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Development and validation of a stability indicating LC method for the analysis of chlordiazepoxide and trifluoperazine hydrochloride in the presence of their degradation products

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ABSTRACT

A novel, accurate, and specific stability-indicating RP-HPLC method for determining Chlordiazepoxide (CLR) and Trifluoperazine HCl (TFP) in drug substances and drug products has been developed. A forced degradation study was performed as per the ICH guideline for both drugs. The degradation of chlordiazepoxide and trifluoperazine HCl in bulk and formulation was tested under a variety of stress conditions, including acidic, alkaline, neutral, oxidative, thermolytic, and photolytic conditions. The Separation was done using a C_{18} (250 mm × 4.6 mm, 5µm) column as a stationary phase and 70:30%(v/v) Acetonitrile: Phosphate buffer (pH 5.5) adjusted with 0.1% Triethaylamine (TEA) as isocratic mobile phase. The flow rate was 1ml/min and the wavelength for detection was 262 nm. The retention time was 4.1min and 7.1min for Chlordiazepoxide and Trifluoperazine HCl respectively. The developed method was validated as per the ICH guideline Q2(R1). Specificity, linearity, accuracy, precision, LOD, LOQ, robustness, and system suitability were checked to meet specified criteria. Specificity, linearity, precision, accuracy, LOD, LOQ,

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robustness, system suitability, and other criteria were analyzed, Chlordiazepoxide and Trifluoperazine HCl were susceptive to degradation in photolytic and thermal stress conditions. The method was proven to be appropriate for use in the analysis of Chlordiazepoxide and Trifluoperazine HCl formulations in quality-control laboratories.

Keywords: chlordiazepoxide, trifluoperazine hydrochloride, HPLC, stability indicating, degradation products

INTRODUCTION

Chlordiazepoxide

Chlordiazepoxide is a long-acting benzodiazepine approved by the FDA for adults suffering from mild-moderate to severe anxiety, preoperative anxiety, and alcohol withdrawal. It is one of the safer psychopharmacological benzodiazepine compounds. Chlordiazepoxide (CLR) structure is shown in Figure 1, and the IUPAC name is 7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine 4-oxide having molecular formula is $C_{16}H_4ClN_3O$ and the molecular weight is 299.75g/mol, melting point is 135-138°C. CLR is soluble in water and alcohol[1-3].

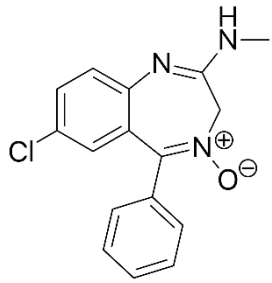

Figure 1. Structure of Chlordiazepoxide

Trifluoperazine hydrochloride

Trifluoperazine, a typical antipsychotic medication, not only blocks dopamine D2 receptors but also stimulates 5-HT2 receptor-mediated behavior. Trifluoperazine was discovered to be superior to a placebo for the treatment of generalized anxiety disorder in a randomized, double-blind, placebo-controlled study. Trifluoperazine also suppresses human purinergic receptor P2X7 responses, which relate inflammation to depression. This action is comparable to that of paroxetine. Trifluoperazine Hydrochloride (TFP) structure is shown in Figure 2, and the IUPAC name is 10 - [3 - (4 - methyl piper zine - 1 - yl) propyl] - 2

- (trifluoromethyl) phenothiazine, hydrochloride. TFP belongs to the phenothiazine class and is used as an antipsychotic for treating schizophrenia and anxiety[4-6].

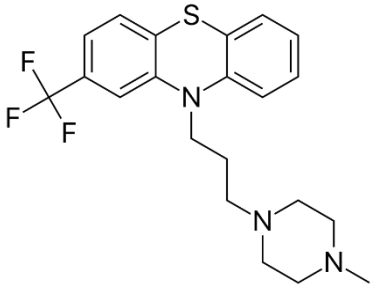

Figure 2. Structure of Trifluoperazine HCL

This two-drug combination is used in the treatment of mental disorders such as schizophrenia, and psychotic disorders. In this combination CLR is acted by enhancing the action of GABA, a chemical messenger that overcomes abnormal and excessive activity of nerve cells in the brain and TFP is an antipsychotic, it acts by blocking the action of dopamine, which affects thoughts and mood.

The literature review revealed that many UV spectrophotometric[7-10] and chromatographic methods[11-18] are available for the estimation of both drugs either alone or in combination with other drugs. One UV visible spectrophotometric method[18] and two RP-HPLC methods[19-21] are available for the determination of both drugs in combination. But there is no reported stability indicating the RP-HPLC method for Simultaneous estimation of CLR and TFP in the combined dosage form. The present RP-HPLC method is specific for the simultaneous quantification of chlordiazepoxide and trifluoperazine HCl in both formulation and bulk in presence of its degradation products in various stressed conditions[22].

METHODOLOGY

Instrumentation

Chromatographic measurement was performed on a Shimadzu corporation LC-2010 HT system (Shimadzu Corporation, Tokyo), consisting of a quaternary pump LC-2010, and a UV-Visible detector LC-2010. For drug substance chromatographic separation, a reverse-phase Phenomenex Luna C18 analytical column (4.6mm×250mm) was used. Chromatographic analysis and data integration were recorded on a Windows computer system using a Shimadzu LC-2010HT LC system with software LC-solution (1.25).

