_t of degradation products (min)
Acid, 0.1 N HCl, refluxed	CHLO	6 h	83.26	DP1 (11.53 %), DP2 (5.53%)	2.547, 2.760
	FLUR	6 h	79.90	DP1 (19.59 %)	7.000
Base, 0.1 N NaOH, refluxed	CHLO	24 h	97.34	DP1 (2.66 %)	2.527
	FLUR	24 h	99.48	Not detected	-
Peroxide (3%, *v/v*), refluxed	CHLO	4 h	77.62	-	-
	FLUR	4 h	79.15	-	-
Dry heat (60 °C)	CHLO	6 h	80.46	DP1 (19.22 %), DP2 (0.2%)	1.413, 2.540
	FLUR	6 h	85.04	DP1 (14.58%)	6.572
Photo	CHLO	4 h	90.48	DP1 (2.00 %), DP2 (7.51%)	2.533, 3.227
	FLUR	4 h	99.13	-	-

*DP1 and DP2 are the degradation products obtained in the forced degradation studies.

Acid-induced degradation study

Chloramphenicol and Flurbiprofen sodium, both were found to undergo acid degradation very rapidly. The reaction in 0.1 M HCl at 60 °C under reflux for 6 h showed extensive degradation for chloramphenicol with additional peaks at R_t values of 2.547, 2.760 (about 11.53 %, 5.34 % degradation), respectively. For Flurbiprofen sodium, additional peaks were observed with R_t values 7.000 (about 19.54% degradation), respectively as shown in **Figure 4**, suggesting significant degradation of both drugs.

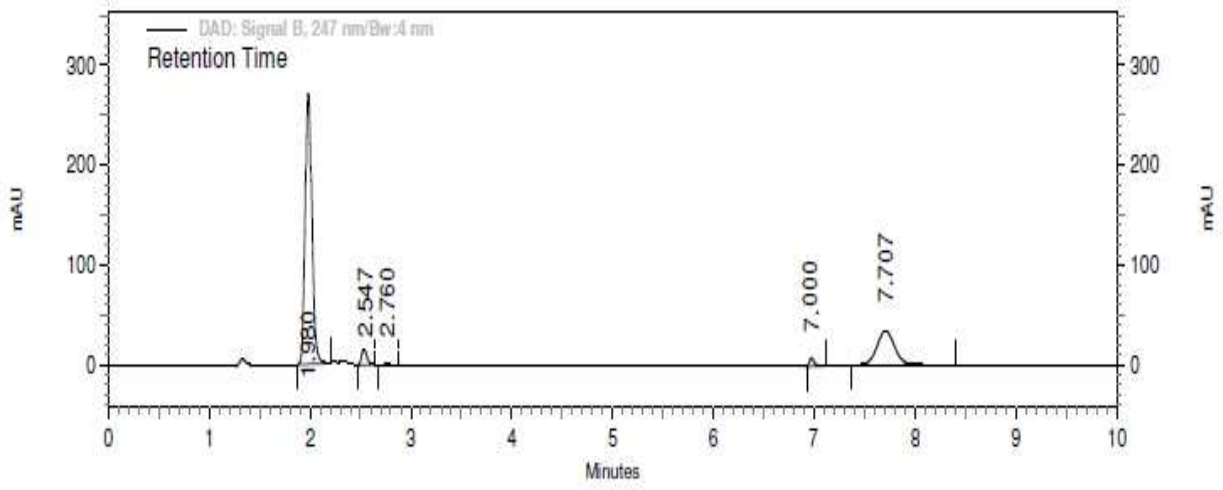


Fig. 4. Chromatogram of acid degraded Chloramphenicol and Flurbiprofen sodium

Base-induced degradation study

In base induced degradation study, drugs did not show any additional peak other than the standard Chloramphenicol and Flurbiprofen sodium peak at R_t 1.980 min and 7.693 min respectively. Drug recovery at the level of 97.34 % and 99.48 % for Chloramphenicol and Flurbiprofen sodium respectively, suggested that the drug is stable toward the alkali as shown in **Figure 5**.

