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Degradation Pathway of Caffeine in Bulk Drug and Pharmaceutical Dosage Form

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Abstract

A simple, robust, precise, and sensitive reverse-phase high-performance liquid chromatographic method to determine the degradation pathway of caffeine in bulk drug, dosage form, and soft drinks has been developed and validated. Separation was achieved using X-bridge C_{18} (250×4.6 mm, *i.d.*5 µm) analytical column. The mobile phase used was Water: Methanol: Glacial acetic acid (79.98:20:0.02) and the run time was 10 min having a flow rate of 1 mL/min. Detection was carried out at 273 nm using a UV detector. Linearity of caffeine, theophylline, and theobromine at concentration range 8-12 µg/mL. The same optimized method was utilized for the identification of degradation products. Caffeine in bulk and tablet were subjected to stress conditions such as hydrolytic, oxidative, photolytic, and thermal stress conditions as per the recommendation of the International Conference on Harmonization (ICH) guideline Q1A (R2).

Keywords: Caffeine, Method development, Degradation studies, Theophylline, Theobromine, RP-HPLC.

1] Introduction

Human body consists of different routes of administration, in which oral route is one of the popular routes for administering the dosage form into the body. Tablets, syrups, capsules and so on are the more dosage which are taken by the patients via oral routes with the prescription of physicians. In our dosage form the API consist of caffeine that is belongs to the class of xanthine, which is a natural occurring compound consist of the derivatives like the bromine, theophylline and the theobromine which are bronchodilators and are also act as the CNS and respiratory stimulants and as well as the also act as the mild diuretics [1].

The mechanism of action behind the caffeine is that it stimulates center like the respiratory centre, vasomotor and the vagal nerve that promotes the bradycardia and vasoconstriction which are responsible for the increases the respiratory action, the reason that increases the respiratory rate is to be done by the

increase in the AMP intracellular cyclic and by the inhibit of phosphodiesterase it decreases the cyclic AMP. Phosphodiesterase is an enzyme that degrades the cyclic AMP. So, by the use of xanthine such as caffeine it acts as the antagonists at the adenosine receptors in the cell followed by plasma [2,3]. Similarly, Theobromine that is (3,7-dimethylxanthine) and Theophylline which is (1,3-dimethylxanthine) are the naturally occurring antioxidants, that is naturally presents in the natural occurring compounds such as the marijuana which consist of caffeine in the large amount and act as a CNS stimulant. Caffeine is used in the most of the beverages, and also in the many dietary sources that can be widely used for the CNS stimulants but the most use of caffeine is used in many soft drinks that can be act as a flavoring agent[4,5]. The amount of added caffeine in the in the product should be the limited, intake of excess amount of caffeine can causes the various disorders like it can rise the release of the acid from the stomach, malfunctioning in the stomach and can also disturb the central nervous system such ass the irregular seizure and delirium [6].

Mostly the caffeine that is consist of theophylline and theobromine can be used in the large amount in the bronchodilator's drugs, the use of these drugs can be act against the acute as well as the chronic asthma [7,8]. This the use of excess amount of the drugs can be determined by the various analytical methods [9, 10], In this research paper we have determined the Caffeine, Theophylline and the Theobromine can be determined by using the analytical techniques like HPLC, UV spectroscopy in the pharmaceutical dosage form and this method can also be validated by the ICH guideline Q2R1, with this analytical technique LOD and the LOQ lower limit of detection and the lower limit of the quantitation can also be determined that is given below in the results and discussions[11,12].Also, the chemical structure of the drugs are given below in the **Figure 1**

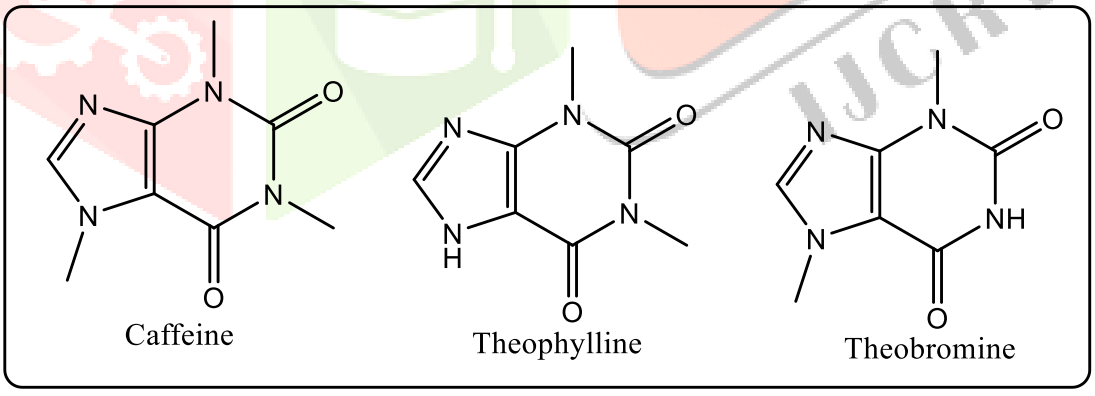

Figure 1. Chemical Structure of Caffeine, Theophylline and Theobromine

2. EXPERIMETAL WORK

2.1. Drug authentication

2.1.1. Authentication of Caffeine, Theophylline, and Theobromine

The identification of standard drugs was carried out by physical appearance, UV (λ_{max}), and I.R Spectroscopy. The purity of the standard drugs complied with British Pharmacopoeia (2008) [BP; 2008] and Clarke's Analysis of Drugs and Poisons (2005).

2.1.2. Physical appearance

The physical appearance of all standard drugs was checked by visual observation, dispersing the drug on clean butter paper. The observations performed for the colour and texture of drugs.

2.1.3. Determination of λ_{max} using U.V. spectrophotometer

The UV–spectrum of standard solutions of all drugs taken having a concentration of 100µg/ml in methanol: water (40:60). Baseline correction was doneconcerning blank before taking spectra. The spectra taken in duplicate, λ_{max} observed, and calculations performed.

2.1.4. Determination by using IRspectroscopy

IR spectra were recorded for identification and to check the purity of procured drugs, i.e. CAF, TP, and TB. IR transmission spectra of pure obtained using FTIR. The characteristic peaks of the functional groups interpreted for the authenticity of the reference standard. [IP: 2014]

2.2. Calibration curve

2.2.1 Calibration curve of Caffeine, Theophylline, and Theobromine using U.V. spectrophotometer

The calibration curve (CC) of standard solutions were prepared in different solvent systems as per the working range of double beam UV-spectrophotometer.

2.2.2. Preparation of stock solution

About 10 mg of CAF, TP, and TB were weighed accurately and taken in 10 ml volumetric flask and dissolved in 10 ml of water and methanol (60:40) to get the concentration of 1000µg/ml.

2.2.3.Preparation of CC standard solutions

The CC standard solutions were prepared by diluting a suitable quantity of stock solution in methanol: water as diluent. The CC standard solutions of CAF were prepared of concentrations 5,10,15,20, and 25 µg/ml in duplicate. The CC standard solutions of TP and TB were prepared of concentrations 4, 8, 12, 16, 20, and 24 µg/ml in duplicates. Baseline correction was done concerning blank before taking spectra. The absorbance was measured at $\lambda_{max}$of the drug in the same solvent mixture against blank solution using UV spectrophotometer. The mean absorbance was measured, and a graph was plotted between concentration (x-axis) and absorbance (y-axis).The line equation and correlation coefficient were calculated for the calibration curve.

2.3. Assay method validation of Caffeine, Theophylline, and Theophylline in bulk drug using U.V. spectrophotometer

Assay method validation was performed for the content determination of CAF, TP, and TB in bulk drug using UV spectrometer as per recommendations of ICH Q2(R1).

2.4. Pre-validation parameters

For assay method validation of CAF, TP, and TB, MeOH was used as a solvent. The drugs had shown a good linear relationship between concentration and absorbance when the calibration curve is drawn in selected diluents. For the current method, 10 µg/ml was taken as test concentration for CAF, TP, and

TB because it can be serially prepared from the stock solution. CAF, TP, and TB had shown maximum absorbance (λ_{max}) of 273, 271, and 272 nm, respectively.

2.5. Assay validation parameters

The validation parameters viz. specificity, linearity, and range, the limit of detection and limit of quantification, precision, accuracy, and robustness were exercised for assay of CAF, TP, and TB in bulk drugs.

2.5.1 Specificity

For specificity study observed the blank solvent spectrum for the interferences at the λ_{max} of diluted CAF, TP, and TB standard solutions.

2.5.2 Range

For assay method validation, the range should be 80-120% of test concentrations. The test concentrations of 10µg/ml used for CAF, TP, and TB. Therefore, the range was 8-12 µg/ml.

2.5.3. Linearity

2.5.3.1 a Preparation of the stock solution:

About 10 mg of CF, TP, and TB was weighed and taken in 10 ml volumetric flask and dissolved in 4 ml MeOH with gentle shaking and volume was made up to 10 ml with distilled water to get a concentration of 1 mg/ml.

2.5.3.2b Preparation of linearity standard solutions

Methanol and water (40:60) was used as diluent. The linearity standard solutions of CAF were prepared of concentration 5, 10, 15, 20 and 25 µg/ml, in duplicate. The linearity standard solutions of TP and TB were prepared of concentrations 4, 8, 12, 16, 20,and 24 µg/ml in duplicate.

2.5.4. Precision

2.5.4.1 a) System precision

For system precision, suitable quantity of a stock solution of CAF, TP, and TB was taken in 10mlvolumetric flask and diluted upto the mark with water and methanol (60:40) as the solvent system to get the concentration of 10µg/ml.The solutions were observed in six repetitions to check the absorption at the particular λ_{max} of CAF, TP, and TB, and the mean absorbance and %relative standard deviation (%R.S.D.) was calculated.

2.5.4.2. b) Method precision

For method precision, suitable quantity of stock solutions of CAF, TP and TB were taken in10ml volumetric flasks and diluted up to the mark with water and methanol (60:40) as a solvent to get the concentration of 10 µg/ml for CAF, TP, and TB. Six replicates of each concentration were made, and the absorbance of each replication was observed, and the mean absorbance and %relative standard deviation (%R.S.D.) was calculated.

2.5.4.3 c) Intermediate precision

The intermediate precision was determined by different analysts and on different days using the same

instrument. The six replicate of CAF, TP and TB standard solution containing test concentration of 10 µg/ml were measured at respected λ_{max} of CAF, TP and TB using UV spectrophotometer as schedule given in **Table** 1.

Table 1. Schedule for intermediate precision

Concentration of CAF	Concentration of TP	Concentration of TB	Name of analysts
10 µg/ml	10 µg/ml	10 µg/ml	Koushal Billowria
10 µg/ml	10 µg/ml	10 µg/ml	Navjot Kaur Sandhu

The mean absorbance and %R.S.D. for all replicate were calculated. The mean absorbance of analyst-1 was compared with analyst -2, and %R.S.D. was calculated.

2.5.5 Accuracy

The accuracy of the method was measured by recovery studies. The recovery study procedure for the CAF, TP, and TB by U.V.spectrometer was as follows

2.5.5.1 a) Preparation of CAF, TP and TB standard solution (For recovery studies)

Standard solution of CAF, TP, and TB was prepared with water and methanol (60:40) as a solvent. The standard solutions containing 80%, 100% and 120% of the test concentration of CAF, TP, and TB were prepared in duplicate.

For recovery studies, samples were prepared by spiking standard solution of 100 % with 80%, 100%, and 120% test concentration solution, in duplicate. The UV absorbance for fortified and unfortified samples was measured using the following formula-

$$80\% = \frac{MeanAbs.of\ 180\%\ fortifiedsample - MeanAbs.of\ 80\%\ unfortified}{MeanAbsofunfortifiedstandardsolutionof\ 100\%\ testconcentration} \times 100$$

$$100\% = \frac{MeanAbs.of\ 200\%\ fortifiedsample - MeanAbs.of\ 100\%\ unfortified}{MeanAbsofunfortifiedstandardsolutionof\ 100\%\ testconcentration} \times 100$$

$$120\% = \frac{MeanAbs.of\ 220\%\ fortifiedsample - MeanAbs.of\ 120\%\ unfortified}{MeanAbsofunfortifiedstandardsolutionof\ 100\%\ testconcentration} \times 100$$

The mean recovery for the method and %R.S.D. was calculated.

2.5.6. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limits of detection and quantitation were calculated by a method based on standard error (σ) and slope (S) of regression of linear plot using the formula:

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

2.5.7. Robustness

2.5.7.1. a) Change in λ_{max} of CAF, TB, and TP

The absorbance of CAF,TB, and TP standard solution containing test concentration was measured at $\lambda_{max}$of CAF,TB and TP and $\lambda_{max}\pm$ 2nm of CAF,TB, and TP, in triplicate. The mean absorbance and

%R.S.D. for all standard preparation were calculated.

2.5.7.2. b) Solution stability of CAF, TB, and TP

The absorbance of triplicate CAF, TB, and TP standard solution containing test concentration was measured after 24 h from the preparation and changes measured by calculating mean absorbance and %R.S.D. for all standard preparations.

