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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF TRAMADOL & CAFFEINE IN BULK

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ABSTRACT

Objective: The aim & objectives of this research work was to develop & validate a simple, selective, linear, precise, and accurate RP-HPLC method for the quantitative determination of Tramadol & Caffeine in bulk.

Methods: Optimized liquid chromatographic condition was obtained by performing many trials for the selection of mobile phase and column, for the separation of Tramadol & Caffeine in RP-HPLC. The method development was conducted with C18 column (250 X 4.6 mm, 5µ particle size) with the flow rate of 1mL/min. the optimized mobile phase conditions were 1% Triflouroacetic acid, Acetonitrile and methanol in the ratio of 70:20:10 (v/v). The method was validated as per ICH guidelines.

Results: The method found to be linear, accurate, rugged and robust for validated parameters. The linearity range was determined by external standard calibration method in the concentration range of 1µg/ml to 10µg/ml. The amount of recovery was calculated as 94% – 101% and it was

observed that all the values are within the limits. The Retention time for Tramadol & Caffeine is observed as 3.01 and 7.39 minutes respectively. Further the precision of the method was confirmed by the repeatable analysis of sample. The results were found to be precise due to low values of the % RSD. It indicated that the method has good precision. Limit of detection for Tramadol & for Caffeine 0.077µg/ml and 0.006µg/ml respectively. Similarly limit of quantification for Tramadol & for Caffeine 0.233µg/ml and 0.017µg/ml respectively. In the robustness study %RSD obtained for change of flow rate and wavelength and ruggedness for change of analyst was found to be below 2, which was within the acceptance criteria. So, simple, sensitive, accurate, precise RP- HPLC methods were developed and validated for the simultaneous estimation of Tramadol & Caffeine.

Keywords: RP-HPLC, Method development, Validation, Accuracy, Tramadol & Caffeine

INTRODUCTION

Quantitative analysis of any drug is an important tool in an industry. It is important to determine that the raw material, intermediate products as well as final products meet its specifications and are of required quality **[1]**.

The number of drugs and drug formulations introduced into the market are increasing at an alarming rate. These drugs or formulations may be either new entities or partial structural modification of the existing ones or novel dosage forms. High Performance Liquid Chromatography which is also known as High Pressure Liquid Chromatography. It is a popular analytical technique used for the separation, identification and quantification of each constituent of mixture. HPLC is an advanced technique of column liquid chromatography **[2-3]**.

Tramadol is a synthetic codeine analogue, used as a narcotic analgesic for severe pain, it can be addictive and weakly inhibits norepinephrine and serotonin reuptake. Tramadol half-life is 5-6 hours while the M1 metabolite presents a half-life of 8 hours. The bioavailability of tramadol has ranged from 75 to 90% **[4-9]**.

Caffeine is a Central Nervous System Stimulant and Methylxanthine. The physiologic effect of caffeine is by means of Central Nervous System Stimulation. The chemical classification of caffeine is Xanthines. The half-life is approximately 5 to 6 hours. Caffeine is rapidly absorbed after oral or parenteral administration, reaching peak plasma concentration within 30 minutes to 2 hours after administration **[10-14]**.

An UV-Visible spectrophotometric and HPLC method is considered to be most suitable for the simultaneous estimation of two drugs present in multicomponent dosage forms. Deep literature survey reveals that there are various analytical methods available for estimation of Tramadol and Caffeine separately either alone or in combination with other drugs. Literature survey does not reveal any analytical method for simultaneously estimation of Tramadol and Caffeine. So, it was thought of interest to develop a UV-Visible spectrophotometric and RP-HPLC method of estimation of combination of Tramadol and Caffeine.

The Aim of study to develop a cost effective and accurate RP-HPLC method for the estimation of Tramadol and Caffeine in their combination.

To validate the developed methods according to ICHQ2R1 guidelines to ensure their precision, accuracy, repeatability, reproducibility and other analytical method validation parameters.

METHODS [15-26]

Reagents and chemicals

Tramadol, Caffeine& Reagents and chemicals was obtained Gangamai College of Pharmacy, Nagaon, Dhule (MS) India & from Oniosome health care Private Limited, Mohali, Punjab.

