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HPLC Method Development and Validation for Simultaneous Estimation of Tramadol and Piroxicam

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Abstract
The suggested new RP-HPLC method for the quantitative measurement of piroxicam and tramadol in bulk was found to be speedy, accurate, sensitive, straightforward, and exact. It was discovered that the pure drug's melting points for tramadol and piroxicam are between 178.42°C and 199.21° Tramadol and Piroxicam in methanol showed a wide band at 273 nm and 334 nm in their UV spectra. The method was developed using a Force C18 Column (5 µm, 250 × 4.6 mm) at a flow rate of 1 mL/min. The optimal acetonitrile to glacial acetic acid (0.1%) ratio for the mobile phase conditions was 50:50 v/v. According to Simultaneous data, the retention length of Tramadol Amount of Acid Hydrolysis Degradation for 5 Hours at 40°C for Tramadol and Piroxicam: 3.95% and 1.40%, respectively. Tramadol and Piroxicam's base hydrolysis amount for a 5-hour period at 40°C were 7.85% and 4.77%, respectively. Quantity of Tramadol and Piroxicam's oxide degradation over a 0.5-hour period at 37°C was 17.16% and 1.28%, respectively. Tramadol and Piroxicam's UV degradation percentages after 0.5 hours are 23.72% and 0.73%, respectively. Tramadol and Piroxicam's respective amounts of thermal degradation after two hours at 105°C were 35.93% and 1.16%, respectively.

Keywords: Tramadol, RP-HPLC Tramadol and Piroxicam.

Introduction:

Tramadol is a rhizomic mixture of two pharmacologically active enantiomers that are transformed into active metabolites and each of which contributes differently to the analgesic effect of the drug: Both (+)-tramadol and its primary metabolite (+)-O-dimethyl-tramadol (M1) are agonists of the µ opioid receptor, despite the fact that (+)-tramadol inhibits serotonin reuptake and (-)-tramadol inhibits norepinephrine reuptake. The way that these pathways cooperate and support one another improves tramadol's ability to regulate pain perception and response.

It has also been shown that tramadol affects a number of additional central nervous system pain modulators, immune mediators, and non-neuronal inflammatory markers. Numerous pain conditions, such as neuropathic pain, post-operative pain, lower back pain, labour pain, osteoarthritis, fibromyalgia, and cancer pain, have all been demonstrated to respond effectively to tramadol. Considering the variety of targets involved in pain and inflammation, this is not surprising. Because of its SNRI activity, tramadol has anti-shivering, anxiolytic, and depressive effects in addition to pain relief.
Because of its high tolerability profile and multimodal mechanism of action, tramadol is generally considered a lower-risk opioid option for the treatment of moderate to severe pain. It is considered a Step 2 alternative on the WHO pain scale, with a potency around a tenth of that of morphine. Tramadol differs from other traditional opioid medications in that it functions as a μ-opioid agonist on its own and also alters the actions of neurotransmitters such as norepinephrine and serotonin, which trigger descending pain inhibitory pathways and influence monoamines. Tramadol functions similarly to other SNRI antidepressants like venlafaxine and duloxetine in terms of how it affects serotonin and norepinephrine.

Piroxicam is a nonsteroidal oxicam derivative that has anti-inflammatory, analgesic, and antipyretic properties. The non-selective NSAID piroxicam binds to and chelates both isoforms of cyclooxygenases (COX1 and COX2), hence blocking the activity of phospholipase A2 and the rate-limiting cyclooxygenase enzyme step that converts arachidonic acid into precursors of prostaglandins. As a result, prostaglandin synthesis is inhibited. Piroxicam's overall anti-inflammatory qualities are enhanced by an additional, independent effect that inhibits neutrophil activation.
Osteoarthritis, rheumatoid arthritis, bursitis, tendinitis, and ankylosing spondylitis (Bechterew's disease) are among the moderate-to-severe inflammatory illnesses that can be treated with piroxicam, a non-steroidal anti-inflammatory medicine (NSAID) produced from oxicam. It is also used to treat pain that originates from sources other than the musculoskeletal system, like post-operative discomfort and primary dysmenorrhea. PC is classified as a Class II drug by the biopharmaceuticals Drug Classification system, meaning that it has poor solubility and high permeability.