2.5.8. Assay of marketed formulation of CAF (tablet) by UV spectroscopy

Twenty tablets of marketed formulation of CAF were weighed accurately and grounded to obtain a fine powder. Accurately weighed powder sample equivalent to 10 mg of CAF was dissolved in a 100 ml volumetric flask containing diluent. The solution was sonicated for 20 min and filtered through whatman filter paper no.41. An aliquot of this solution was diluted to produce a concentration of 10 µg/ml of CAF (n=6).The absorbance of the sample solution at 273 nm was measured, and the amount of drug present in the solution was calculated. Assay of the marketed formulation was done by comparing the absorbance of a tablet with that of standard drug. The assay was calculated based on absorbance using the following formula:-

$$\text{Assay} = \frac{\text{Absorbance of tablet solution}}{\text{Absorbance of standard solution}} \times 100$$

2.5.9. Analytical method development for simultaneous estimation of CAF, TP and TB in laboratory mixture using RP-HPLC

RP-HPLC method development and validation for simultaneous estimation of CAF, TP, and TB in laboratory mixture were carried out by taking numbers of trial, and different chromatographic conditions were optimized for the proposed method.

3. RESULT AND DISCUSSION

The main aim of the present study was to determine the degradation products of caffeine in bulk, pharmaceutical dosage form and soft drinks by using sophisticated analytical UV and HPLC methods, for determination of CAF, TB, and TP method developed, optimized and validated as per the ICH guideline.

3.1 Drug authentication

3.1.1. Identification tests for authentication of CAF, TP, and TB

Identification tests are performed for the determination of physical and chemical characteristics of the drug. As per IP 2010, the procured drugs were tested for their identity by physical properties, melting point, solubility, IR analysis, and assay determination.

3.1.2. Physical appearance

By visual observation, the physical appearances of all drugs were recorded.CAF, TP, and TB were a white crystalline powder. CAF was found to freely soluble in boiling water, sparingly soluble in water and ethanol, slightly soluble in ether, TP was found to be slightly soluble in water, ethanol, ether, chloroform and where as TB was found to be slightly soluble in water, ethanol, ether, chloroform.

3.2. UV- visible spectroscopy of selected drugs

UV-visible spectra for all drugs were taken in water: methanol (60:40) as shown in **Figure** 2-4 with their respective absorption maxima (λ_{max}).

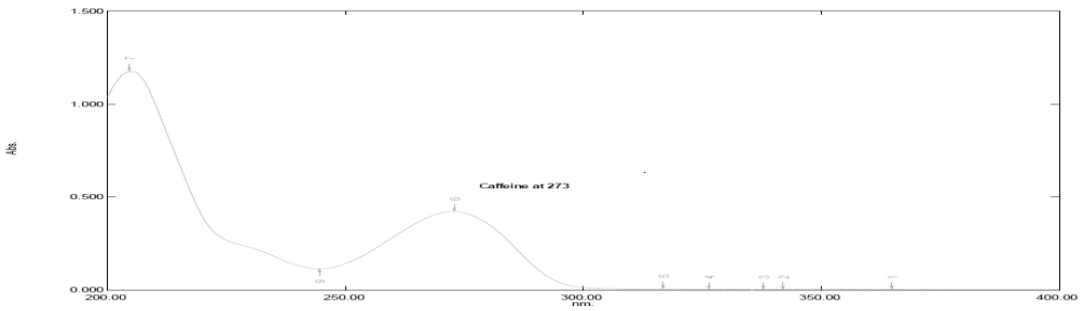

Figure 2 UV-visible spectra of CAF

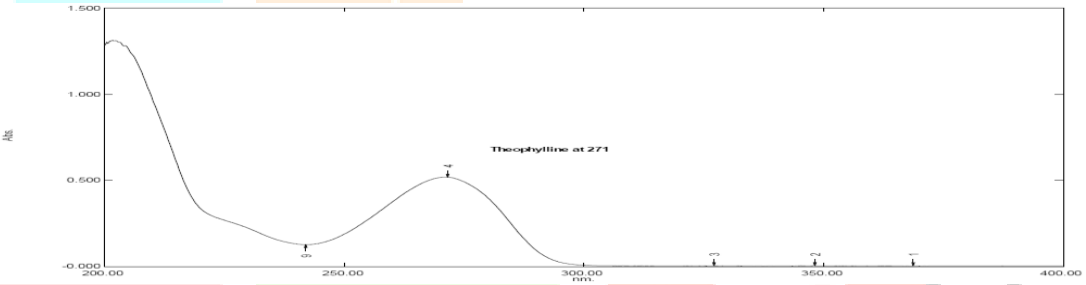

Figure 3 UV-visible spectra of TP

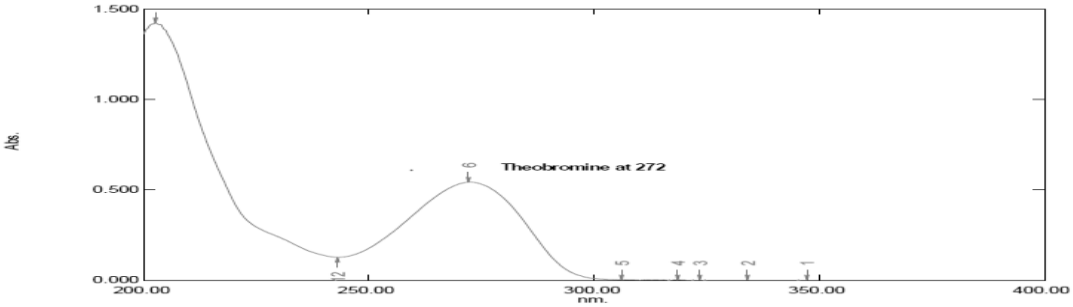

Figure 4 UV-visible spectra of TB

3.3. Assay method validation

3.3.1. Assay method validation of CAF, TP, and TB in bulk drug using UV spectrophotometer

Assay method validation was performed for the content determination of CAF, TP, and TB in bulk drug using UV spectrophotometer as per recommendations of ICH Q2 (R1).

3.4. Assay validation parameters

The validation parameters viz. specificity, linearity, and range, the limit of detection and limit of quantification, precision, accuracy, and robustness were exercised for assay of CAF, TP, and TB in bulk drugs.

3.4.1 Specificity:

For specificity,the study observed the blank solvent spectrum for the interferences at the λ_{max} of diluted CAF, TP, and TB standard solutions. In specificity studies, interference with non-drug component of the matrix should be evaluated and separated from the instrumental signal (whatever may be) of the analyte/s of interest. All the spectrums of standard drugs solution obtained did not show any kind of interference with diluent.

3.4.2 Linearity and Range

For any quantitative method, it is necessary to determine the range of analyte concentrations values over which the method may be applied. For the assay method validation, 10 µg/ml of all the drugs was taken as a test concentration. Linearity range of CAF, TP, and TB were established in the concentration range of 5-25µg/ml, 4-20µg/ml, and 4-20 µg/ml respectively using water and methanol in the ratio of 60:40 as a solvent. The linearity standard solutions were prepared in duplicate. The absorbance was taken in duplicate and meant absorbance was calculated. Linearity spectrums overlay of all drugs given in **Figure** 5-7**,** and linearity plot given in **Figure** 8-10.Data generated from the linearity curve of all drugs were given in **Table** 5.4