Reagents and materials

Reference Standards for trifluoperazine and chlordiazepoxide were procured from MANUS AKTTEVA BIOPHARMA LLP. Formulations of LIBRA CHEM-T tablets with the labelled dosages of 1 mg and 10 mg of CLR and TFP each were purchased from the local market. All HPLC-grade solvents were bought from Merck (Mumbai, India) and all AR-grade chemicals were bought from Loba Chemie Pvt Ltd.

Chromatographic conditions

Several isocratic elution strategies were employed to assess the optimization of chromatographic parameters. At pH 5.5, the optimised mobile phase was found to be 70:30% (v/v) Acetonitrile: Phosphate buffer adjusted with 0.1% TEA. The wavelength of detection was 262nm, and the flow rate was 1ml/min. The optimized chromatogram gives a sharp and symmetric peak with retention times of 4.17 minutes for CLR and 7.17 minutes for TFP.

Standard solution preparation

A standard solution of CLR (1000µg/ml) and TFP (1000ug/ml) was prepared by dissolving an accurately weighed quantity of CLR 10 mg and TFP 10 mg in 10 mL of mobile phase, and then the same solvent was used to dilute 1 mL of the resultant solution to 10 mL.

Sample solution preparation

Twenty tablets were weighed and then finely powdered (CLR 10 mg, TFP 1 mg). In a 10ml volumetric flask, tablet powder containing 10 mg of CLR and 1 mg of TFP was transferred. In order to create "Sample Stock1," 5ml of methanol was added, sonicated for 10 minutes, diluted with methanol to volume, and then filtered through Whatman filter paper No. 41. 1 ml of the resultant solution was diluted up to 10 ml with mobile phase to produce "Sample Stock2," which contained 100µg/ml CLR and 10µg/ml TFP. Syringe filters were used to filter Sample Stock 2, which was then injected into the HPLC apparatus. The calibration curve formulae y= 20783x+5041.8 and y= 63950x-2270 were used to determine the concentrations of CLR and TFP in the tablets, respectively.

Forced degradation studies[21]

Preparation of test solution for forced degradation study

From the stock solution of CLR and TFP, 20ml and 2ml were pipetted out respectively and made up to 100ml volumetric flask with the mobile phase.

Acid degradation

Accurately weighed and transferred TFP (1mg) and CLR (10mg) into a 50 ml volumetric flask. 10ml of 0.1M HCl was added and thoroughly mixed, and the volumetric flask was set to reflux at 70°C for 3 hrs. After the time period, the reaction was stopped by neutralizing the mixture with 10 ml of 0.1M NaOH. The solution was then diluted to a volume of 50 ml with the mobile phase. The tablet contained 10mg CLR and 1mg TFP was Taken and transferred into 50 ml of volumetric flask. 10 ml of 0.1M HCl was added and thoroughly mixed in. For 3 hours, the volumetric flask was refluxed at 70°C. Following the time period, the mixture was neutralized with 10 ml of 0.1M NaOH to stop the reaction. The solution was then diluted to a volume of 50 ml with the mobile phase.

Base degradation

Accurately weighed and transferred TFP (1 mg) and CLR (10 mg) into a 50 ml volumetric flask. 10 ml of 0.1M NaOH was added and thoroughly mixed, and the volumetric flask was set to reflux at 70 °C for 3 hrs. After the time period, the reaction was stopped by neutralizing the mixture with 10 ml of 0.1 M HCl. The solution was then diluted to a volume of 50 ml with the mobile phase. The tablet contained 10 mg CLR and 1mg TFP was Taken and transferred into 50 ml of volumetric flask. 10 ml of 0.1 M NaOH was added and thoroughly mixed in. For 3 hours, the volumetric flask was refluxed at 70 °C. Following the time period, the mixture was neutralized with 10 ml of 0.1 M HCl to stop the reaction The solution was then diluted to a volume of 50 ml with the mobile phase.

Neutral degradation

Accurately weighed and transferred TFP (1mg) and CLR (10mg) into 50 ml volumetric flask.10ml of water was added and thoroughly mixed, and the volumetric flask was set to reflux at 70°C for 3 hrs. Following the time period, the mixture was diluted to a volume of 50 ml with the mobile phase. The tablet contained 10mg CLR and 1mg TFP was Taken and transferred into 50 ml of volumetric flask. 10 ml of water was added to it and thoroughly mixed in. For 3 hours, the volumetric flask was refluxed at 70°C. Following the time period, the mixture was diluted to a volume of 50 ml with the mobile phase.

Oxidative degradation

Accurately weighed and transferred TFP (1mg) and CLR (10mg) into a 50 ml volumetric flask. 10 ml of 3% H_2O_2 was added and mix well. The volumetric flask was refluxed at a temperature of 70°C for 3 hrs. Following the time period, the mixture was diluted to a volume of 50 ml with the mobile phase. The

tablet contained 10mg CLR and 1mg TFP was Taken and transferred into 50 ml of volumetric flask. 10 ml of 3% H_2O_2 was added to it and mix well. The volumetric flask was refluxed at a temperature of 70° C for 3 hrs. Following the time period, the mixture was diluted to a volume of 50 ml with the mobile phase.