Chromatographic Conditions for Simultaneous Estimation for RP-HPLC of Tramadol & Caffeine

HPLC method Selection of Detection Wavelength

The sensitivity of RP-HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. In the present study the Tramadol & Caffeine solution of 10µg/ml was prepared in Methanol. This solution was then scanned in the UV region of 200-400 nm and a spectrum was recorded.

Selection of Mobile Phase and Optimization of Chromatographic condition

- **Stationary Phase:** C_{18}, 250 × 4.6 mm, 5µm particle size, Phenomenex
- **Elution mode:** Low pressure gradient mode (70:20:10 v/v)
- **Mobile phase:** Solvent A was 1 % Triflouroacetic acid; Solvent B was Acetonitrile and Solvent C was methanol
- **Detector:** UV
- **Absorption maxima:** 232 nm
- **Column Temperature:** 30 °C
- **Flow rate:** 1 ml/min.
- **Injection volume:** 20 µl

- **Diluent:** Water
- **Run time:** 10 minutes

Standard Stock Solution Preparation (1000 µg/ml)

Blank

Diluent was filtered through 0.22 µ Millipore membrane filters and injected in HPLC system.

Standard solution preparation

An accurately weighed quantity of about 10 mg of Tramadol and 10mg of caffeine were taken in eppendorf and dissolved in 1 ml of diluents (as mentioned above) to obtain a solution of 1000 µg/ml as stock and sonicate to dissolve.

Preparation of calibration curve of Tramadol & Caffeine

From the standard stock solution, 0.01 ml was pipette out in 1ml Eppendorf tube and 1ml dilution are made with mobile phase to obtain working standard solution of concentration ranges from 1µg/ml to 10µg/mland filtered through 0.22 µ Millipore membrane filters and injected in RP-HPLC system RP-HPLC Chromatogram was recorded of each concentration and the calibration curve was plotted (area vs. concentration). The regression equation and correlation coefficient were obtained.

HPLC Chromatogram of standard

On HPLC analysis of standard, chromatogram was optimized in which Retention time of drugs.

Validation of RP-HPLC method as per ICH guidelines [15-26]

Linearity and Range

Selected linearity range for Tramadol & Caffeine was 1-10µg/ml. All the dilutions were filtered through 0.22 µ filter and injected.

LOD and LOQ

The LOD and LOQ of developed method were studied as per ICH guidelines. Several approaches for determining the LOD & LOQ are possible, depending on the procedure i.e., a non-instrumental or instrumental. Among them here employed method was,

LOD= 3.3σ/S and

LOQ= 10σ/S

Where, σ = the standard deviation of intercept

S = mean of slope in calibration curve.

Accuracy

Recovery studies were carried out by addition of standard drug solution at the 3 concentration levels 80%, 100% and 120% in pre-analyzed sample. In this method the known concentration of standard drug was added to the assay sample.

Precision

The inter-day and intra-day variation for determination of the drug was carried out with a concentration in the same day (replicates) and six consecutive days (single injection each day) where repeatability was determined with lower concentration and injected six times and % RSD was calculated.

Ruggedness

The ruggedness was studied by analyzing the same samples of Tramadol& Caffeine concentration 5µg/ml by changing analyst. The changes in the responses of Tramadol& Caffeine (5µg/ml) were noted in terms of %RSD.

Robustness

The robustness was studied by analyzing the sample of lower concentration with deliberate variation in the method parameters. The change in the responses of drugs was noted in terms of % RSD. Robustness of the method was studied by change in wavelength and change in flow rate.

RESULT AND DISSCUSSION

HPLC Method

Determination of chromatogram of Blank & Standard (Tramadol & Caffeine)

On HPLC analysis of Blank and standard solution of Tramadol and Caffeine (10µg/ml) chromatogram was optimized & analyzed as per the proposed method. HPLC analysis of blank and standard chromatogram was shown in **Figure 1 & 2**.

Preparation of standard curve of Tramadol by RP-HPLC (Table 1)

The calibration curve for Tramadol was obtained by using the 1 to 10 µg/ml solution. The area was measured at 232 (Isosbestic point). The calibration curve as shows in graph indicated the regression equation Y = 59059x + 2990and R^2 value 0.999 which shows good linearity as shown in **Figure 3**.