Materials And Method

- **Equipments:**

S. No.	Instruments	Manufacturer
1	UV/VIS Spectrophotometer,	Shimadzu, Japan
2	Digital Weighing balance, (CY220)	Shimadzu, Japan
3	RP-HPLC instrument equipped with PDA detector	Shimadzu, Japan
4	Ultrasonicator	PCi analytics, India
5	Vortex mixer	Remi Scientific Instruments, Mumbai
6	Hot air oven	P. L. Tandon & Co, Delhi
7	Melting Point Apparatus	Remi Scientific Instruments, Mumbai
8	Infrared red spectrophotometer (FTIR)	Bruker Alpha, Berlin, Germany
9	Microcentrifuge	Remi Scientific Instruments, Mumbai
10	Vacuum pump	Suguna single phase, Chennai, India
11.	Nylon 0.22 μm membrane filter	Pall corporation, Mumbai

- **Materials:**

S. No	Materials	Source
1	Piroxicam	UniChem Laboratories Ltd.
2	Tramadol	Sun Pharma, India
3	Methanol	Fisher Scientific India Pvt. Ltd.
4	Orthophosphoric acid HPLC grade	Merck, Mumbai
5	Acetonitrile HPLC grade	Merck, Mumbai

Preparation Of Stock Solution of Tramadol & Piroxicam

1. In the HPLC system, Diluent was filtered through 0.22 µ Millipore membrane filters
2. **Standard solution preparation:** In a 10 ml volumetric flask, an accurately weighed quantity of about 10 mg of Tramadol and 10 mg of Piroxicam were added. They were then mixed in 10 ml of diluents (as previously mentioned) to produce a stock solution of 1000 µg/ml, which was then sonicated to dissolve.
3. **Preparation of calibration curve of Pravastatin sodium & Fenofibrate:** To create a working standard solution with concentration ranges of 15µg/ml to 90µg/ml, 1.5 ml, 3 ml, 4.5 ml, 6 ml, 7.5 ml, and 9 ml were pipetted out of the standard stock solution into a 10 ml volumetric flask. A 10 ml dilution was then made with mobile phase and filtered through 0.22 µ Millipore membrane filters before being injected into an HPLC system.

Validation Of Method

Using an HPLC and a UV spectrophotometer, the approach was verified in accordance with the ICH guideline. The parameters that were examined were linearity, accuracy, precision, limit of detection, limit of quantification, robustness.

Linearity and Range

The capacity of an analytical method to yield test findings that are exactly proportionate to the analyte concentration in the sample within a specified range is known as its linearity. The interval between the highest and lower levels of analyte that have been shown to be determined within an appropriate degree of precision, accuracy, and linearity is known as the analytical method's range. For Tramadol and Piroxicam, the chosen linearity range was 1–10 µg/ml. After being filtered using a 0.22 µ filter, each dilution was then injected.

Accuracy

The method's accuracy was assessed using the standard recovery percentage. Analysis was done at three different concentration levels: 50%, 100%, and 150%.

Precision

The drug's concentration was determined both inside and between days using a single injection on the same day. The repeatability of the injection was assessed using a 60g/ml concentration and two injections, and the percentage RSD was computed.

- Repeatability
- Inter-day

- Intraday

Limit of Detection (LOD) and Limit of quantification (LOQ)

In accordance with ICH recommendations, the developed method's LOD and LOQ were examined. There are various methods available for figuring out the LOD and LOQ, depending on whether an instrumental or non-instrumental procedure is used. One of the techniques used here was,

LOD= 3.3σ/S and

LOQ= 10σ/S

Where, σ = the standard deviation of intercept

S = mean of slope in calibration curve.

Robustness

By examining the sample with a lower concentration and purposefully changing the procedure parameters, the resilience was investigated. The percentage RSD was used to indicate how the responses of the medicines changed. The method's robustness was examined using wavelength and flow rate variations.