Thermal degradation

Accurately weighed and transferred TFP (1mg) and CLR (10mg) into a petri dish. The Petri dish was then placed in the hot air oven for 12 hours at a temperature of 110 o C. A heated drug sample was transferred to and dissolved in a mobile phase in a 50ml volumetric flask. The heated drug sample was dissolved in the mobile phase in a 50ml volumetric flask. Volume was made up to the mark using the mobile phase. The tablet contained 10mg CLR and 1mg TFP was Taken, powdered, and transferred into a petri dish. The Petri dish was then placed in the hot air oven for 12 hours at a temperature of 110 ° C. A heated drug sample was transferred to and dissolved in a mobile phase in a 50ml volumetric flask. Volume was made up to the mark using the mobile phase.

Photolytic degradation

Accurately weighed and transferred TFP (1mg) and CLR (10mg) into a petri-dish and for 24 hours, a petri dish was placed within the UV chamber. The drug sample was dissolved in a mobile phase in a 50ml volumetric flask. Volume was made up to the mark using the mobile phase. The tablet contained 10mg CLR and 1mg TFP was Taken, powdered, and transferred into a petri dish. A petri dish was put inside the UV chamber for 24 hours. In a 50ml volumetric flask, a UV-Exposed drug sample was transferred and dissolved in the mobile phase. Volume was made up to the mark using the mobile phase.

Method validation

Specificity

It is the ability to analyses unequivocally samples in the presence of other components which are expected to exist or present which can be impurities, degradants, or matrices. Specificity was determined by injecting diluents, standard solution, and sample preparation. Forced degradation was performed on the drug product in addition to establishing specificity.

Linearity and range

Six solutions were prepared in the mobile phase. The range was 50-300 µg/ml and 5-30 µg/ml for CLR and TFP respectively. The calibration plot is obtained

which determines the slope, coefficient correlation and intercept providing the required statistics for linearity.

Accuracy

The accuracy of the method was determined by calculating the recoveries of CLR and TFP by the method of standard addition. The recovery was assessed at three levels 80%,100%, and 120%. The % recovery was calculated.

Precision

The precision was performed by repeatability and inter-day or intra-day precision. For repeatability 6 replicates were injected for CLR and TFP and %RSD was calculated. For inter-day and intra-day precision 3 solutions with different concentration levels solution were prepared for the two drugs and %RSD values were calculated accordingly.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD is defined as "the lowest or smallest concentration of component or sample which can be detected for a specified experimental condition of an analytical method.

LOQ is defined as "an ability to detect and precisely and accurately quantify the least or lowest concentration of compound or sample under the stated experimental parameters of an analytical method.

Robustness

It is a measurement of the ability of any analytical technique to remain unaffected or unchanged by deliberate or known variation in method parameters like in HPLC involving column or sample temperature, flow rate, pH, mobile phase ratio, and injection volume.

System suitability test

It is performed to prove the suitability and reproducibility of the developed method. The test solution was taken in the concentration of CLR and TFP 100µg/ml, and 10µg/ml respectively. Then six replications were injected into the system. Various parameters used for these tests including capacity factor (K NMT 2), resolution (NLT 1.5), tailing factor (NMT 2), column efficiency or number of theoretical plates (N more than 2000), relative standard deviation (% RSD NMT 2%) and separation or relative retention (NLT 2).

RESULTS and DISCUSSION

Development of LC method

The LC method was developed with good peak shape and resolution. Peak characteristics like symmetry and theoretical plates were used to determine the mobile phase. Table 1 shows the optimized conditions, and Figure 3 shows the chromatogram.

Table 1. Optimized LC conditions for the analysis of CLR and TFP

SR NO.	Parameter	Results
1	Mobile phase	Acetonitrile : Phosphate buffer (10mM) (70:30 v/v) (pH-5.5) pH adjust with 0.1%TEA
2	Stationary phase	Luna C_{18} column (4.6mm×250mm)
3	Column oven temperature	40°C
4	Wavelength	262nm
5	Flow rate	1ml/min
6	Elution mode	Isocratic
7	Injection volume	10µl

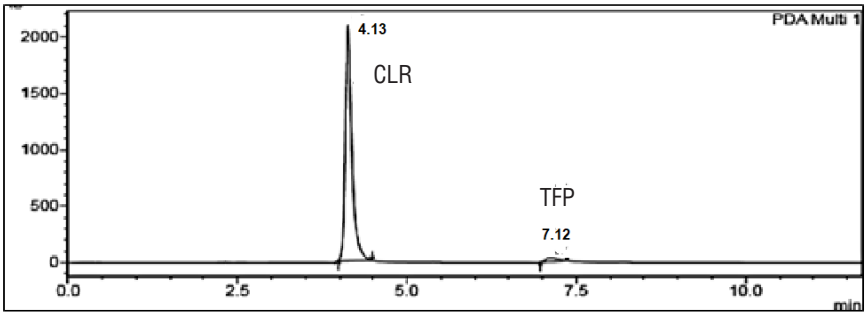

Figure 3. Standard chromatogram of CLR and TFP for degradation study

Forced degradation study

Degradation was observed under various stress conditions such as acidic, basic, photolytic, thermal, oxidation, and neutral. CLR and TFP samples were degraded into acid (Figure 4), base (Figure 5), photolytic (Figure 6), oxidative (Figure 7), Thermal (Figure 8), and neutral (Figure 9) conditions and formed polar impurities. The CLR and TFP sample peaks are homogeneous under all evaluated stress situations, according to peak purity data. The tablet's unaffected sample assay demonstrates the method's accuracy in showing stability (Table 2).