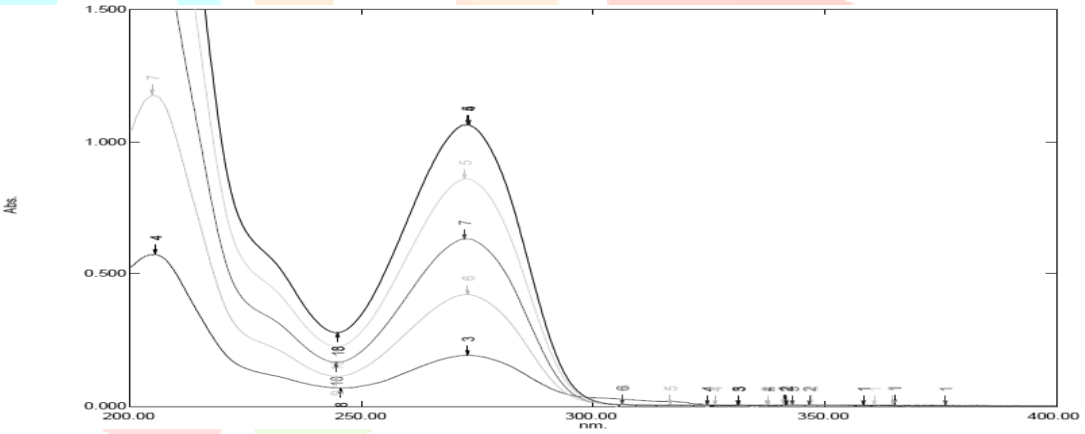

Figure 5 Linearity overlay absorption spectra of CAF

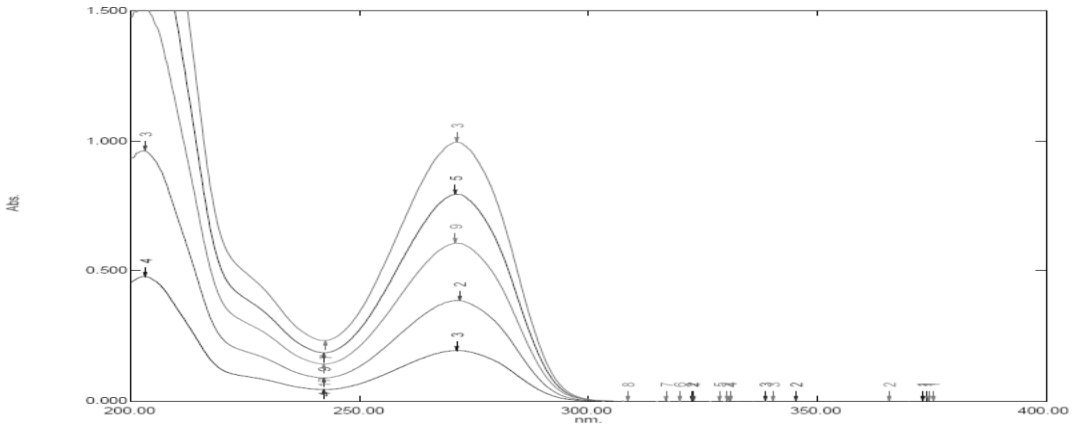

Figure 6 Linearity overlay absorption spectra of TP

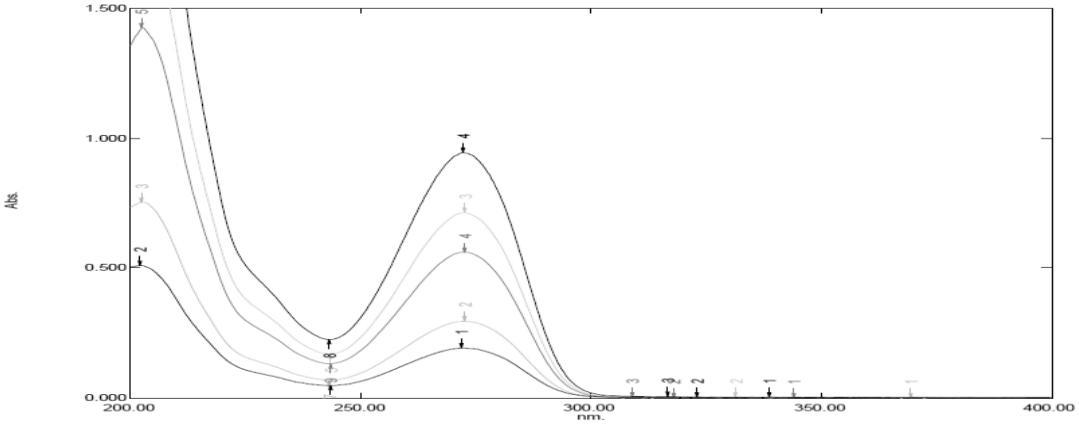

Figure 7 Linearity overlay absorption spectra of TB

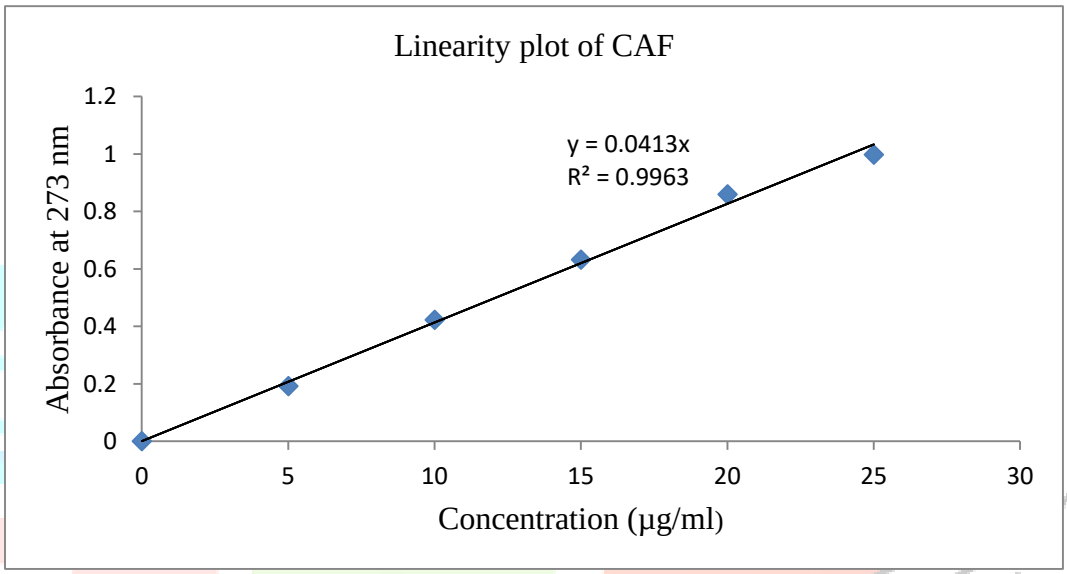

Figure 8 Linearity plot of CAF

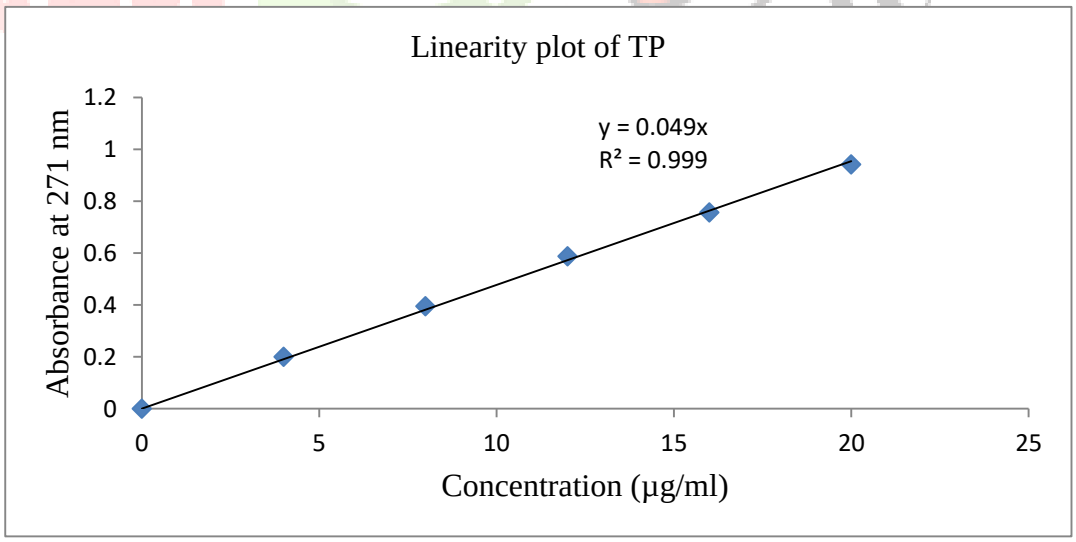

Figure 9 Linearity plot of TP

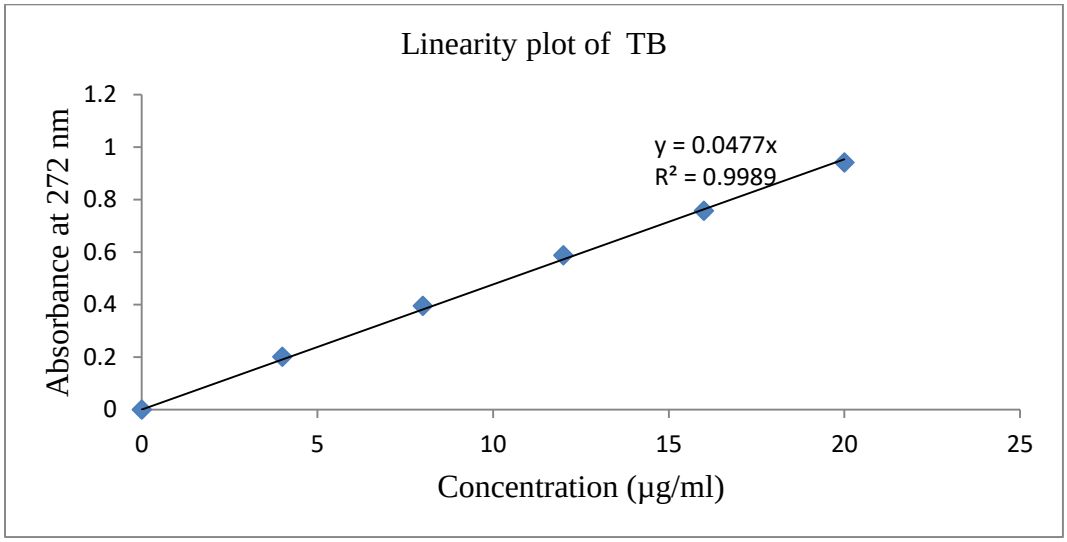

Figure 10 Linearity plot of TB

3.4.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

Detection and quantification limits (LOD and LOQ) are two major parameters of method validation. LOD and LOQ calculated from the calibration curve by using formula

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

Several approaches are recommended by the ICH for determining the detection and quantitation limit of the sample depending on the instrument used for analysis, nature of the analyte, and suitability of the method. The acceptable approaches are visual evaluation, signal to noise ratio, the standard deviation of the response, and the standard deviation of the slope of linearity plot. The LOD and LOQ based on standard deviation of the slope of the linearity plot are summarized in **Table** 2

Table 2 Summary of resulting parameters of linearity, LOD and LOQ method

Validation Parameters	CAF	TP	TB
Absorbance maxima(λ_{max}),nm	273	271	272
Linearity range(µg/ml)	5-25	4-20	4-20
Coefficient of determination (r^2)	0.996	0.999	0.998
Regression equation (y)	y = 0.041x	y = 0.049x	y = 0.047x
Slope (b)	0.041	0.049	0.047
Limit of Detection (µg/ml)	1.27	0.636	0.689
Limit of Quantification(µg/ml)	3.872	2.121	2.297

3.4.4. Precision:

3.4.4.1. a) Repeatability

The precision of the analytical procedure represents the closeness between a series of measurements got from multiple sampling of the same homogenous sample under the same analytical conditions.

The system precision was performed at the test concentration by taking six repetitive absorbances of the same solution. The %R.S.D. of absorbance for six repetitive spectra of CAF, TB, and TP, test concentration was 10 µg/ml of all drugs. The %R.S.D. for system precision was ≤ 2, which is within the limit.

The method precision was performed at the test concentration by preparing the CAF, TP and TB standard solution of test concentration and measuring absorbance at the λ_{max} of the respective standards as shown in **Table** 3-5.%R.S.D. of absorbance for method precision of CAF, TB, and TP,test concentration was 10 µg/ml of all drugs. The %R.S.D. for method precision was within the limit.

Table 3 System and method precision studies of CAF at the test concentration

Conc.(µg/ml)	System precision absorbance	Conc. (µg/ml)	Method precision Absorbance
10	0.437	10	0.44
10	0.438	10	0.434
10	0.438	10	0.425
10	0.439	10	0.426
10	0.438	10	0.425
10	0.439	10	0.423
Mean	**0.438**	**Mean**	**0.428**
±S.D	**0.0007**	**±S.D**	**0.006**
% R.S.D.	**0.171**	**% R.S.D.**	**1.55**

Table 4 System and method precision studies of TP at the test concentration

Conc.(µg/ml)	System precision absorbance	Conc. (µg/ml)	Method precision absorbance
10	0.502	10	0.504
10	0.502	10	0.508
10	0.502	10	0.517
10	0.502	10	0.51
10	0.504	10	0.502
10	0.506	10	0.502
Mean	**0.503**	**Mean**	**0.507**
±S.D.	**0.0017**	**±S.D**	**0.005**
%R.S.D.	**0.333**	**% R.S.D.**	**1.14**

Table 5 System and method precision studies of TB at the test concentration

Conc.(µg/ml)	System precision absorbance	Conc. (µg/ml)	Method precision absorbance
10	0.460	10	0.460
10	0.462	10	0.464
10	0.462	10	0.477
10	0.463	10	0.464
10	0.462	10	0.462
10	0.464	10	0.462
Mean	**0.462**	**Mean**	**0.464**
±S.D	**0.0013**	**±S.D**	**0.0061**
% R.S.D.	**0.287**	**% R.S.D.**	**1.322**

3.4.4.2. b)Intermediate precision

Intermediate precision, the method was tested on multiple days, instruments, analysts, etc. Among these all for intermediate precision, a similar procedure was employed using two different analysts. Intermediate precision studies of CAF, TP, and TB at test concentration as shown in **Table** 6-8.

Table 6 Intermediate precision studies of CAF at the test concentration

S.N.	Concentration (µg/ml)	Analyst-1 absorbance	Analyst-2 absorbance
1.	10	0.434	0.428
2.	10	0.425	0.429
3.	10	0.425	0.434
4.	10	0.431	0.431
5.	10	0.436	0.436
6.	10	0.440	0.436
Mean		**0.432**	**0.437**
S.D.		**0.006**	**0.007**
%R.S.D.		**1.401**	**1.637**

Table 7 Intermediate precision studies of TP at the test concentration

S.N.	Concentration (µg/ml)	Analyst-1 absorbance	Analyst-2 absorbance
1.	10	0.502	0.504
2.	10	0.502	0.502
3.	10	0.512	0.516
4.	10	0.508	0.512
5.	10	0.509	0.502
6.	10	0.515	0.508
Mean		**0.508**	**0.507**
S.D.		**0.005**	**0.006**
%R.S.D.		**1.034**	**1.133**

Table 8 Intermediate precision studies of TB at the test concentration

S.N.	Concentration (µg/ml)	Analyst-1 Absorbance (nm)	Analyst-2 Absorbance (nm)
1.	10	0.468	0.464
2.	10	0.460	0.460
3.	10	0.460	0.462
4.	10	0.462	0.462
5.	10	0.464	0.445
6.	10	0.448	0.450
Mean		**0.461**	**0.457**
S.D.		**0.007**	**0.008**
%R.S.D.		**1.465**	**1.690**

3.4.5. Accuracy

The accuracy of the method was measured by calculating the percentage recovery of the standard. Recovery studies were carried out by the addition of standard drug solution at the concentration of 80,100 and 120 % concerning test concentration to pre-analyzed sample. The samples were prepared in duplicate, and % recovery was calculated. The %recovery of CAF, TP, and TB were obtained are shown in **Table** 9, the fortified and unfortified sample spectra are given in **Figure** 11-13.

Table 9 Result of recovery studies by UV method

Drugs	Unfortified sample		Fortified sample		%Recovery
	Conc. (µg/ml)	Mean absorbance	Conc.(µg/ml)	Mean absorbance	
CAF	8	0.346	10+8	0.747	98.28
	10	0.408	10+10	0.821	101.22
	12	0.525	10+12	0.926	98.28
TP	8	0.383	10+8	0.893	101.53
	10	0.502	10+10	0.995	98.28
	12	0.607	10+12	1.099	98
TB	8	0.375	10+8	0.838	100.21
	10	0.462	10+10	0.93	101.29
	12	0.529	10+12	0.998	101.51

The %mean recovery of CAF,TP, and TB was found within the limit (98-102%).