Preparation of standard curve of Caffeine by RP-HPLC (Table 4)

The calibration curve for Caffeine was obtained by using the 1 to 10 µg/ml solution. The area was measured at 232 (Isosbestic point). The calibration curve as shows in graph indicated the regression equation Y = 25459x - 628.8and R^2 value 0.999 which shows good linearity as shown in **Figure 4**.

Validation of RP-HPLC method as per ICH guidelines

Linearity and RangeLinearity and Range of Tramadol

A calibration curve was plotted over a concentration range of 1 to 10µg/ml for Tramadol. Accurately measured working stock solution of Tramadol (1, 2, 3, 4, 5, 6, 7 and 10) and all the dilutions were filtered

through 0.22 µ filter and injected. The area of all solution was taken at their respective wavelength. The Linearity was constructed by plotting concentration against area where each reading.

Linearity and Range of Caffeine

A calibration curve was plotted over a concentration range of 1 to 10µg/ml for Caffeine. Accurately measured working stock solution of Caffeine (1, 2, 3, 4, 5, 6, 7 and 10) and all the dilutions were filtered through 0.22 µ filter and injected. The area of all solution was taken at their respective wavelength. The Linearity was constructed by plotting concentration against area where each reading **(Table 7)**.

Accuracy

Accuracy of the method was determined in terms of % recovery of standard. Recovery studies were carried out by addition of standard drug solution at the 3 concentration levels 50%, 100% and 150% in pre-analyzed sample. In this method the known concentration of standard drug was added to the assay sample **(Table 8)**.

The results (**Table 9**) indicate that the recoveries are well within the acceptance range of 94% – 101%, indicating a good degree of sensitivity of the method towards detection of analytes in sample. Therefore, method is accurate and it can be used for the estimation of drug.

Precision

Standard solution of Tramadol and Caffeine (10 + 10 μg/ml) was prepared and analyzed as per the proposed method **(Table 10)**. The method was found to be precise due to low values of the % RSD.

LOD and LOQ

The Limit of detection and limit of quantification of the method were calculated basing on standard deviation of the response and the slope (s) of the calibration curve at approximate levels of the limit of detection and limit of quantification. The results obtained were within the limit **(Table 11)**.

Robustness

The robustness was studied by analyzing the sample of lower concentration with deliberate variation in the method parameters. The change in the responses of drugs was noted in terms of %RSD. Robustness of the method was studied by change in wavelength& change in flow rate **(Table 12)**.

The Percentage RSD should not be more than 2. The %RSD obtained for change of flow rate and wavelength was found to be below 2, which was within the acceptance criteria. Hence the method was robust (**Table 16**).

Ruggedness

The ruggedness was studied by analyzing the same samples same drug by changing analyst. The change in the responses of drugs was noted in terms of % RSD **(Table 17)**.

The Percentage RSD should not be more than 2. The % RSD obtained for change of analyst was found to be below 2, which was within the acceptance criteria. Hence the method was rugged.