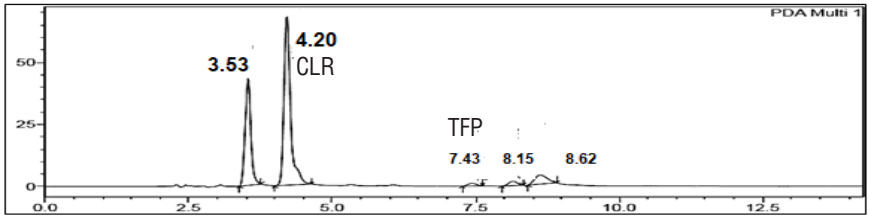

Figure 4. Chromatogram of acidic degradation

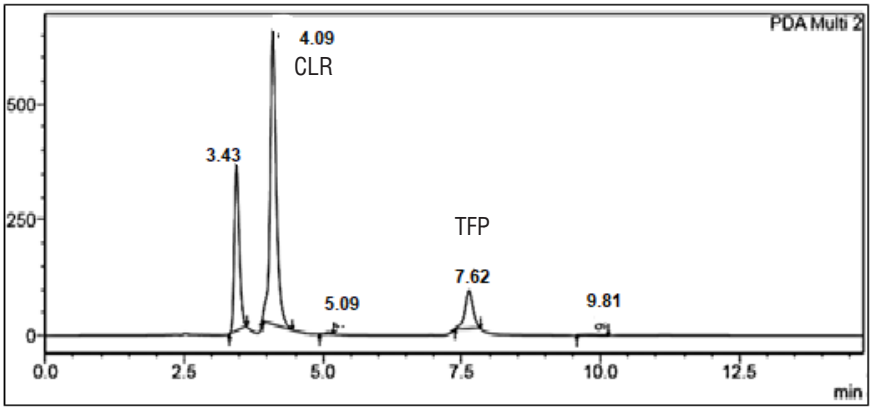

Figure 5. Chromatogram of basic degradation

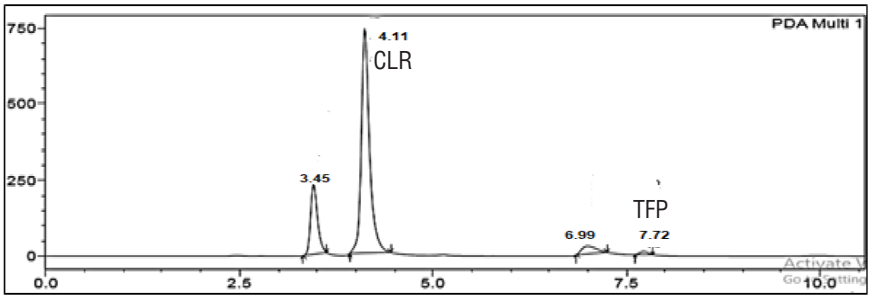

Figure 6. Chromatogram of photolytic degradation

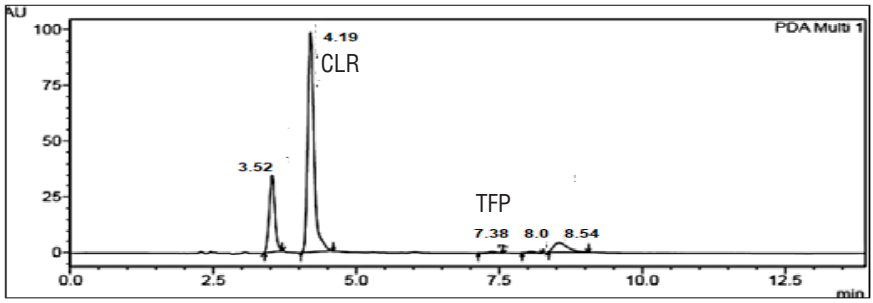

Figure 7. Chromatogram of oxidative degradation

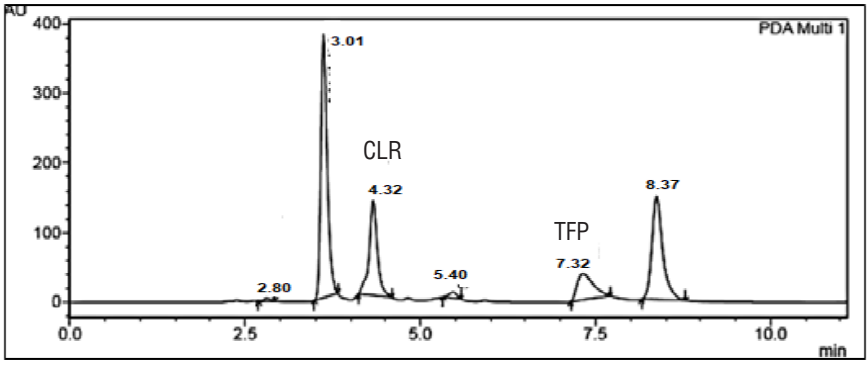

Figure 8. Chromatogram of thermal degradation

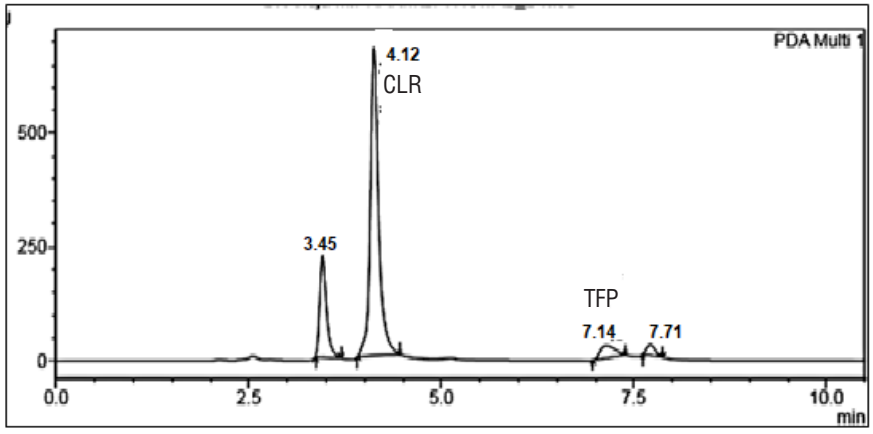

Figure 9. Chromatogram of neutral degradation

Table 2. Summary of force degradation study (CLR and TFP at different stress conditions)

Stress condition	Drugs	Standard area	Degradation area	% degradation
Acid degradation	CLR	14246076	10549619	25%
	TFP	402231	309961	22%
Alkali degradation	CLR	14246076	11356452	20.8%
	TFP	402231	315621	21.5%
Photolytic degradation	CLR	14246076	10452358	26%
	TFP	402231	322345	20%
Oxidative degradation	CLR	14246076	10754411	24.5%
	TFP	402231	316524	21.3%
Thermal degradation	CLR	14246076	10117544	28.2%
	TFP	402231	300979	25.1%
Neutral degradation	CLR	14246076	11558677	18.5%
	TFP	402231	313275	22.1%

Method validation

Method validation is carried out as per ICH guideline Q2(R1). Method validation is a process to ensure that the method was reliable and reproducible.

Specificity