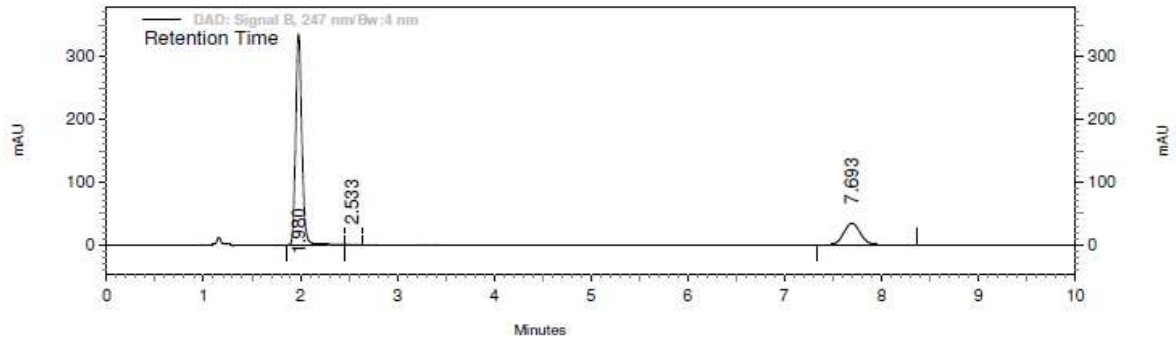


Fig. 5. Chromatogram of alkali degraded Chloramphenicol and Flurbiprofen sodium

Oxidative induced degradation study

In the oxidative degradation study, it was found that the area of the H_2O_2-degraded product peaks of Chloramphenicol and Flurbiprofen sodium was found to be extremely small than the area of standard drug concentration and drug recovery at the level of 77.62% and 79.15% for Chloramphenicol and Flurbiprofen sodium respectively suggest that significant degradation of both drugs in oxidative induced degradation. The chromatogram of peroxide degraded CHLO and FLUR is shown in **Figure 6**.

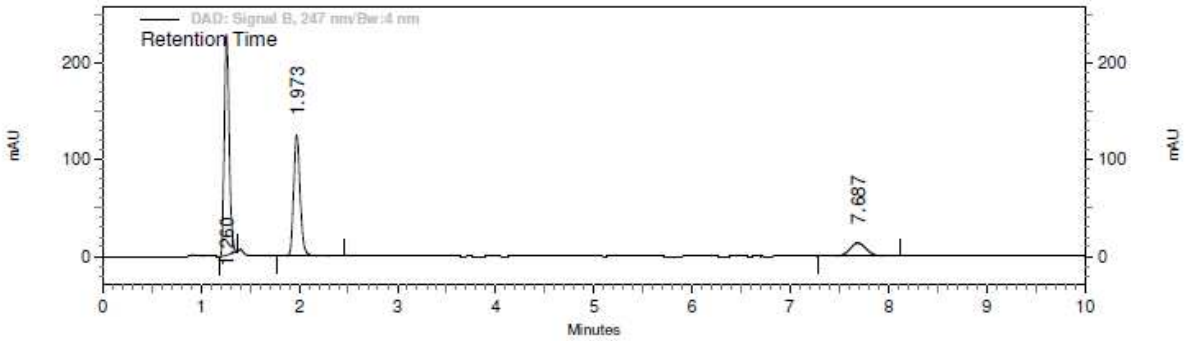


Fig. 6. Chromatogram of peroxide degraded Chloramphenicol and Flurbiprofen sodium

Thermal degradation study

In the heat degradation study, Chloramphenicol and Flurbiprofen sodium showed additional peaks at R_t value at 1.413 (about 19.22% degradation) and 6.687 (about 14.58% degradation), respectively as shown in **Figure 7**, suggesting significant degradation of both drugs in thermal degradation.