Results and Discussion

- **HPLC Method:**

Determination of chromatogram of Blank & Standard (Tramadol & Piroxicam):

The chromatogram on HPLC analysis of the Blank and standard solution of Piroxicam (60µg/ml) and Tramadol was optimized and evaluated in accordance with the suggested procedure. The HPLC analysis of the standard and blank chromatograms is displayed in Figures: 1.1 & 1.2

Figure 1.1: Chromatogram of blank

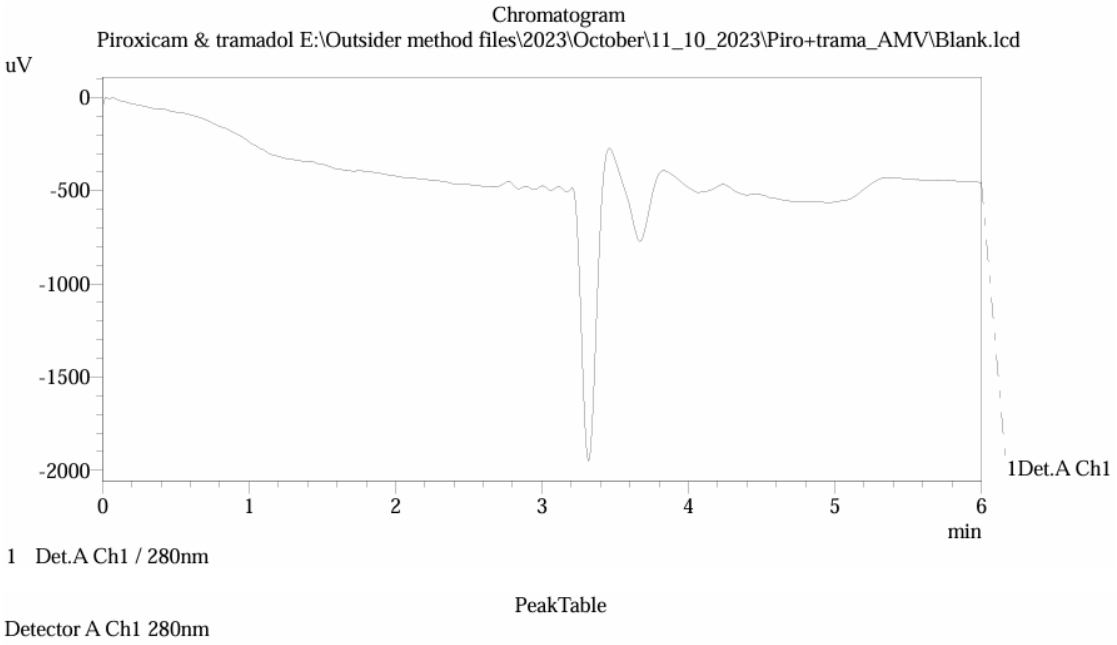
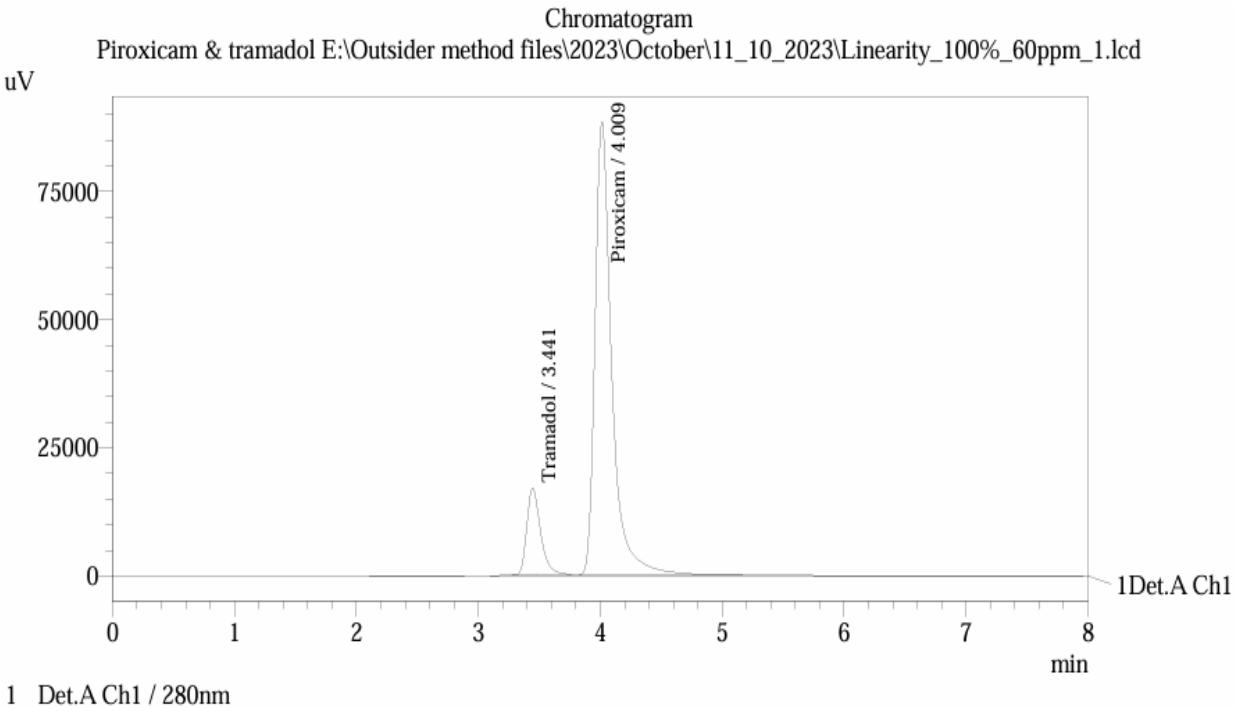