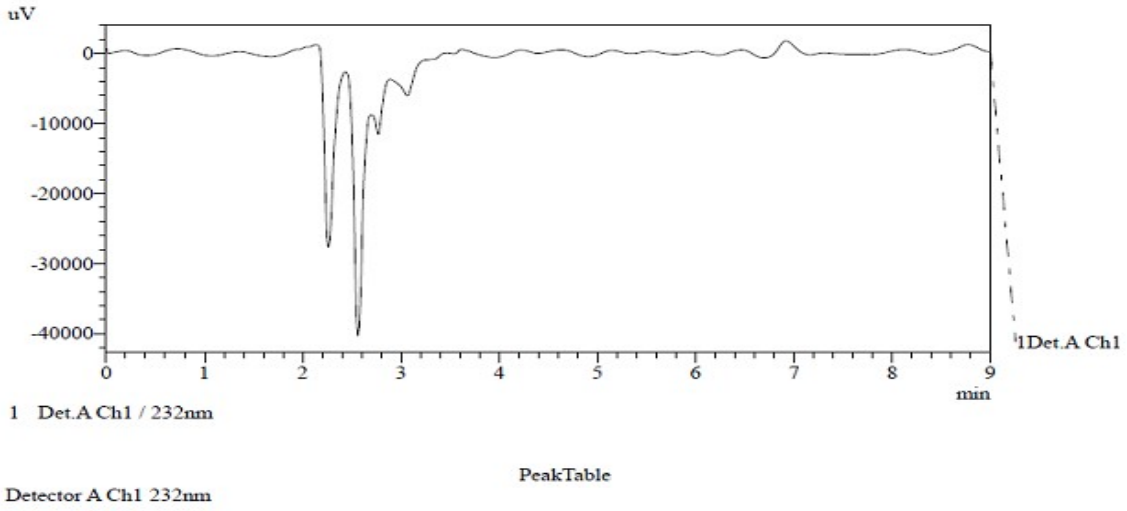

Figure 1: Chromatogram of blank

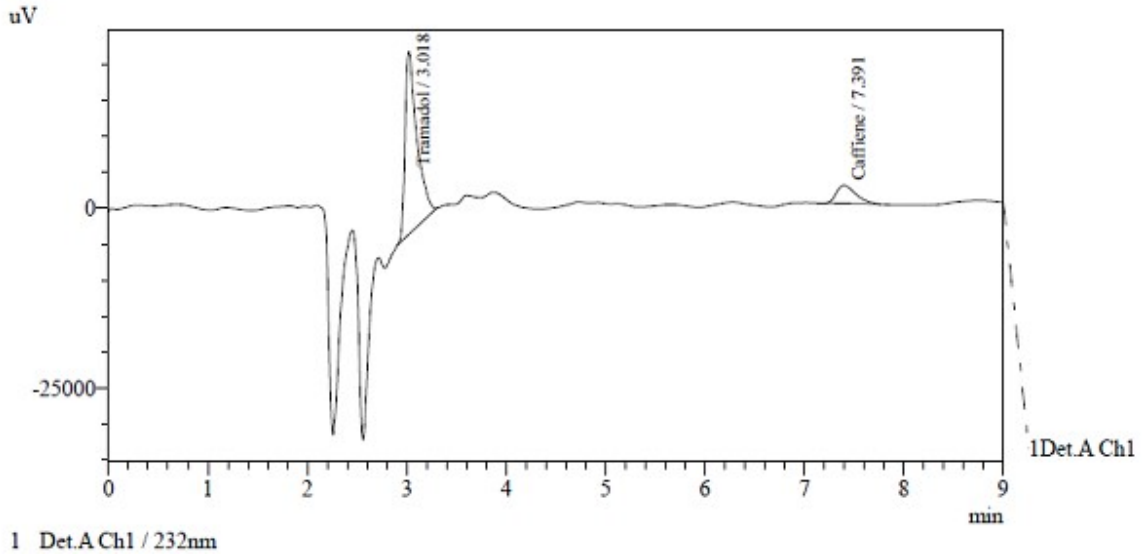

Figure 2: Simultaneous chromatogram of standard Tramadol and Caffeine (5µg/ml)

Table 1: Data of Simultaneous Tramadol and Caffeine (5ug/ml)

S. No.	Compound Name	Retention time	Area
1	Tramadol	3.018	298737
2.	Caffeine	7.39	128451

Table 2: Calibration curve of Tramadol

Sr. No.	Concentration µg/ml	Area
1	1	61705.00±526.30
2	2	111646.00±1341.09
3	3	184059.00±2847.6
4	4	242587.33±3524.27
5	5	299534.67±1379.87
6	6	361797.33±3240.76
7	8	418836.67±1900.01
8	10	587989.33±4913.27