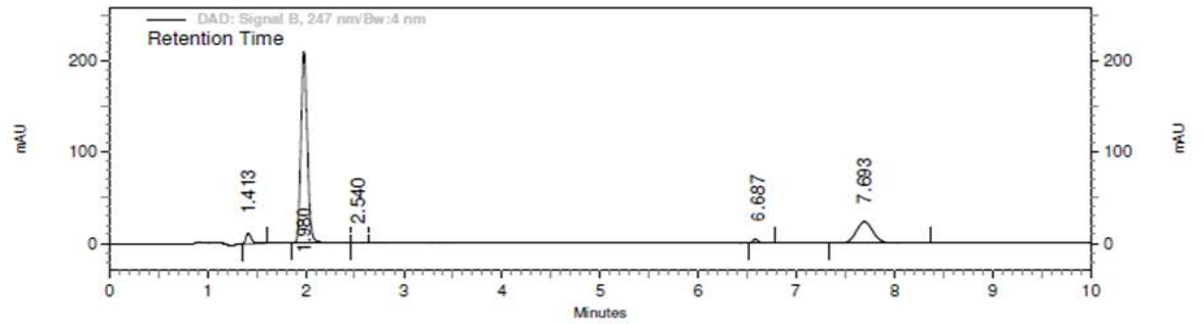


Fig. 7. Chromatogram of thermal degraded Chloramphenicol and Flurbiprofen sodium

Photo-degradation study

In the oxidative degradation study, it was found that the area of the photodegraded product peaks of Chloramphenicol and Flurbiprofen sodium was found to be extremely small than the area of standard drug concentration and drug recovery at the level of 90.48% and 99.13% for Chloramphenicol and Flurbiprofen sodium respectively, suggesting the stability of FLUR toward photodegradation as shown in **Figure 8**.

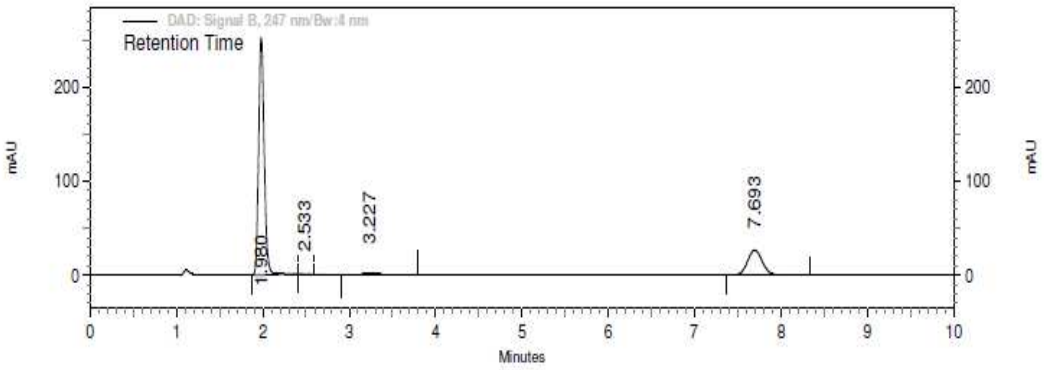


Fig. 8. Chromatogram of photo degraded Chloramphenicol and Flurbiprofen sodium

CONCLUSION

Stability indicating high-performance liquid chromatography (HPLC) method was developed and validated for the determination of Chloramphenicol and Flurbiprofen sodium in co-formulations on HPLC column using acetonitrile: water: glacial acetic acid (50:49:1 *v/v/v*) as the mobile phase with PDA detection at 247 nm. The developed method was found to be simple, rapid, selective, sensitive and suitable for the simultaneous determination of Chloramphenicol (CHLO) and Flurbiprofen sodium (FLUR). The stability indicating properties established the following recommendations of ICH guidelines also indicated that the drugs could be evaluated in presence of their degradation products and thereby can be employed for the simultaneous estimation of Chloramphenicol and Flurbiprofen sodium and their degradation products in stability samples in the industry.

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