Figure 1.2: Simultaneous chromatogram of standard Piroxicam and Tramadol



- **Linearity of Piroxicam**

For Piroxicam, a calibration curve was drawn across a concentration range of (15 µg/ml, 30 µg/ml, 45 µg/ml, 60 µg/ml, 75 µg/ml, and 90 µg/ml). were precisely measured, and each dilution was filtered through a 0.22 µ filter before being injected.

Conc.	Linearity 1	Linearity 2	Linearity 3
15	217074	218886	208414
30	425738	413575	410148
45	646719	632061	633487
60	841674	863119	860779
75	1072959	1067608	1066674
90	1281508	1271847	1273158

Table 1.3: Linearity of Piroxicam

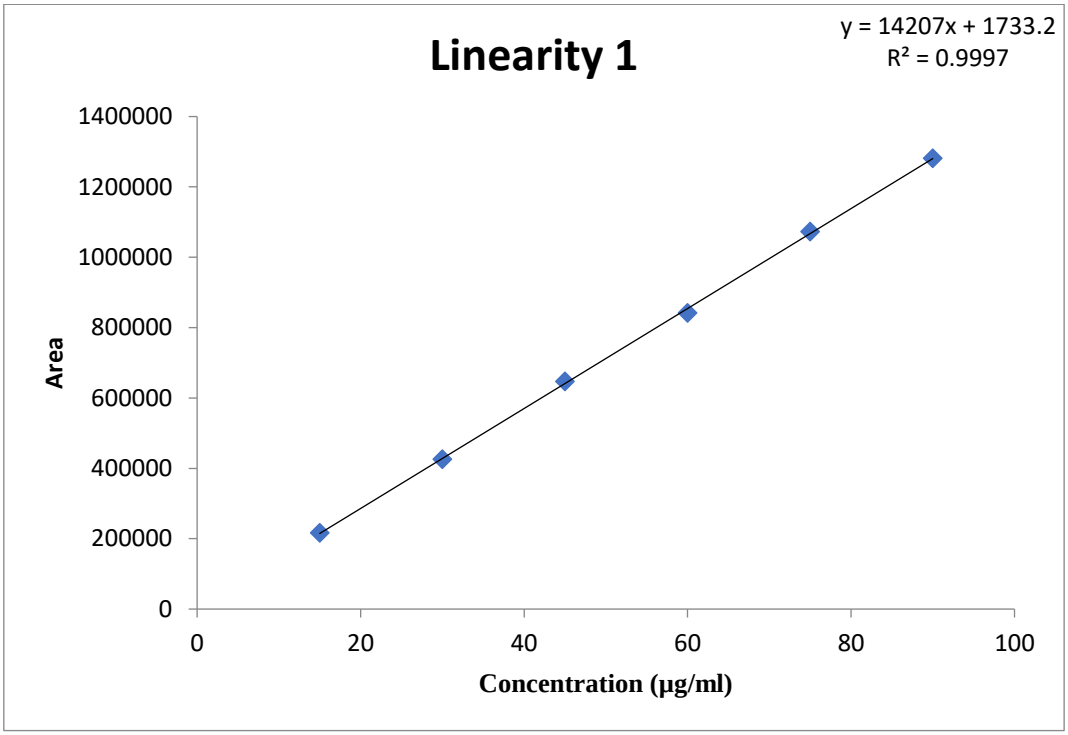


Figure 1.3.1: Linearity 1 Graph of Piroxicam

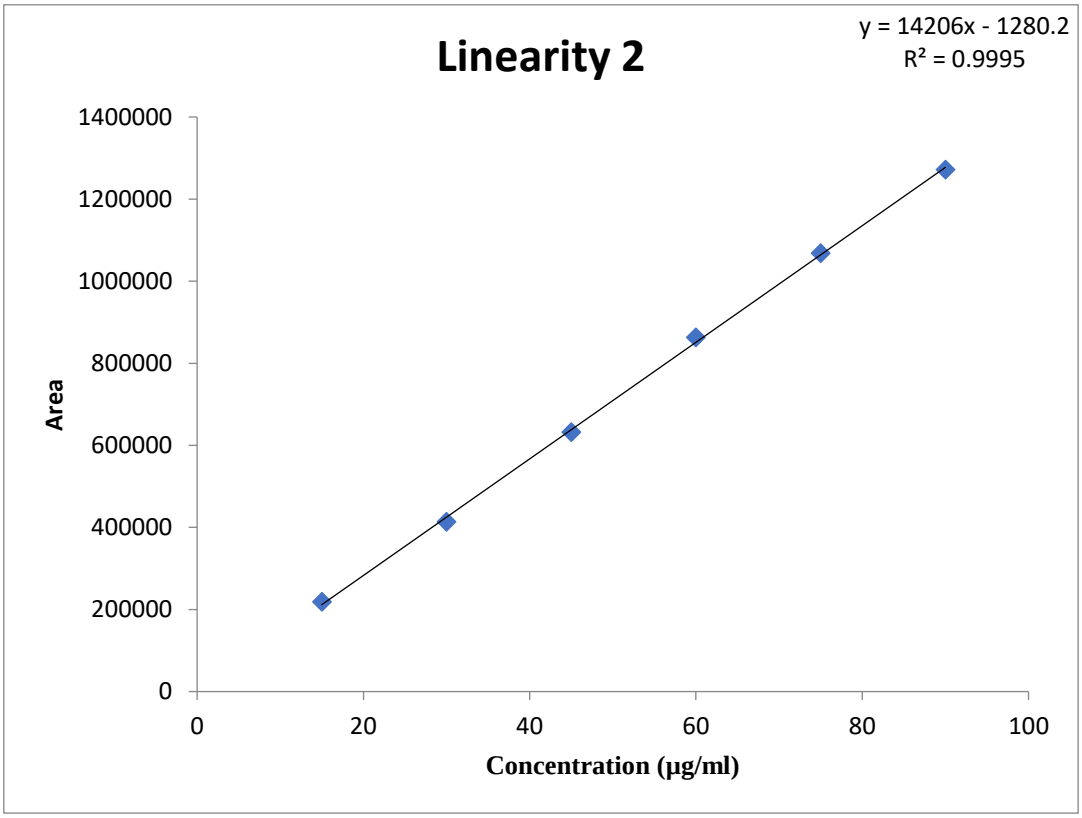


Figure 1.3.2: Linearity 2 Graph of Piroxicam

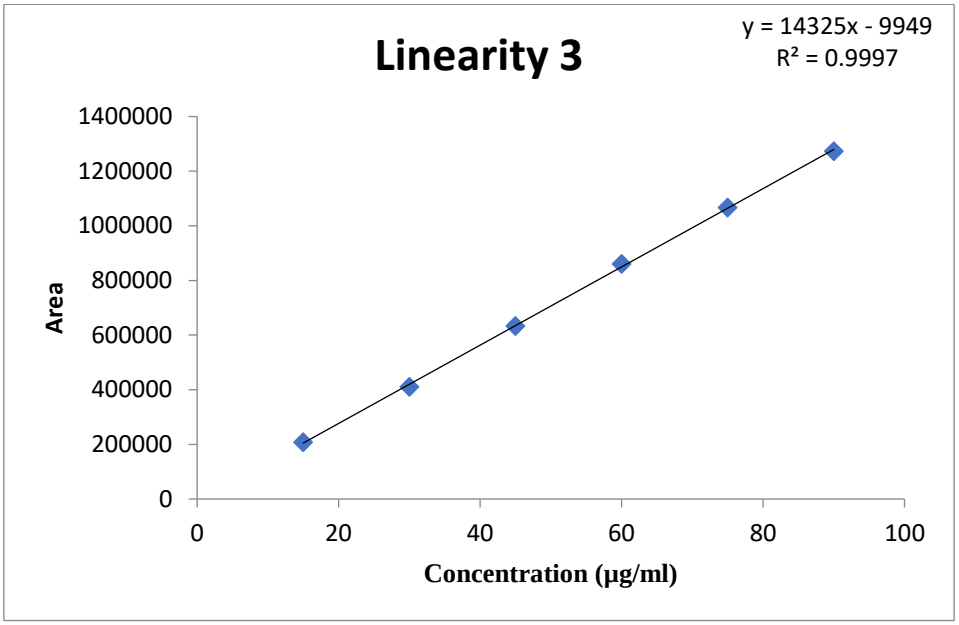


Figure 1.3.3: Linearity 3 Graph of Piroxicam

- **Linearity of Tramadol**

For Tramadol, a calibration curve was drawn across a concentration range of (15 µg/ml, 30 µg/ml, 45 