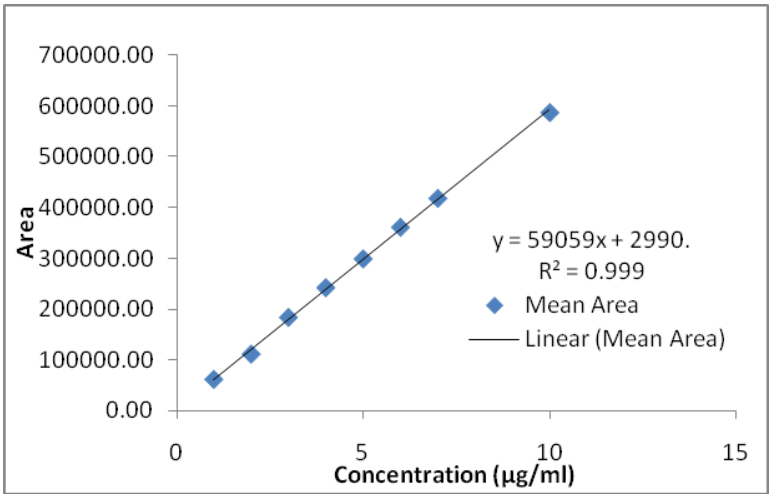

Figure 3: Graph of standard calibration curve of Tramadol by RP-HPLC

Table 3: Result of Statistical parameters for estimation of Tramadol

Statistical parameters	Results
Regression equation: y=mx+C	Y= 59059x + 2990
Slope (m)	59059
Intercept (C)	2990
Correlation coefficient (r^2)	0.999

Table 4: Calibration curve of Caffeine by RP-HPLC

Sr. No.	Concentration µg/ml	Area
1	1	25269.33±174.127
2	2	48134.000±237.502
3	3	78256.33±112.447
4	4	101258.67±98.053
5	5	128426.00±285.323
6	6	152170.00±68.352
7	7	173050.00±614.291
8	10	255834.33±603.291

Table 5: Result of Statistical parameters for estimation of Caffeine

Statistical parameters	Results
Regression equation: y=mx+C	Y=25459x - 628.8
Slope (m)	25459
Intercept (C)	628.8
Correlation coefficient (r^2)	0.999

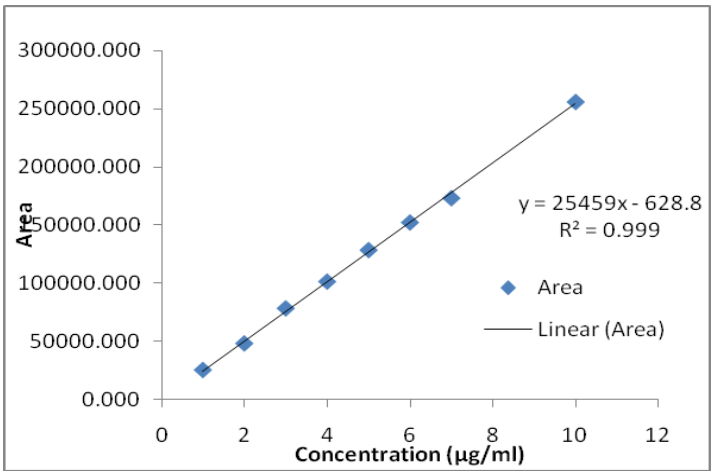

Figure 4: Graph of standard calibration curve of Caffeine by RP-HPLC

Table 6: Linearity of Tramadol

Conc. (µg/ml)	Area-1	Area-2	Area-3
1	62034	61098	61983
2	112456	112384	110098
3	182341	182490	187346
4	239985	246598	241179
5	298737	301128	298739
6	359837	365538	360017
7	416645	420019	419846
10	583829	586729	593410