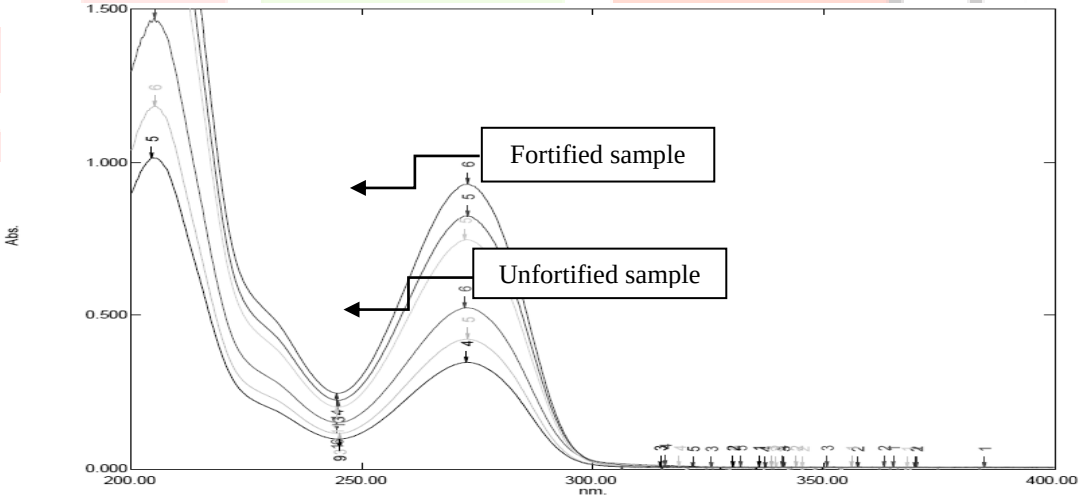

Figure 11 Overlay UV spectra of CAF fortified and unfortified samples

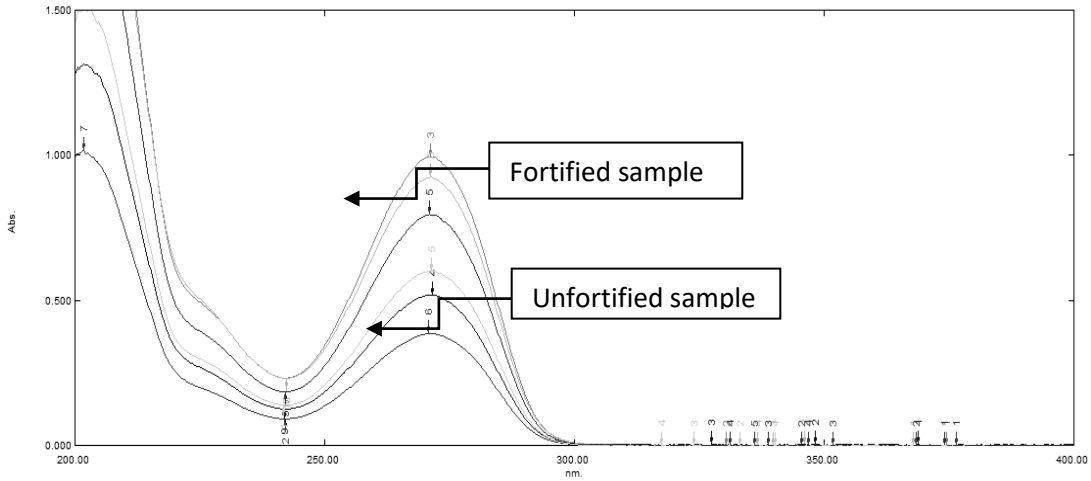

Figure 12 Overlay UV spectra of TP fortified and unfortified samples

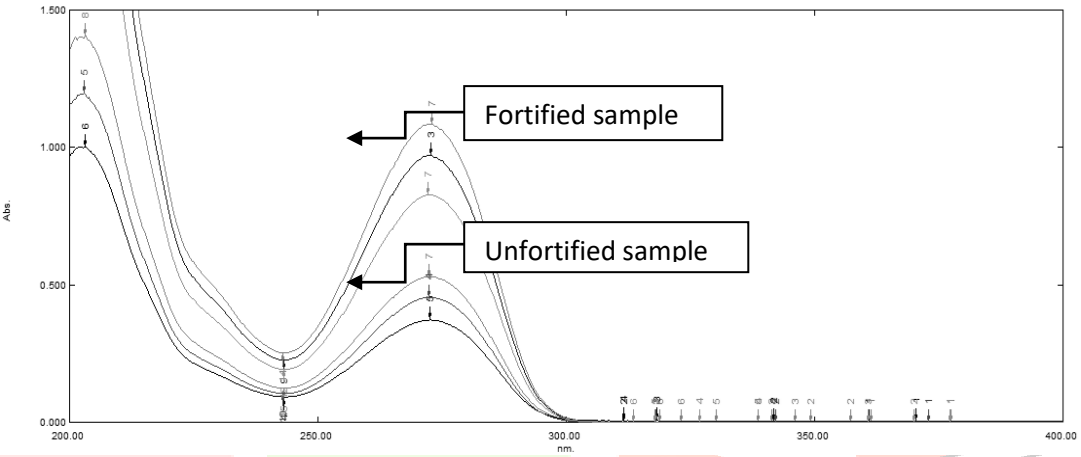

Figure 13 Overlay UV spectra of TP fortified and unfortified samples

3.4.6.Robustness

It is the measurement of the effectiveness of the analytical method, in case of any small, deliberate change in a procedure such as a change in absorbance maxima of the drug by ±2 nm. The standard drug solution stability checked after 24h and %R.S.D.as shown in **Table** 10-12.

Table 10 Stability and change in $\lambda_{max}$of CAF spectrum

S.N.	Conc. (µg/ml)	Stability		λ_{max}(nm)		
		0 h	24 h	271	273	275
1.	10	0.446	0.448	0.458	0.461	0.456
2.	10	0.467	0.452	0.463	0.467	0.460
3.	10	0.466	0.447	0.461	0.466	0.458
Mean		**0.464**	**0.449**	**0.460**	**0.464**	**0.458**
S.D.		**0.003**	**0.002**	**0.0025**	**0.0032**	**0.002**
%R.S.D.		**0.691**	**0.481**	**0.546**	**0.691**	**0.436**

Table 11 Stability and change in $\lambda_{max}$of TP spectrum

S.N.	Conc. (µg/ml)	Stability		λ_{max}(nm)		
		0 h	24 h	269	271	273
1.	10	0.517	0.520	0.510	0.517	0.512
2.	10	0.504	0.509	0.498	0.504	0.501
3.	10	0.502	0.504	0.495	0.502	0.499
Mean		**0.508**	**0.511**	**0.501**	**0.508**	**0.504**
S.D.		**0.0081**	**0.0082**	**0.0079**	**0.0082**	**0.007**
%R.S.D.		**1.604**	**1.601**	**1.584**	**1.604**	**1.388**

Table 12 Stability and change in $\lambda_{max}$of TB spectrum

S.N.	Conc. (µg/ml)	Stability		λ_{max} (nm)		
		0 h	24 h	269	271	273
1.	10	0.517	0.520	0.510	0.517	0.512
2.	10	0.504	0.509	0.498	0.504	0.501
3.	10	0.502	0.504	0.495	0.502	0.499
Mean		**0.508**	**0.511**	**0.501**	**0.508**	**0.504**
S.D.		**0.0081**	**0.0082**	**0.0079**	**0.0082**	**0.007**
%R.S.D.		**1.604**	**1.601**	**1.584**	**1.604**	**1.388**

3.5. Assay of marketed formulation of CAF by UV method

Direct absorption method: For estimation of CAF in tablet dosage form by UV spectrophotometer was carried out by carrying out three replicating assay determination of tablet and data are given below **Table** 13

Table 13 Assay of marketed formulation of CAF by UV Method

Drug	Label Claim (mg)	Amount Found (mg)	%Assay
CAF	100	105.6	105.6

3.5. RP-HPLC method development

3.5.1. Optimized chromatographic condition

After taking several trials by a different combination of mobile phase with different composition of the organic phase and aqueous phases, the final method selected for the simultaneous estimation of CAF, TP and TB in laboratory mixture given below:

Table 14. Final method for simultaneous estimation of CAF, TP and TB

Stationary phase	C_{18} column
Mobile phase	Solvent A –Water Solvent B – Methanol Solvent C –GAA
Mobile phase ratio	(79.98:20:0.02) (A:B:C)
Diluent	Solvent A –Water Solvent B – Methanol
Diluent ratio	(60:40) (A:B)
Detection wavelength	273 nm
Flow rate	1 ml/min
Temperature	Room temperature
Sample size	20µL

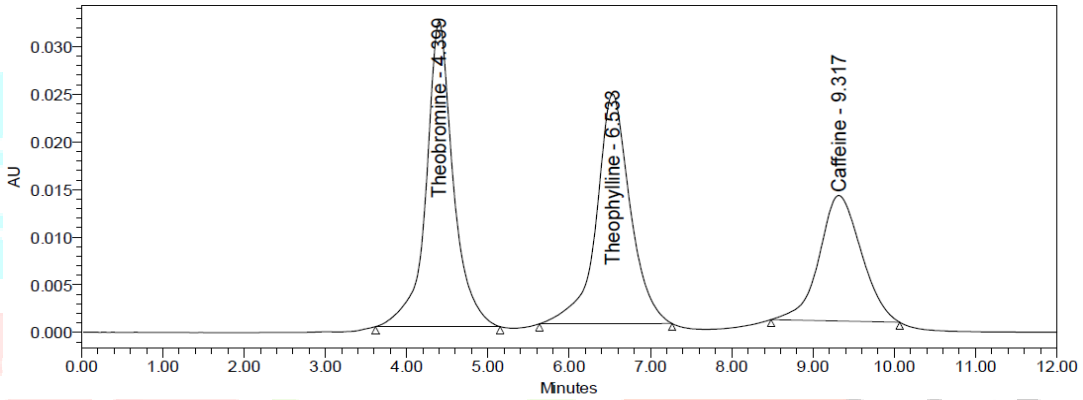

Figure 14 Chromatogram of optimized condition

3.5. System suitability

System suitability is an integral part of the analytical procedure. System suitability testing originally believed by the industry of pharmaceuticals to decide whether a chromatographic system is being utilized day today in a routine manner in pharmaceutical laboratories where the quality of results is most important which is suitable for a definite analysis.

For testing system suitability six replicate of 100% test concentration of CAF,TP, and TB were prepared and injected in HPLC and results were used to calculate the system suitability **Table** 15.

Table 15 System suitability studies of CAF, TP, and TB in optimized RP-HPLC compared with USP Pharmacopoeia limits

System suitability parameter	Limit	TB	TP	CAF
Retention time	-	4.374	6.56	9.364
% R.S.D. of R_t	-	0.375	0.4	0.831
Mean peak area	-	744348	686948	546923.7
% RSD of peak area	≤ 2.0	0.212	0.415	0.276
Peak asymmetry factor (at 10% peak height)	≤ 2.0	0.875	1	1.25
Resolution	> 1.5	-	1.94	2.315
Mean number of theoretical plates	≥ 2000	3439	2735.708	2170

3.6. Analytical method validation for simultaneous estimation of CAF, TP and TB in laboratory mixture using RP-HPLC

Assay method validation was performed for the content determination of CAF, TB, and TP in bulk drug using RP-HPLC as per recommendations of ICH Q2 (R1).

Analytical method validation is performed to ensure that analytical methodology is simple, accurate, specific, robust, and reproducible in specific range of analytes to be analyzed. Method validation an ensure the reliability of methods and also provide documented evidence that method is suitable for the intended use.

3.6.1 Specificity

Specificity was performed for the analytical method to differentiate and quantify the analyte in complex mixtures. Specificity was conducted during the determination of impurities and validation of identification tests.

For specificity study, blank (diluent) was injected, and the chromatogram was recorded in **Figure** 15.Any interference at the R_t of diluted CAF, TB, and TP standard solution was checked given in **Figure** 16.

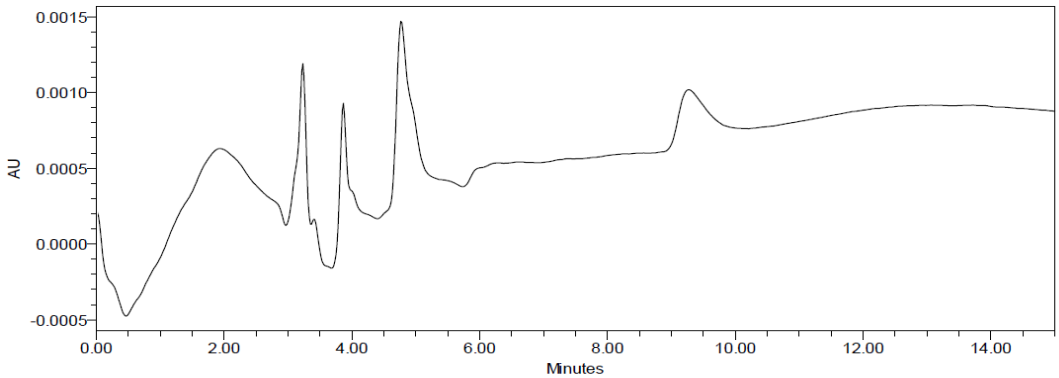

Figure 15 Chromatogram of blank

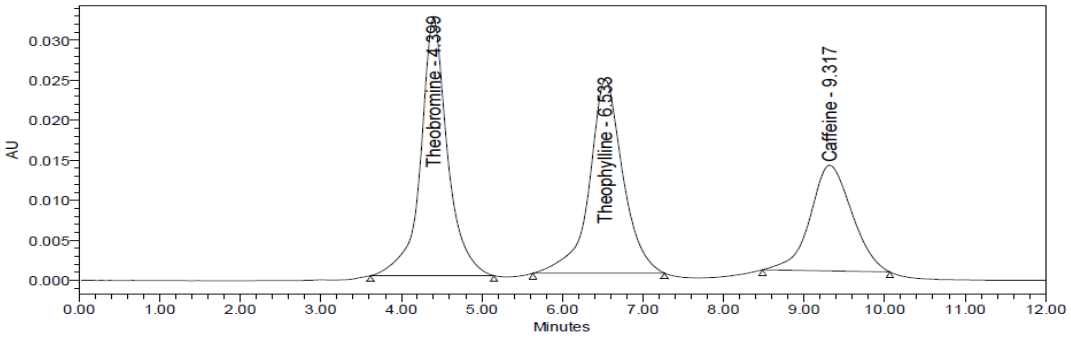

Figure 16 HPLC-chromatogram of diluted CAF, TP and TB standard solution

3.6.2 Linearity and Range

The linearity of the method is assessed by preparing five different concentrations in replicate ranging from 80-120% of the test concentration of CAF, TP, and TB. The obtained results were shown in **Table** 16 and **Figure** 17-19

Table 16 Result of linearity of CAF

% Test concentration	Concentration (µg/ml)	Average Area
0	0	0
80%	8	438536
90%	9	494455
100%	10	548452
110%	11	619660
120%	12	677013

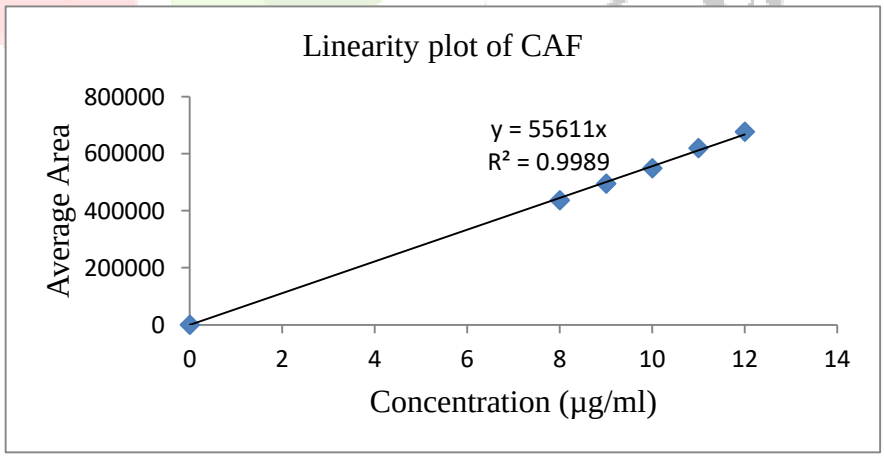

Figure 17 Linearity curve of CAF

Table 17 Result of linearity of TP

% Test concentration	Concentration (µg/ml)	Average Area
0	0	0
80%	8	524531
90%	9	598178
100%	10	685365
110%	11	751447
120%	12	818429

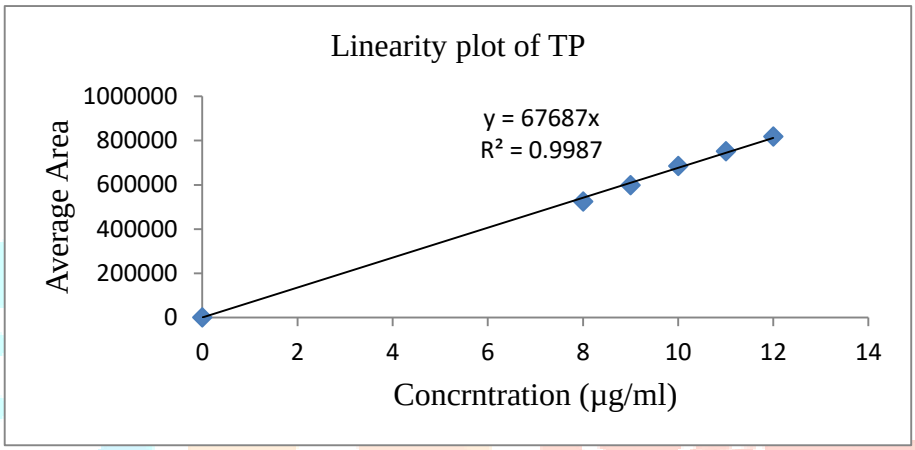

Figure 18 Linearity Curve of TP

Table 18 Results of linearity TB

% Test concentration	Concentration (µg/ml)	Average Area
0	0	0
80%	8	587302
90%	9	665824
100%	10	743616
110%	11	836644
120%	12	908769

The calibration curve of average peak area v/s concentration for TB is shown in **Figure**5.26.