The specificity of the method indicates, there is no interference in the analyte peak. So, there is no other peak was interfering with the standard chromatogram in under different stress conditions and blank. The specificity of the method was tested by subjecting the analyte to various stress conditions, such as light, acid, base, oxidation, heat and determining the extent of degradation and the ability of the method to measure the analyte accurately in the presence of its degradation. Standard chromatogram of CLR and TFP and specificity chromatogram (Blank) are given as Figure 10-11.

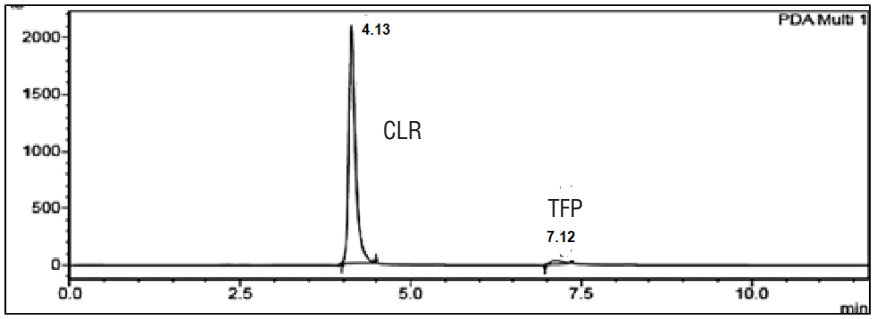

Figure 10. Standard chromatogram of CLR and TFP

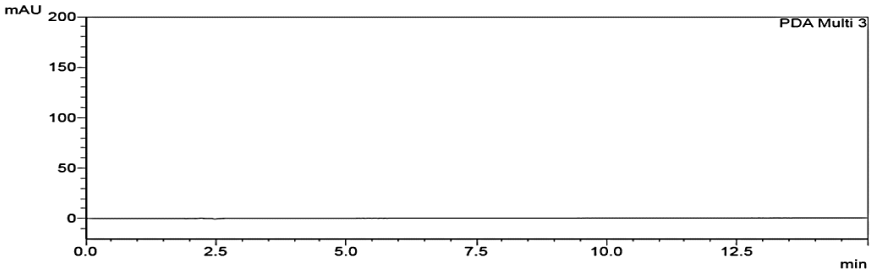

Figure 11. Specificity chromatogram (blank)

Linearity and range

Linear correlation was found between area versus concentration of CLR and TFP in concentration ranges of (50-300) and (5-30) μg/ml respectively. Calibration curves of CLR and TFP are shown in Figures 12 and 13 respectively. The R^2 values were 0.998 and 0.997 for CLR and TFP respectively. So, the method was considered linear at the above-mentioned concentration ranges for the two analytes. Specificity chromatograms belong to photolytic degradation, oxidative degradation, thermal degradation and neutral degradation are given as Figure 14,15,16,17 respectively. Calibration curves of TFP and CLR are also given as Figure 18,19 respectively.