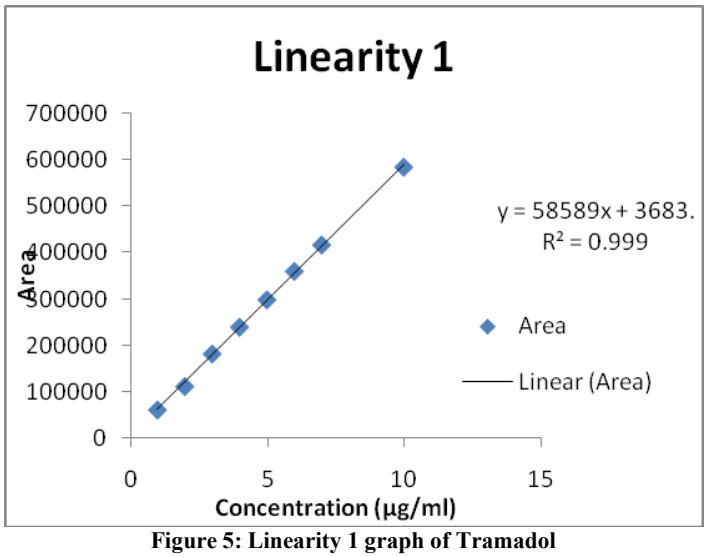

Figure 5: Linearity 1 graph of Tramadol

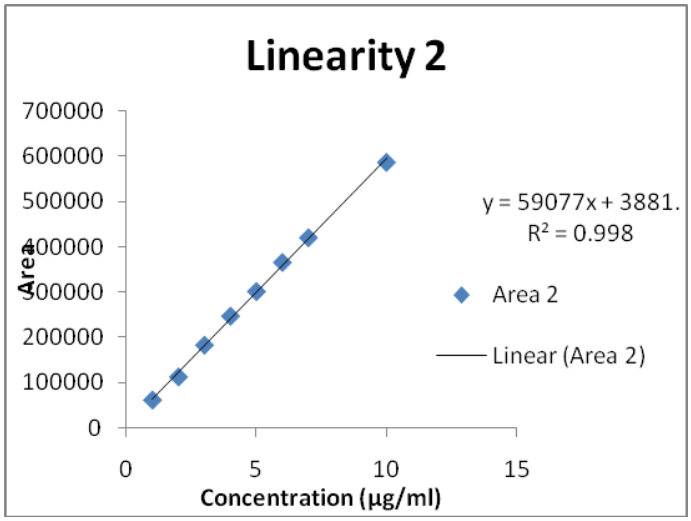

Figure 6: Linearity 2 graph of Tramadol

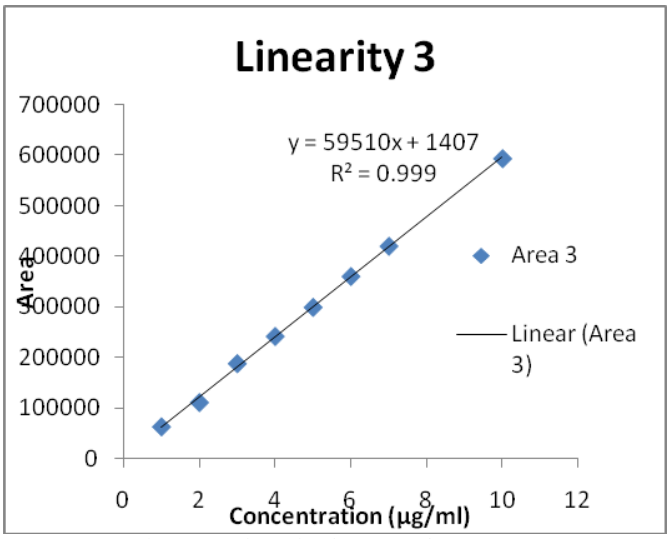

Figure 7: Linearity 3 graph of Tramadol

Table 7: Linearity of Caffeine

Conc. (µg/ml)	Area-1	Area-2	Area-3
1	25457	25238	25113
2	48372	47897	48133
3	78342	78129	78298
4	101233	101367	101176
5	128451	128698	128129
6	152234	152178	152098
7	173423	172341	173386
10	256528	255543	255432