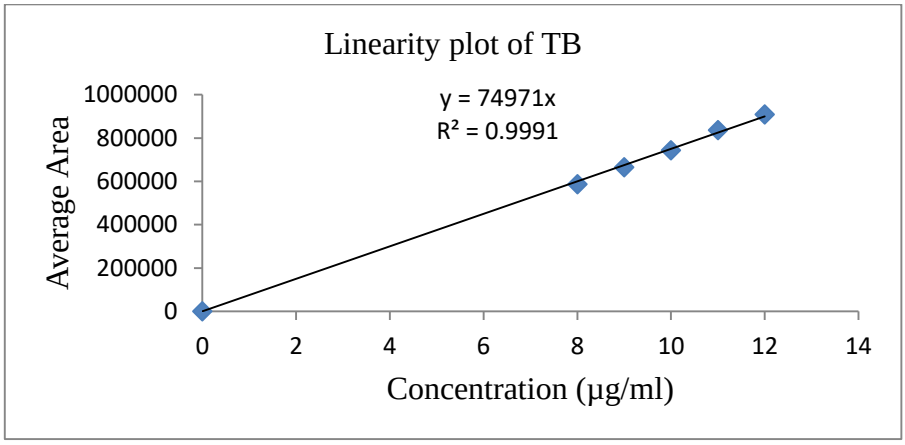

Figure 19 Linearity curve of TB

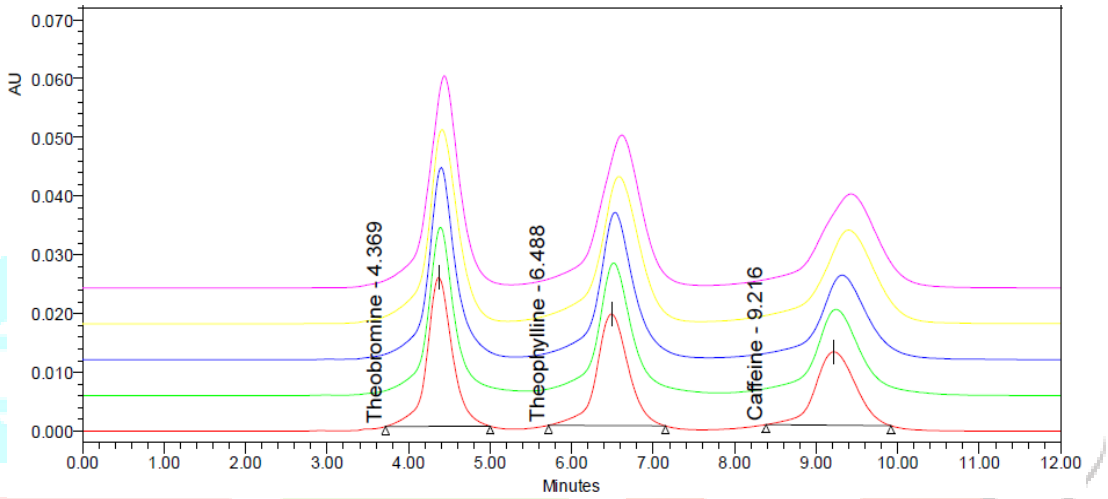

Figure 20 Overlay chromatogram of CAF (8-12µg/ml),TP (8-12 µg/ml) and TB (8-12µg/ml)

3.6.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD is determined by the analysis of samples with the known concentration of analyte and by establishing that minimum level at which the analyte can reliably detect. The detection limit is generally expressed in the concentration of the analyte (ppm or µg/ml) in the sample. Limit of quantitation is the least concentration of drug in a sample which is estimated with appropriate precision and accuracy under the affirmed experimental conditions. In the present method, LOD and LOQ was calculated based on standard error and regression line (Vial Jardy, A., 1999) which was given in **Table** 19.

Table 19 Summary of resulting linearity, LOD and LOQ of RP-HPLC method

Validation Parameter	CAF	TP	TB
Absorbance maxima (λ_{max})	273	271	272
Linearity range (µg/ml)	8-12	8-12	8-12
Coefficient of determination (r^2)	0.998	0.998	0.999
Regression equation (y)	y=55611x	y=67687x	y=74971x
Slope (b)	55611	67687	74971
Limit of detection (µg/ml)	0.475	0.436	0.429
Limit of quantification (µg/ml)	1.582	1.456	1.431

3.6.4 Precision

3.6.4.1 System precision and method precision

System precision was performed by repetitive analysis of the same homogenous solution of 100% test concentration 10µg/ml of each CAF, TP, and TB using the same instrument conditions. For method precision, six replicate of 100% test concentration of CAF, TP, and TB were prepared. The results obtained are shown in **Table** 20-22.

Table 20 System and method precision studies of CAF at the test concentration

Conc. µg/ml	**System precision area**	**Conc. µg/ml**	**Method precision area**
10	548452	10	548452
10	547391	10	547452
10	548782	10	548694
10	548685.5	10	546169
10	546632	10	544962
10	548556	10	545786
Mean	**548083**	**Mean**	**546923.7**
±S.D.	**871.224**	**± S.D.**	**1511.33**
%R.S.D.	**0.159**	**% R.S.D.**	**0.276**

Table 21 System and method precision studies of TP at the test concentration

Conc. (µg/ml)	**System precision area**	**Conc. (µg/ml)**	**Method precision Area**
10	687365	10	687365
10	688675	10	681948
10	688746	10	690154
10	689976	10	686487
10	686897	10	686584
10	688420	10	689154
Mean	**688346.5**	**Mean**	**686948.67**
±S.D.	**1094.767**	**±S.D.**	**2851.799**
% R.S.D.	**0.159**	**% R.S.D.**	**0.415**

Table 22 System and method precision studies of TB at the test concentration

Conc. (µg/ml)	System precision area	Conc. (µg/ml)	Method precision area
10	743616	10	743616
10	742789	10	744954
10	744154	10	741934
10	745428	10	745876
10	743798	10	744348
10	742789	10	746064
Mean	**743795.2**	**Mean**	**744348**
±S.D.	**948.065**	**±S.D.**	**1581.487**
%R.S.D.	**0.127**	**%R.S.D.**	**0.212**

3.6.4.2 Intermediate precision

Intermediate precision was performed by analysis of a homogenous mixture of 100% test concentration of all drugs (CAF,TP, and TB) in six replicate of preparations by two different in two different days. The results obtained given in the following **Table** 23-25

Table 23 Intermediate precision studies of CAF at the test concentration

Conc. (µg/ml)	Analyst 1 (Area)	Analyst 2 (Area)
10	547952	549024
10	546334	549322
10	544546	548874
10	548204	558260
10	549026	550784
10	545808	547828
Mean	**546978.33**	**550682**
±S.D.	**1693.772**	**3832.733**
%R.S.D.	**0.309**	**0.695**

Table 24 Intermediate precision studies of TP at the test concentration

Conc. (µg/ml)	Analyst 1 (Area)	Analyst 2 (Area)
10	684656.5	685042
10	688321	681098
10	687869	686547
10	684615	686144
10	684890	684112
10	688204	689024
Mean	**686425.9**	**685327.833**
±S.D.	**1876.417**	**2655.771**
%R.S.D.	**0.273**	**0.387**

Table 25 Intermediate precision studies of TB at the test concentration

Conc. (µg/ml)	Analyst 1 (Area)	Analyst 2 (Area)
10	746487	748472
10	749375	748951.5
10	743734	747643
10	748105	749672
10	742967	741284
10	745491	744682
Mean	**746026.5**	**746784.1**
±S.D.	**2475.784**	**3202.943**
%R.S.D.	**0.332**	**0.429**

3.6.5. Accuracy

Accuracy of the method was the measurement of the %recovery of the standard. Recovery studies were carried out by spiking of standard drug solutions (80%, 100%, and 120%) to the pre-analyzed sample (assay sample).Results of recovery study were found to be within the acceptance criteria 98-102%, indicating the reliability of the method. The % recoveries of the CAF, TP, and TB are given in following **Table 26**. The overlay of fortified and unfortified samples chromatogram is given in **Figure** 21.

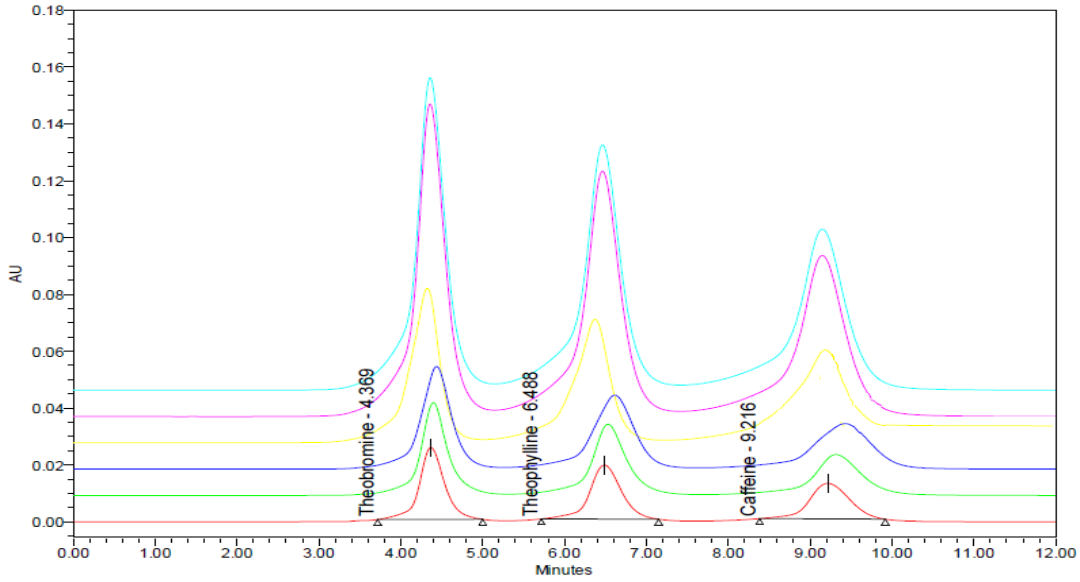

Figure 21 Overlay chromatogram of fortified and unfortified samples

Table 26 Results of recovery studies of HPLC method

Drugs	Unfortified sample		Fortified sample		%Recovery
	Conc. (µg/ml)	Mean peak Area	Conc.(µg/ml)	Mean peak area	
CAF	8	435836	10+8	982446	101.49
	10	548452	10+10	1093409	99.363
	12	677013	10+12	1217552	98.557
TP	8	523531	10+8	1205968	99.283
	10	687365	10+10	1371199	99.486
	12	819429	10+12	1518048	101.637
TB	8	587302	10+8	1341126	101.373
	10	743616	10+10	1501299	101.892
	12	908769	10+12	1644202	98.899

3.6.6. Robustness

Robustness of method is the measurement of the capability of an analytical method to stay unchanged by small, deliberate changes in method parameters. The change in method parameters in the HPLC technique may involve flow rate, column temperature, sample temperature, pH, and mobile phase composition. The effects of change in λmaxand flow rate are shown in **Table** 27-29.