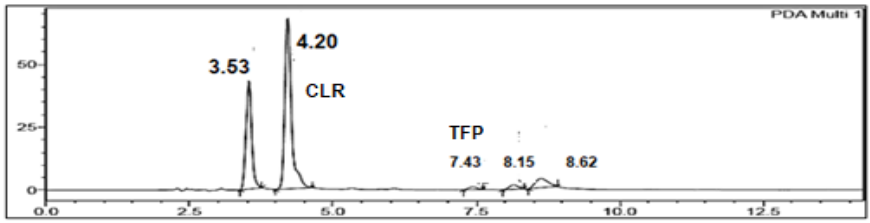

Figure 12. Specificity chromatogram (acid degradation)

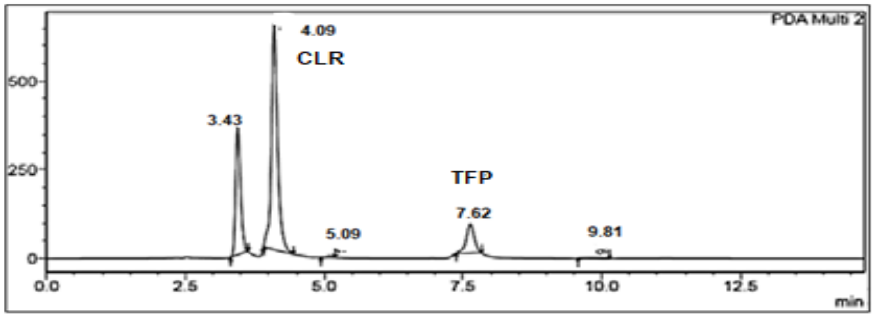

Figure 13. Specificity chromatogram (base degradation)

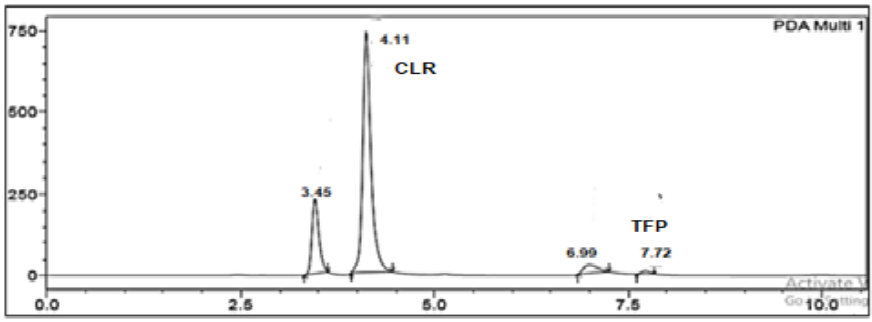

Figure 14. Specificity chromatogram (photolytic degradation)

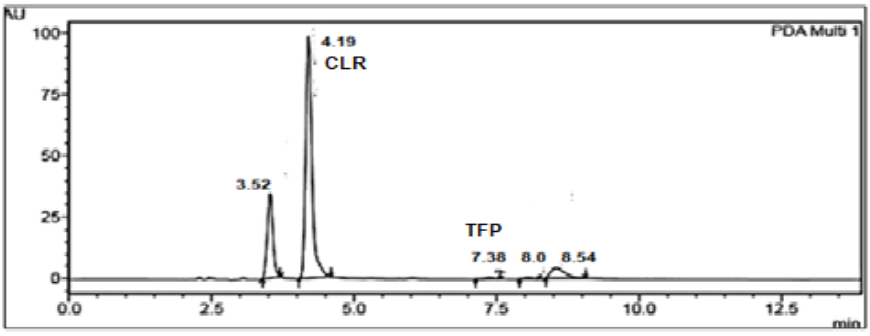

Figure 15. Specificity chromatogram (oxidative degradation)

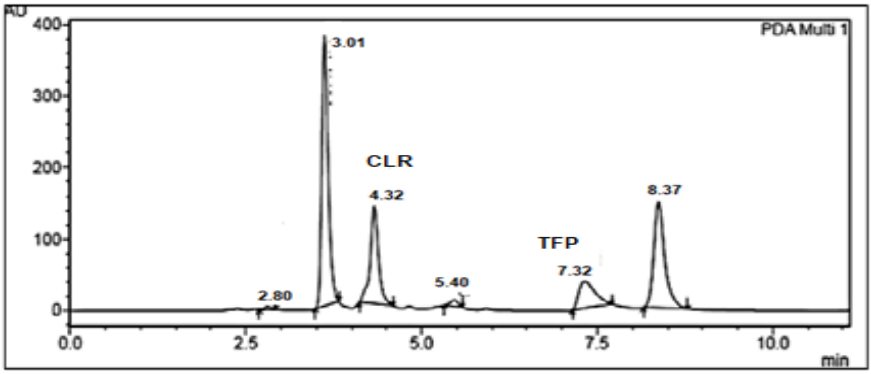

Figure 16. Specificity chromatogram (thermal degradation)