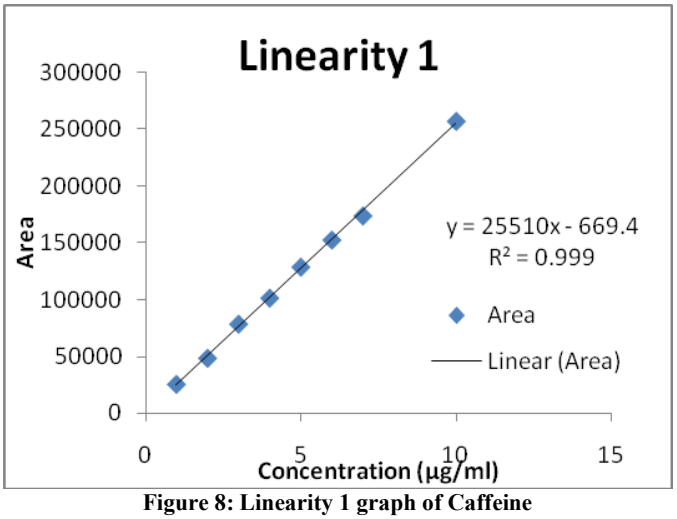

Figure 8: Linearity 1 graph of Caffeine

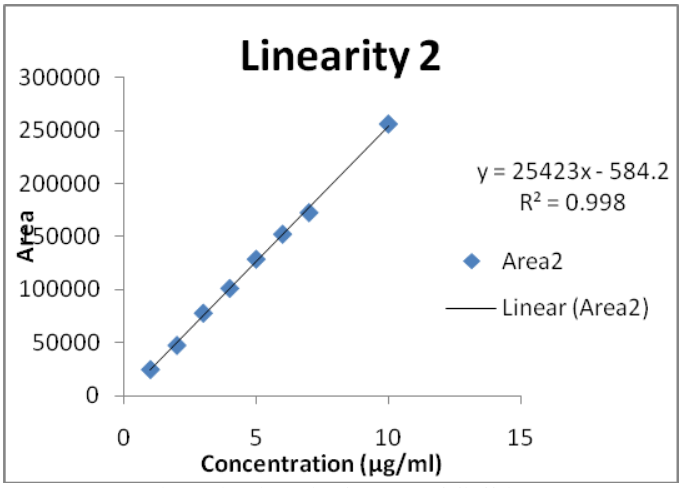

Figure 9: Linearity 2 graph of Caffeine

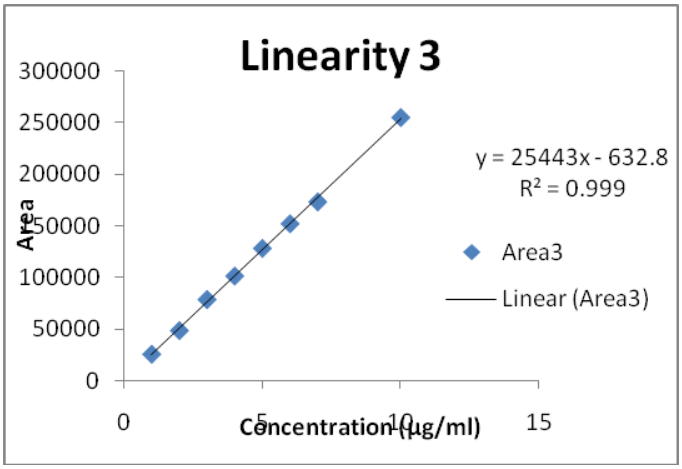

Figure 10: Linearity 3 graph of Caffeine

Table 8: Accuracy Study of Tramadol

Level	Amount added of Tramadol	Percentage recovery of Tramadol	% RSD
80%	8(µg/ml)	101.765±0.189	0.186
100%	10(µg/ml)	98.453±0.373	0.378
120%	12(µg/ml)	100.395±0.296	0.295

Table 9: Accuracy Study of Caffeine

Level	Amount added of Caffeine	Percentage recovery of Caffeine	% RSD
80%	8(µg/ml)	100.610±0.160	0.159
100%	10(µg/ml)	101.999±0.167	0.163
120%	12(µg/ml)	94.582±0.211	0.223

Table 10: Repeatability and inter-intraday precision study

S. No.	Precision	Percentage recovery of Tramadol	% RSD	Percentage recovery of Caffeine	% RSD
1	Repeatability	100.217±0.308	0.308	100.578±0.340	0.338
2	Inter Day	98.859±0.721	0.73	100.987±0.355	0.352
3	Intra Day	96.305±1.589	1.65	102.055±0.488	0.478