Table 27 Results of the robustness of CAF

Conc. (µg/ml)	λ_{max} (nm)			Flow rate (ml/min)		
	271	**273**	**275**	**0.9**	**1**	**1.1**
10	551642	546548	550518	547681.5	546442	542426
10	546646	542446	543642	545774	545774	534822
10	546742	544876	548824	548348	548348	541364
Mean	**548343.3**	**544623.3**	**547661.3**	**548359.2**	**546854.7**	**539537.3**
S.D.	**2857.132**	**2062.639**	**3582.414**	**1864.758**	**1335.698**	**4117.977**
%R.S.D.	**0.521**	**0.378**	**0.654**	**0.34**	**0.244**	**0.763**

Table 28 Results of the robustness of TP

Conc. (µg/m)	λ_{max} (nm)			Flow rate (ml/min)		
	271	**273**	**275**	**0.9**	**1**	**1.1**
10	686892	684652	690148	692568	687692	684894
10	689567	685764	694686	685642	684794	681864
10	690846	687294	691864	691562	686946	684468
Mean	**689101.7**	**685903.3**	**692232.7**	**689924**	**686477.3**	**683742**
S.D.	**2017.655**	**1326.5**	**2291.353**	**3742.279**	**1504.772**	**1640.284**
%R.S.D.	**0.293**	**0.193**	**0.331**	**0.542**	**0.219**	**0.239**

Table 29 Results of the robustness of TB

Conc. (µg/ml)	λ_{max} (nm)			Flow rate (ml/min)		
	271	**273**	**275**	**0.9**	**1**	**1.1**
10	749968	746282	744568	691846	687864	682136
10	745986	742684	743658	6858634	684672	680208
10	745794	742668	741572	689567.5	685982	683564
Mean	**747249.3**	**743878**	**743266**	**690015.8**	**686172.7**	**681969.3**
S.D.	**2356.391**	**2081.94**	**1535.986**	**1652.268**	**1604.519**	**1684.196**
%R.S.D.	**0.315**	**0.279**	**0.207**	**0.239**	**0.234**	**0.247**

3.7. Assay determination of CAF tablet

For marketed formulation, an assay was performed to evaluate the purity of Caffeine drug in the formulation, and % purity of drug was calculated. The obtained results are shown in **Table** 30.

$$\text{Assay} = \frac{\text{Absorbance of tablet solution}}{\text{Absorbance of standard solution}} \times 100$$

Table 30 Assay of marketed CAF Tablet by HPLC method

Drug	The average peak area of standard	The average peak area of the sample	Label claim (mg)	Amount found (mg)	%Assay
CAF	544964	576684	100	105.8	105.82

3.8. Degradation behaviour

The optimized HPLC method was used to study the degradation behaviour of the API and tablet under various stress conditions. The corresponding chromatograms are shown under the respective stress conditions.

3.8.1 Hydrolytic degradation

3.8.1.1. a)Acid hydrolysis*:*

CAF solution of 100µg/ml from API and tablet was exposed to 0.1N HCl at 80°C for a 24h, CAF did not degrade, furthermore the strength of acid increased from 0.1,1, 2N even though the degradation was not observed. The API and tablet were found to be stable under acidic condition. No degradation products were observed on the exposure of API and tablet solution to acid shown in **Figure** 22, 23 & 24.

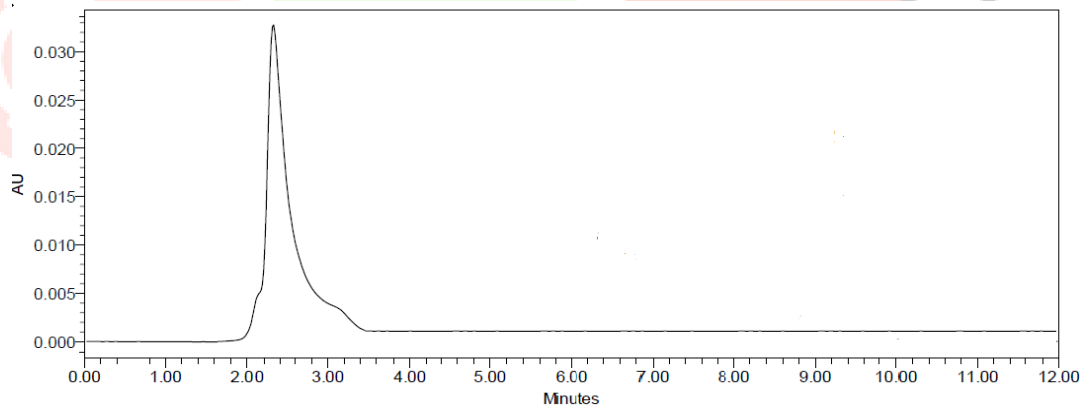

Figure 22 Chromatogram of blank solution for acid degradation

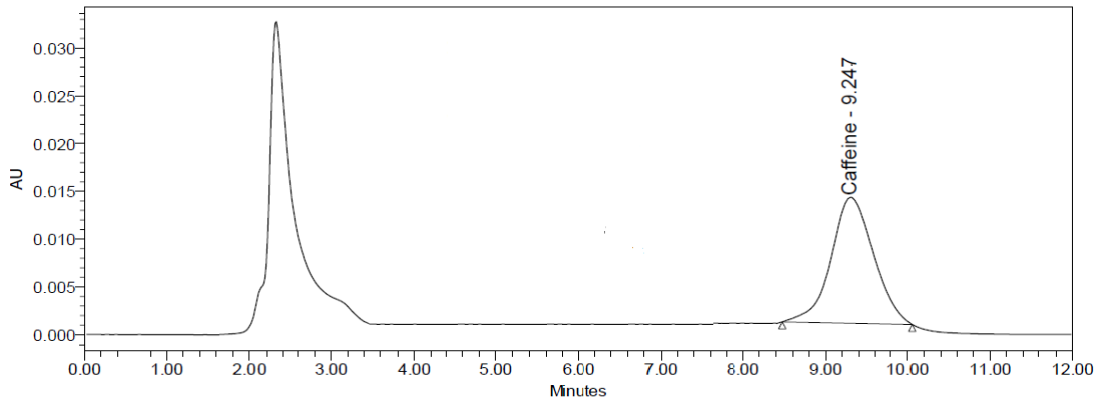

Figure 23 Chromatogram of CAF API (10 µg/ml) for acid degradation

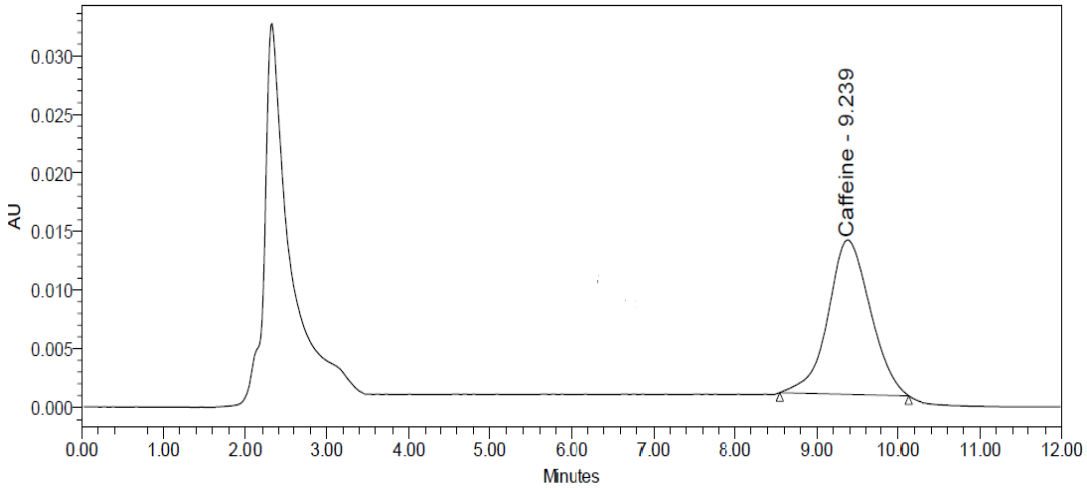

Figure 24 Chromatogram of CAF tablet (10µg/ml) for acid degradation

3.8.1.2. b)Alkaline hydrolysis:

CAF solution of 100µg/ml from API and tablet was exposed to 0.1N NaOH for 24 h at room temperature, but the drug was found to be stable, and no degradation product was observed. In the subsequent step, the strength of NaOH was increased, and the drug was found to show optimum degradation in 1N NaOH when kept at room temperature for 30 min. From the peak area correlation of the unstressed API and Tablet with that of the stressed solutions, it revealed that a total of 11.25% and 12.82% degradation was observed in alkaline condition respectively shown in **Figure** 25, 26 and 27

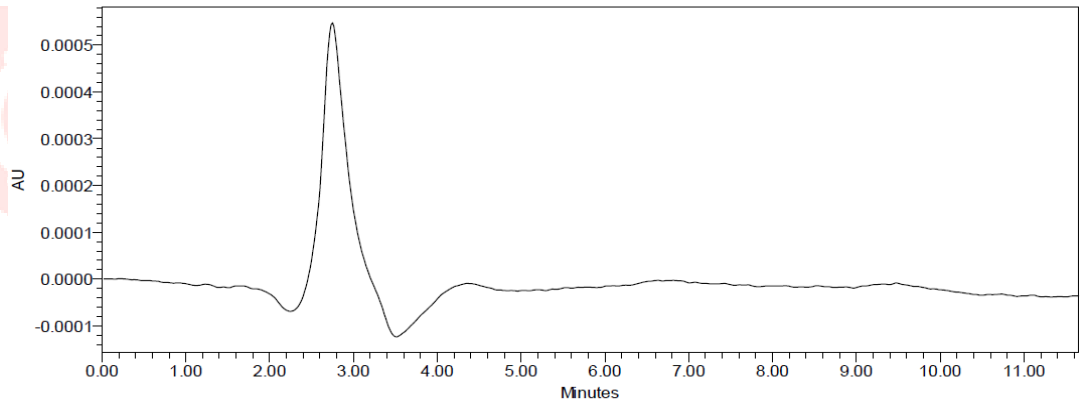

Figure 25 Chromatogram of blank solution for base degradation

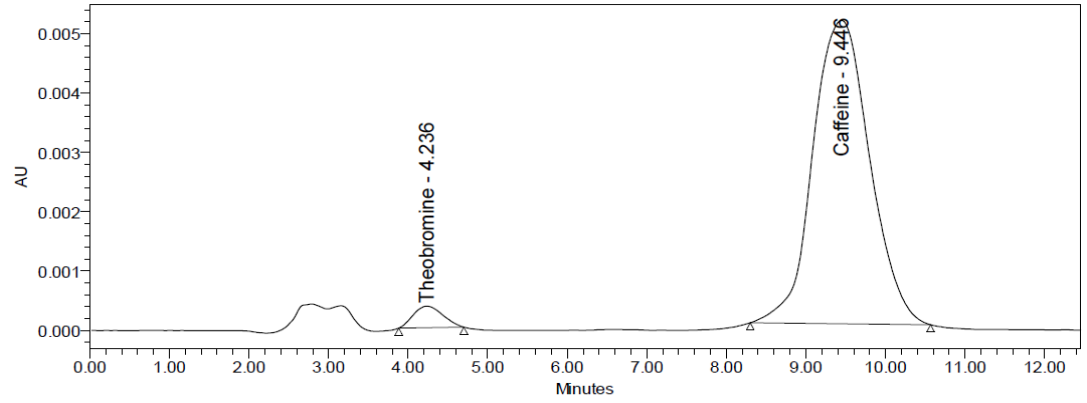

Figure 26 Chromatogram of CAF API (10 µg/ml) for base degradation

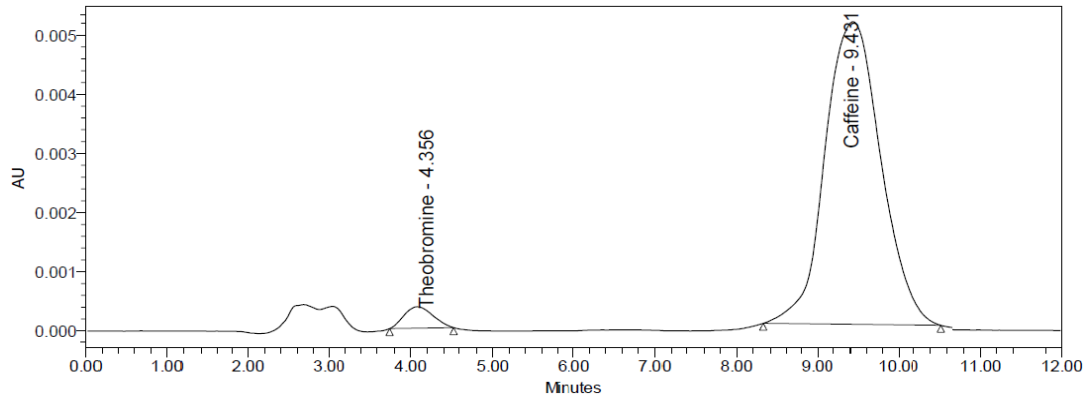

Figure 27 Chromatogram of CAF tablet (10 µg/ml) for base degradation

3.8.2. Oxidative degradation

CAF solution of 100µg/ml from API and tablet was used for oxidative decomposition. Initially, oxidative decomposition was carried out at room temperature by using 3% of hydrogen peroxide, but the drug was found to be stable, and no degradation product was observed. In the next subsequent step, the strength of hydrogen peroxide was increased to 10% at room temperature. Sampling was done day wise even though the CAF was found to be stable in API and tablet. No degradation product was observed on the exposure of API and tablet solution to oxidative conditions shown in **Figure** 28, 29, and 30.