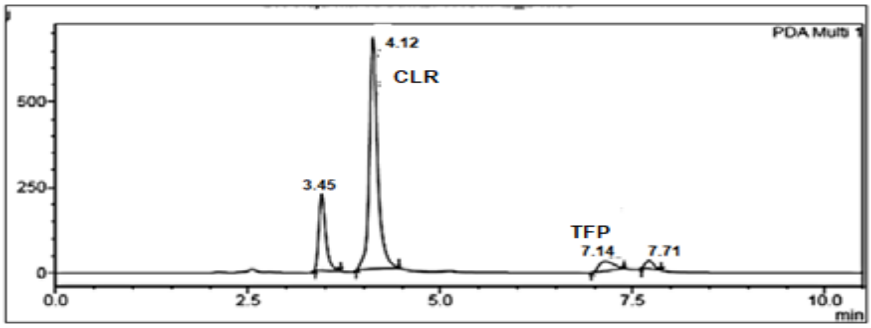

Figure 17. Specificity chromatogram (neutral degradation)

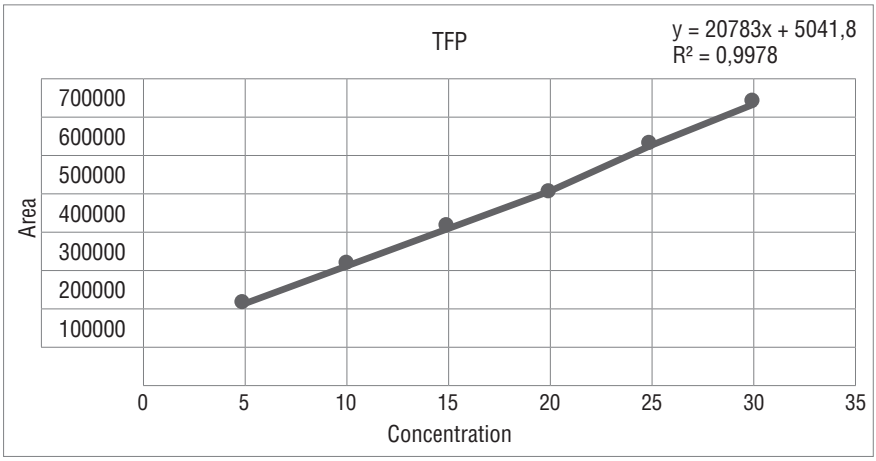

Figure 18. Calibration curve of TFP (5-30 µg/ml)

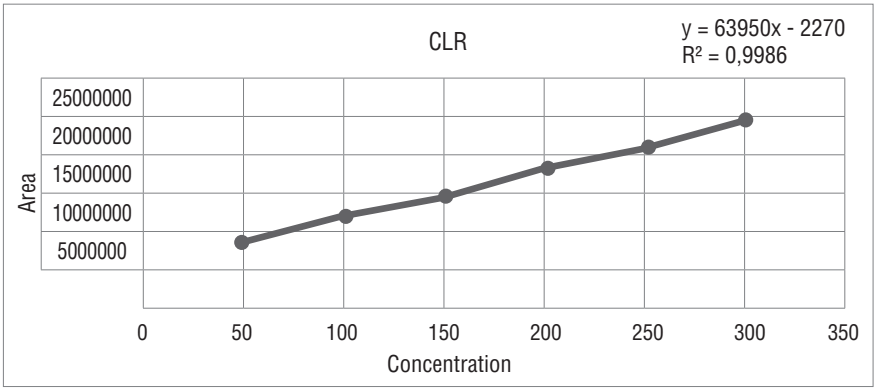

Figure 19. Calibration curve of CLR (50-300 µg/ml)

Accuracy

Accuracy was performed using three different levels 80%, 100%, and 120%. The % recovery was calculated. For both drugs % recovery was 99.8% (Table 3).

Table 3. Accuracy-recovery study of CLR and TFP by standard-addition method

Analyte	Conc. (µg/ml)	Amount of drug added			Amount recovered (µg/ml)	Average area	Average recovery (%)	%RSD
		Level (%)	Spiked amount (µg/ml)	Total conc. (µg/ml)				
CLR	100	80	80	180	179.3	11690936.7	99.6	0.82
		100	100	200	198.9	13038567	99.4	0.62
		120	120	220	219.2	14295238	99.6	0.62
TFP	10	80	8	18	17.9	359346.6	99.8	0.19
		100	10	20	19.8	399812.6	99.4	0.12
		120	12	22	21.8	436232	99.1	0.88

Precision

Repeatability was performed under the same conditions and 6 replicates were injected into the HPLC system. The % RSD was calculated. % RSD value for both drugs was found within the acceptance criteria. The Repeatability Data are shown in Table 4. Interday and intraday precisions were shown in Table 5. The % RSD value less than 2 indicated that the developed method was found to be precise. Table 6 shows data for the inter-day study of CLR and TFP.