Table 11: LOD and LOQ data

Drug	LOD (µg/ml)	LOQ (µg/ml)
Tramadol	0.077	0.233
Caffeine	0.006	0.017

Table 12: Robustness data of Tramadol with deliberate change in wavelength

Conc.(µg/ml)	Wavelength 227nm (Area)	Wavelength 232nm (Area)	Wavelength 237nm (Area)
5	282239	293328	279923
5	282156	297739	280017
5	279924	295629	281120
5	279830	295540	283462
5	283110	291630	281290
5	280126	295629	279939
Mean	281230.8	294915.8	280958.5
SD	1434.9	2130.5	1371.2
%RSD	0.510	0.722	0.488

Table 13: Robustness data of Caffeinewith deliberate change in wavelength

Conc.(µg/ml)	Wavelength 227nm (Area)	Wavelength 232nm (Area)	Wavelength 237nm (Area)
5	184587	127765	217647
5	183498	127329	214899
5	184378	126947	213487
5	185324	127721	212543
5	182624	127811	214343
5	184654	127134	215329
Mean	184177.5	127451.2	214708.0
SD	961.7	366.2	1753.3
%RSD	0.522	0.287	0.817

Table 14: Robustness data of Tramadol with deliberate change in flow rate (ml/min)

Conc.(µg/ml)	Flow rate 0.8ml/min (Area)	Flow rate 1ml/min (Area)	Flow rate 1.2ml/min (Area)
5	274536	293324	289987
5	277863	297153	291126
5	276743	295545	291098
5	277930	295123	293811
5	278937	291630	294530
5	277920	295785	289132
Mean	277321.5	294760.0	291614.0
SD	1531.4	1969.0	2128.0
%RSD	0.552	0.668	0.730

Table 15: Robustness data of Caffeinewith deliberate change in flow rate (ml/min)

Conc.(µg/ml)	Flow rate 0.8ml/min (Area)	Flow rate 1ml/min (Area)	Flow rate 1.2ml/min (Area)
5	117645	127366	133476
5	116539	127320	132398
5	117689	127940	133391
5	116832	127755	134721
5	117722	127832	135540
5	115870	126739	131711
Mean	117049.500	127492.000	133539.500
SD	763.541	446.753	1419.187
%RSD	0.652	0.350	1.063

Table 17: Analyst to Analyst variation data

S. No.		Percentage recovery of Tramadol	% RSD	Percentage recovery of Caffeine	% RSD
1	Analyst 1	100.383± 0.199	0.198	100.777± 0096	0.095
2	Analyst 2	98.633± 0.096	0.098	101.368± 0.330	0.326

CONCLUSION

Various types of HPLC's have been used for simultaneous estimations e.g. normal phase HPLC, reversed phase HPLC, size exclusion HPLC, ion-exchange HPLC, bio-affinity HPLC. Aim & objective of the present research work was to develop simple, selective, linear, precise, accurate and robust RP-HPLC method for the estimation of Tramadol & Caffeine in bulk. As per aim & objective method was developed & validated in accordance with the ICH guidelines.

The method development was conducted with C18 column (250 X 4.6 mm, 5µ particle size) with the flow rate of 1mL/min. the optimized mobile phase conditions were 1% Triflouroacetic acid, Acetonitrile and methanol in the ratio of 70:20:10 (v/v). The method was validated as per ICH guidelines. The method found to be linear, accurate, rugged and robust for validated parameters. The linearity range was determined by external standard calibration method in the concentration range of 1µg/ml to 10µg/ml. The amount of recovery was calculated as 94% – 101% and it was observed that all the values are within the limits. The Retention time for Tramadol & Caffeine are observed

as 3.01 and 7.39 minutes respectively. The correlation coefficients for both the components are close to 1 which shows the accuracy of the method. The results were found to be precise due to low values of the %RSD. It indicated that the method has good precision. Limit of detection for Tramadol & for Caffeine 0.077µg/ml and 0.006µg/ml respectively. Similarly limit of quantification for Tramadol & for Caffeine 0.233µg/ml and 0.017µg/ml respectively. In the robustness study % RSD obtained for change of flow rate and wavelength and ruggedness for change of analyst was found to be below 2, which was within the acceptance criteria. Finally it is concluded that this developed RP- HPLC method is simple, sensitive, accurate, precise methods the simultaneous estimation of Tramadol & Caffeine in bulk.

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