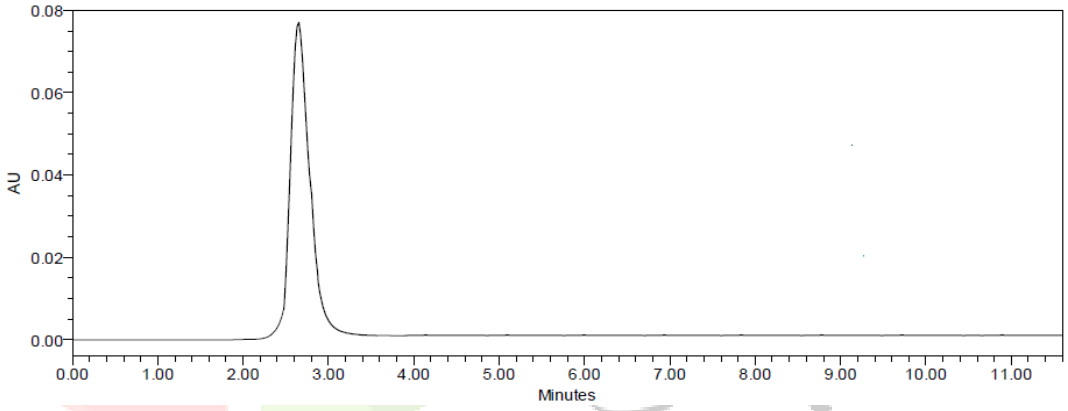

Figure 28 Chromatogram of blank solution for peroxide degradation

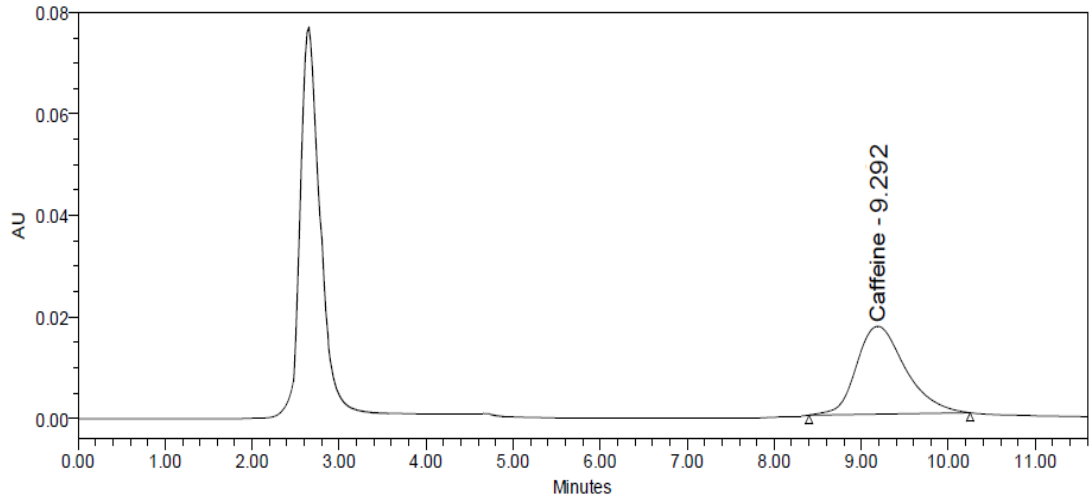

Figure 29 Chromatogram of CAF API (10 µg/ml) for peroxide degradation

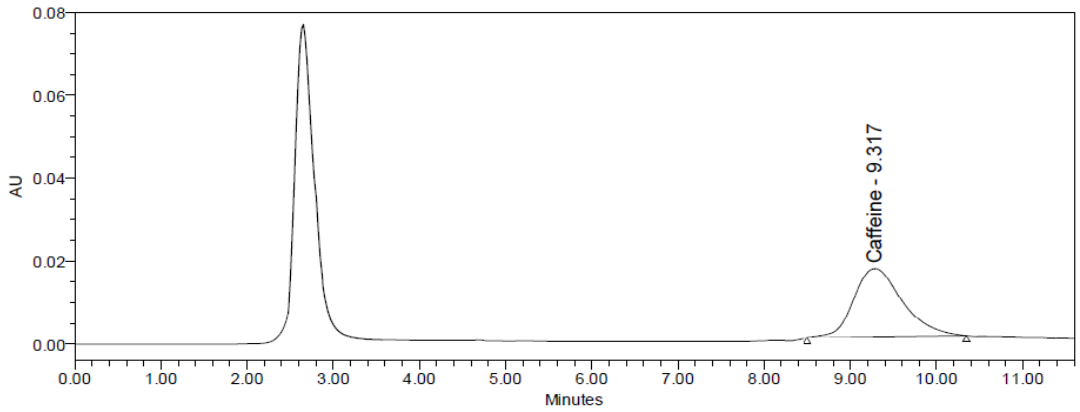

Figure 30 Chromatogram of CAF tablet (10 µg/ml) for peroxide degradation

3.8.3 Photolytic degradation

CAF solution of 100µg/ml from API and tablet was used for photolytic degradation of the drug carried out by exposing it for 60h in day sun light. The CAF was found to be stable under photolytic conditions.No degradation product was observed on the exposure of API and tablet solutions to light shown in **Figure 31, 32** and 33.

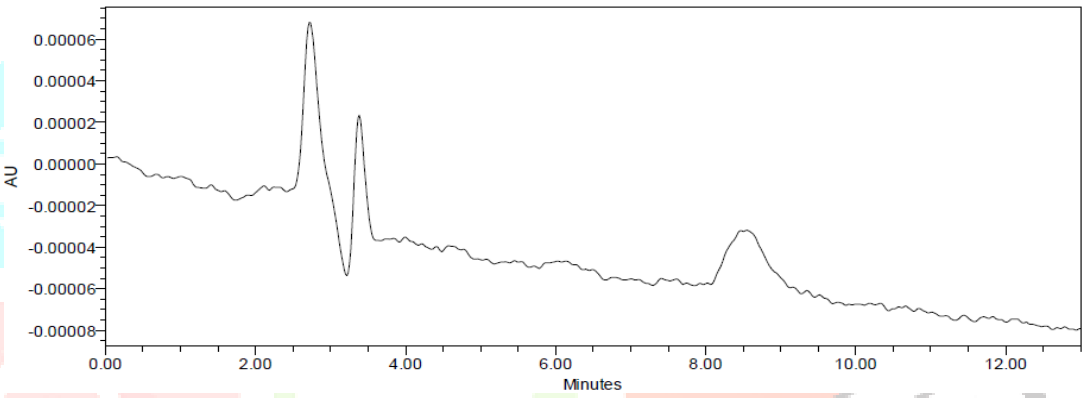

Figure 31Chromatogram of blank solution for photolytic degradation

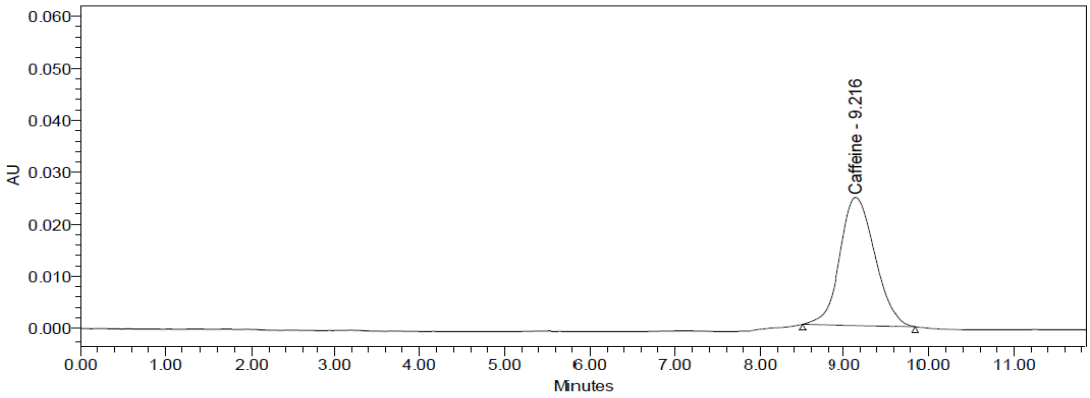

Figure 32 Chromatogram of CAF API (10 µg/ml) for photolytic degradation

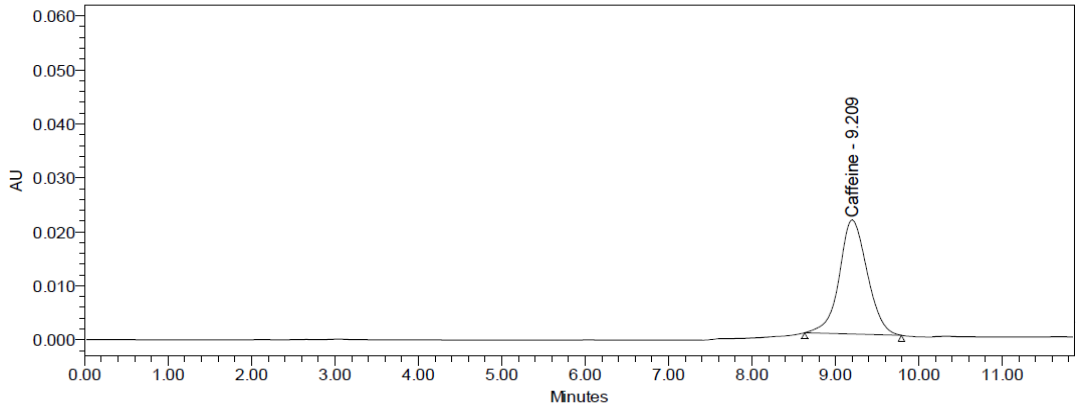

Figure 33 Chromatogram of CAF tablet (10 µg/ml) for photolytic degradation

3.8.4 Thermal degradation

CAF solution of 100µg/ml from API and tablet was placed in a dry air oven at 80°C for 5 days, but no degradation product was observed under this condition. The CAF was stable to thermal stress in the solution state shown in **Figure** 34, 35 and 36.

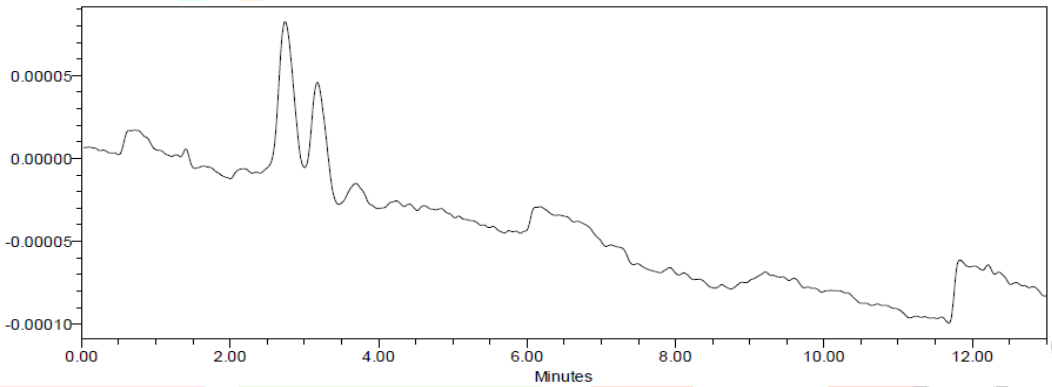

Figure 34 Chromatogram of blank solution for thermal degradation

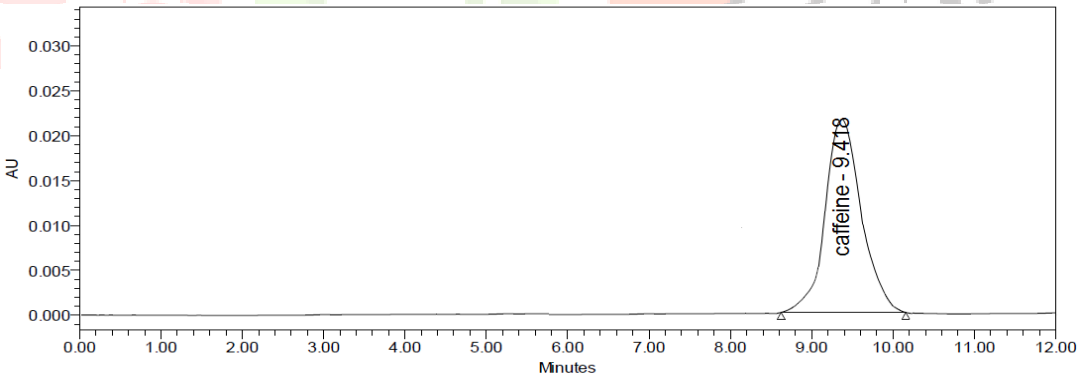

Figure 35 Chromatogram of CAF API (10 µg/ml) for thermal degradation

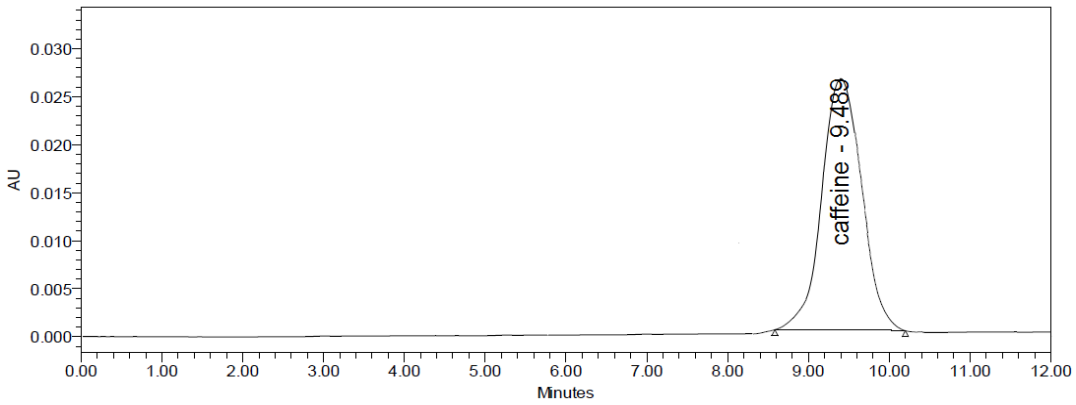

Figure 36 Chromatogram of CAF tablet (10µg/ml) for thermal degradation

3.8.5 Analysis of soft drinks by HPLC

Before performing the analysis of soft drinks, pH of soft drinks was found 2.18 for Coca-cola and 2.46 for Mountain dew. 1 ml soft drinks were diluted with 10 times in diluent (water and methanol in the ratio of 60:40) was injected into HPLC for determination of CAF, but along with CAF,TP and TB also found soft drinks which were shown in **Figure** 37 and 38. It indicates that CAF was degrading into TP and TB.