Table 4. Data for repeatability study of CLR and TFP (n=6)

Parameters	CLR	TFP
Area 1	13127856	402231
Area 2	13154787	405698
Area 3	13246658	405823
Area 4	13168590	415478
Area 5	13478358	401258
Area 6	13254785	405847
Average	13127856	402231
Standard deviation	128063.3	40566.8
%RSD	0.65	0.87

Table 5. Data for intra-day study of CLR and TFP (n=6)

CLR				TFP			
Conc. (µg/ml)	Mean Area	Standard Deviation	%RSD	Conc. (µg/ml)	Mean Area	Standard Deviation	%RSD
100	6408493	2946.6	0.45	10	214154.7	1660.3	0.77
150	9222194	39320.2	0.52	15	303256.3	2430.5	0.80
200	13050371	68038.4	0.52	20	400527.7	3067.3	0.76

Table 6. Data for inter-day study of CLR and TFP (n=6)

CLR				TFP			
Conc. (µg/ml)	Mean Area	Standard deviation	%RSD	Conc. (µg/ml)	Mean Area	Standard deviation	%RSD
100	6463236	37391.7	0.57	10	213065.5	1333.6	0.62
150	9265818	28941.5	0.31	15	309360	2333.9	0.75
200	13077463	67472.3	0.51	20	403931	2044.7	0.50

LOD and LOQ

Limit of detection and Limit of quantitation were calculated from standard calibration curves. LOD was 1.83 µg/ml and 1.12µg/ml for CLR and TFP respectively. LOQ was 5.54 µg/ml and 3.41µg/ml for CLR and TFP respectively (Table 7). Sensitivity was calculated using following formula:

LOD= 3.3* δ/S

where

δ = standard deviation of response (intercept of calibration/linearity plot)

S=the slope of linearity plot

LOQ= 10* δ/S

where

δ = standard deviation of response (intercept of calibration/linearity plot)

S=the slope of linearity plot

Table 7. LOD and LOQ study CLR and TFP

Parameter	CLR(µg/ml)	TFP(µg/ml)
LOD	1.83	1.12
LOQ	5.54	3.41

Robustness

Robustness was performed by changes in different parameters like wavelength, flow rate, temperature, mobile phase ratio, and pH. The %RSD was calculated. The percentage relative standard deviation was less than 2% which indicates the method was robust (Tables 8-9).

Table 8. Results of robustness study of CLR and TFP

Parameter	Actual value	Changed value(+)	Changed value(-)	%RSD	
				CLR	TFP
Wavelength	262 nm	264 nm	260nm	0.16	0.35
Flow Rate	1ml/min	1.2ml/min	0.8ml/min	0.62	0.74
Temperature	40ºC	45ºC	35ºC	0.19	0.38
Mobile phase ratio	70:30v/v	80:20v/v	60:40v/v	0.25	0.88
pH	5.5	5.7	5.3	0.32	0.74

Table 9. Assay of pharmaceutical formulation

Drugs	Label Claim (mg)	% Amount found ±SD	%RSD
CLR	100	98.21±0.147	0.14
TFP	10	98.80±0.113	0.11

System suitability tests

System suitability parameters such as theoretical plates, peak area, tailing factor, and resolution were investigated, followed by the calculation of % RSD values. Obtained results were found to be close to the system suitability criteria, which indicated that the system was suitable and precise for analysis (Table 10).

Table 10. System suitability results of the proposed HPLC method for separation of CLR and TFP

Parameters	Active pharmaceutical drugs			
			TFP	
Retention time	Average	4.23	Average	7.14
	SD	0.085	SD	0.043
	%RSD	1.04	RSD%	0.76
Peak area	Average	6441595	Average	204185
	SD	49157	SD	2105
	%RSD	0.76	RSD%	1.03
Tailing factor	Average	1.32	Average	1.43
	SD	0.026	SD	0.032
Theoretical plates	Average	6405	Average	3165
Resolution	Average	10.4		
	SD	0.121		
	%RSD	1.16		

Table 11. Assay of CLR and TFP

Tablet (LIBRA CHE-T)	Label Claim (mg)	Conc. (µg/ml)	Mean area	%Assay ±SD	%RSD
CLR	10	100	6490131.6	98.21±0.147	0.14
TFP	1	10	205787.3	98.80±0.113	0.11

Chlordiazepoxide and Trifluoperazine HCl are effectively estimated using an isocratic stability-indicating RP-HPLC method in a combined pharmaceutical formulation. In acid and base degradation, maximum degradation was observed. The drug was also susceptive to degradation under photolytic and thermal conditions. Forced degradation studies indicated that both drugs and degradation products are separated from each other. The method was also validated according to ICH Q2(R1) guidelines. All-important analytical parameters were investigated and found within the Acceptance limit. So, the developed RP-HPLC method is accurate, précised, and robust. Therefore, the proposed method can be successfully employed in routine analysis of these drugs in bulk as well as in pharmaceutical formulation.

STATEMENT OF ETHICS

This study does not require any ethical approval.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

All authors contributed to data collection, processing, writing, revision of the draft, reading and approval of the final manuscript.

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