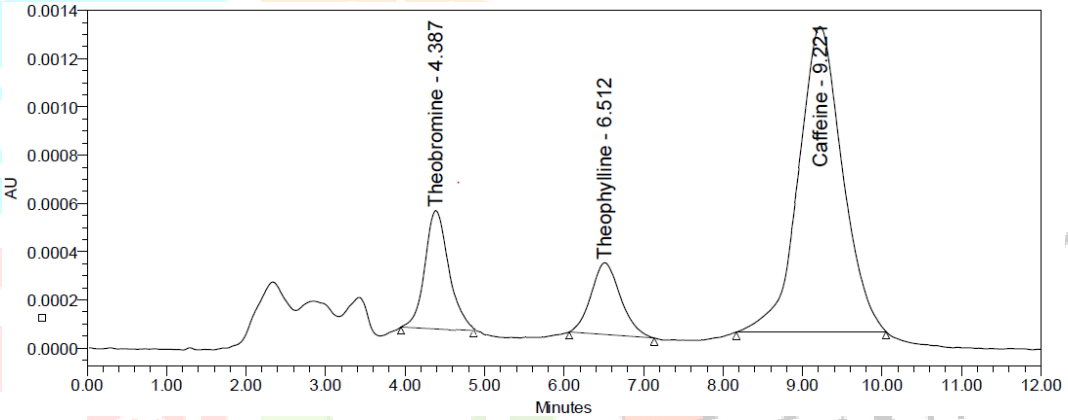

Figure 37 Chromatogram of diluted soft drink (Coca Cola)

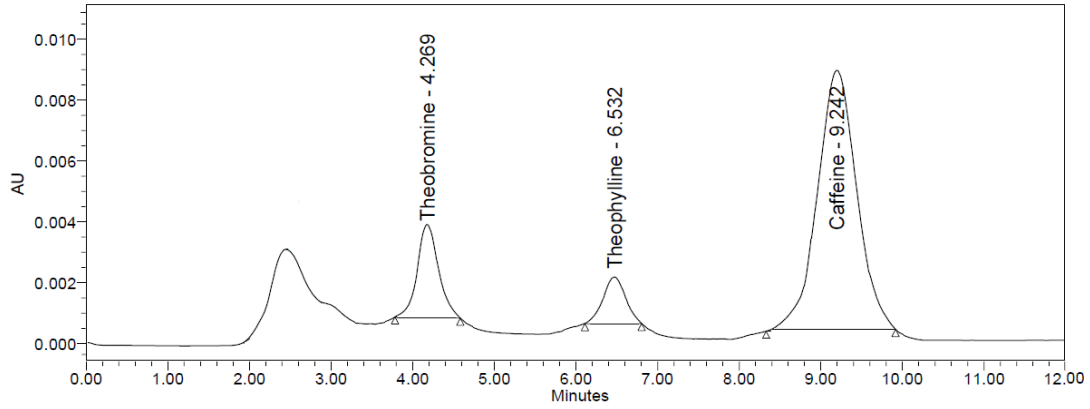

Figure 38 Chromatogram of diluted soft drink (Mountain Dew)

3.8.6 Photolytic degradation of CAF in soft drinks

For photolytic decomposition, solutions of soft drinks sample were exposed to day sun light for 10h.About 7% of CAF of Coca Cola was under gone into degradation in the photolytic condition shown in **Figure**5.46, and 4.5% of CAF in Mountain Dew was under gone into degradation shown in **Figure 39 and 40**.

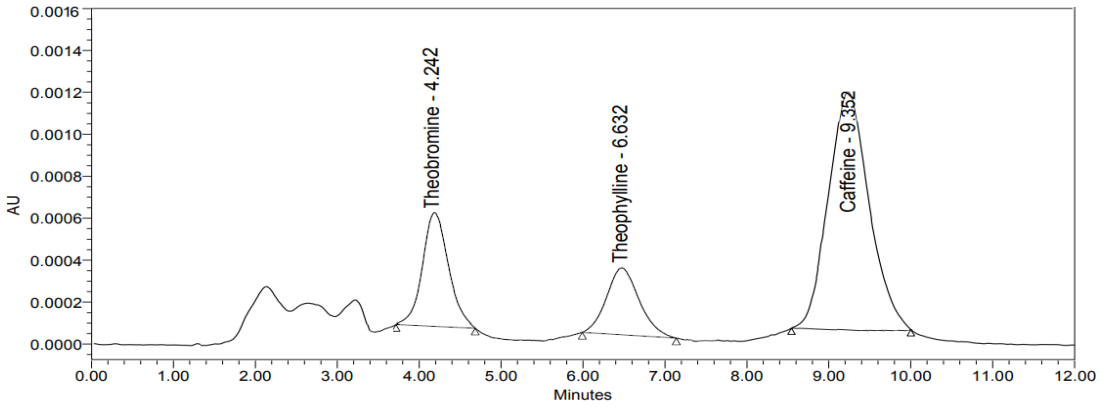

Figure 39 Chromatogram of soft drink (Coca Cola) for photolytic degradation

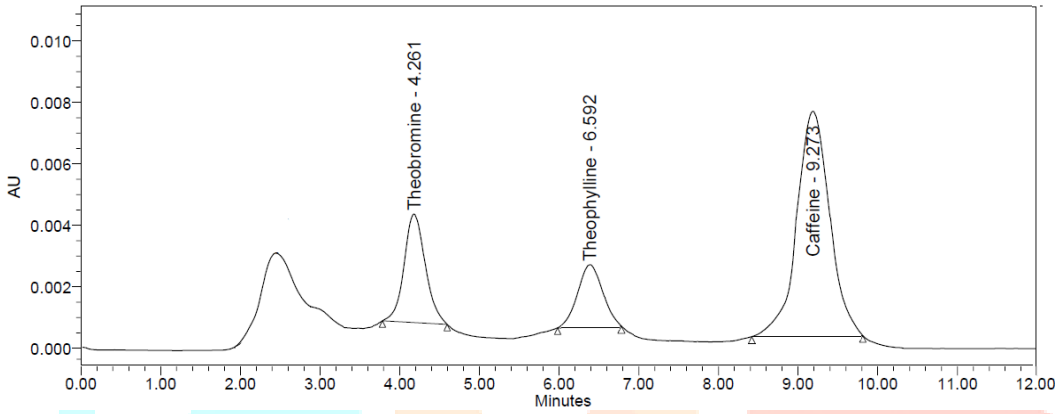

Figure 40 Chromatogram of soft drink (Mountain Dew) for photolytic degradation

Table 31 Summary of the degradation behaviour of Caffeine in bulk drug, pharmaceutical dosage form, and soft drinks.

Caffeine	Stress condition	Concentration of stressor	Exposure condition	Duration	%Degradation
API	Base	1N NaOH	RT	30 min	11.25
Tablet	Base	1N NaOH	RT	30 min	12.82
Coca Cola	Photolytic	-	Day light	10 h	7
Mountain Dew	Photolytic	-	Day light	10 h	4.5

3.9.Postulated degradation pathway of Caffeine

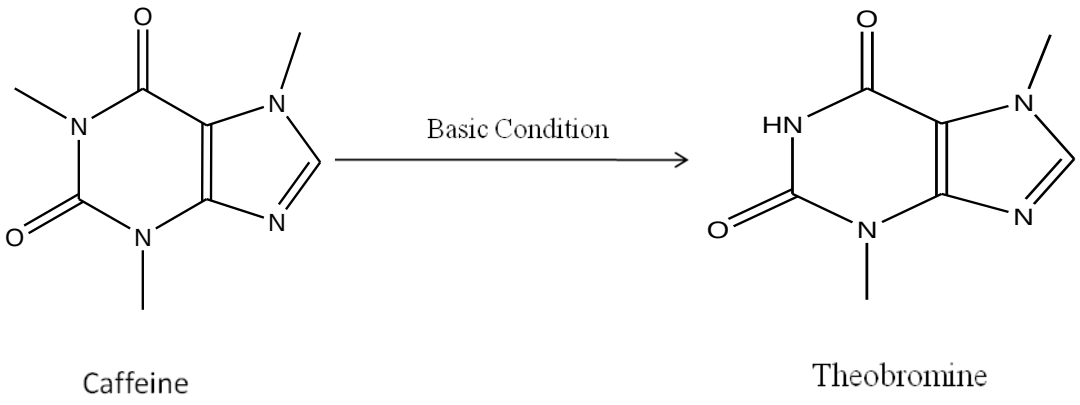

Figure 41 Degradation pathway of Caffeine in API and tablet in basic conditions.

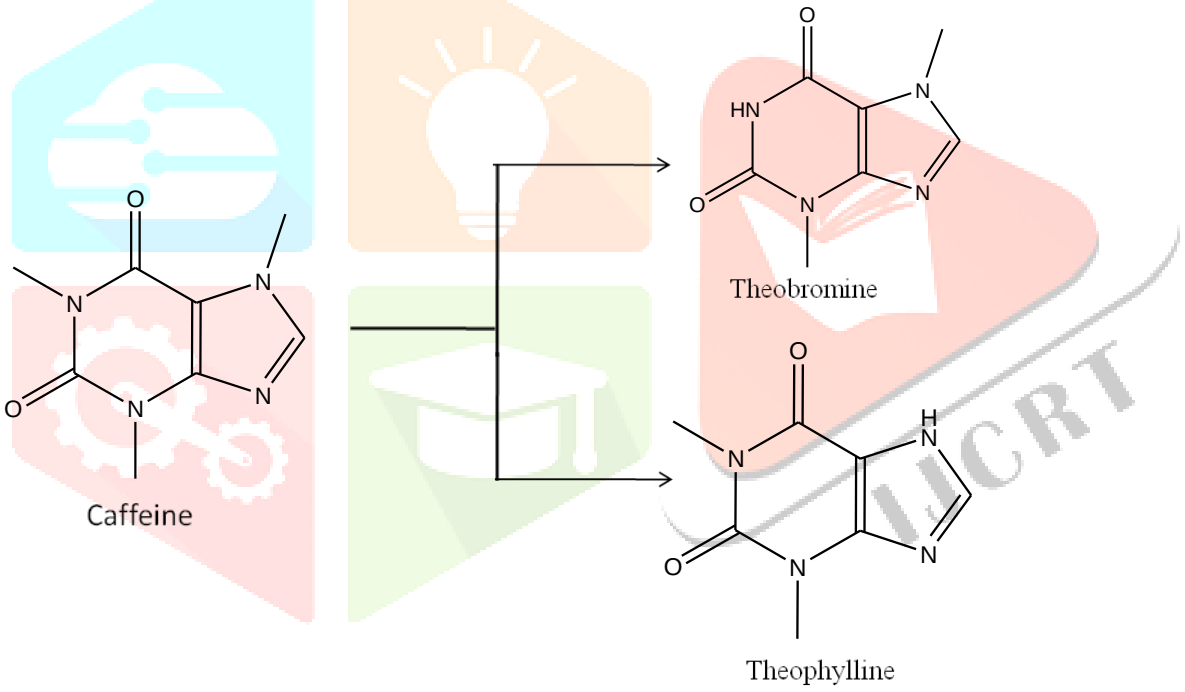

Figure 42 Degradation pathway of Caffeine in soft drinks in photolytic conditions.

Table 32 Correlation of retention time of standard drugs (CAF, TP, and TB) with that of degraded products for their identification.

S.N.	Standard drugs	Standard drugs R_t (min.)	Base degraded product of caffeine in API and caffeine, R_t (min)	Base degraded product of caffeine in tablet and caffeine, R_t (min)	Photolytic degraded product of caffeine in soft drinks and caffeine R_t (min)	
					Coca Cola	Mountain Dew
1.	CAF	9.364	9.446	9.431	9.352	9.273
2.	TP	6.560	-	-	6.632	6.6592
3.	TB	4.374	4.236	4.356	4.242	4.261

4. Conclusion

In this research paper various different analytical techniques are performed for the method development of the Caffeine, Theophylline and theobromine by the use of the Infrared spectroscopy in which the determination of the active pharmaceutical can be done and was found the obtained API were the pure, and also the another spectroscopic techniques also be performed for the method development of the drugs, similarly the another analytical technique that is performed it RPHPLC method development in which the firstly the method of the drugs and the marketed formulation will be developed and also be validated as per the ICH guidelines Q2R1. And the obtained results were shown the developed method is a highly precised, accurate and linear. The founded percentage relative standard deviation will be the less than 2 and this shows the accuracy of the method. After the method development and the validation, the stability of the method will also be performed on the API and also on the marketed products and was founded that the developed method will not be affected by the certain parameters like the thermal, alkaline or other degradation method, and this develop method were meet with all the requirements that are given by the ICH Q2R1 guidelines and this will find that the method will be the linear and the precised.

Acknowledgement

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