µg/ml, 60 µg/ml, 75 µg/ml, and 90 µg/ml). were precisely measured, and each dilution was filtered through a 0.22 µ filter before being injected.

Table 1.4: Linearity of Tramadol

Conc.	Linearity 1	Linearity 2	Linearity 3
15	31572	34101	34284
30	66083	62823	62014
45	99876	98085	98228
60	130513	131384	131755
75	165865	163589	163005
90	192106	197128	197021

Figure 1.4.1: Linearity 1 graph of Tramadol

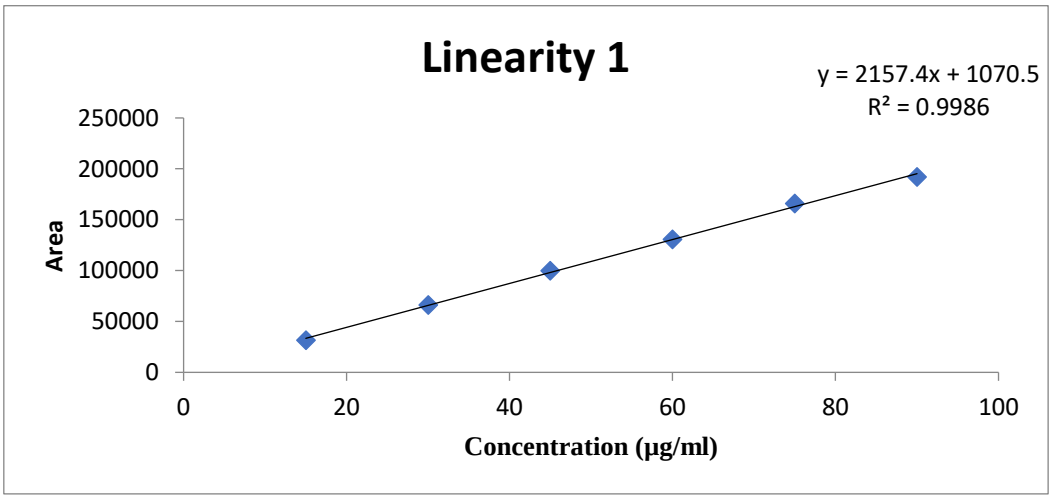


Figure 1.4.2: Linearity 2 graph of Tramadol

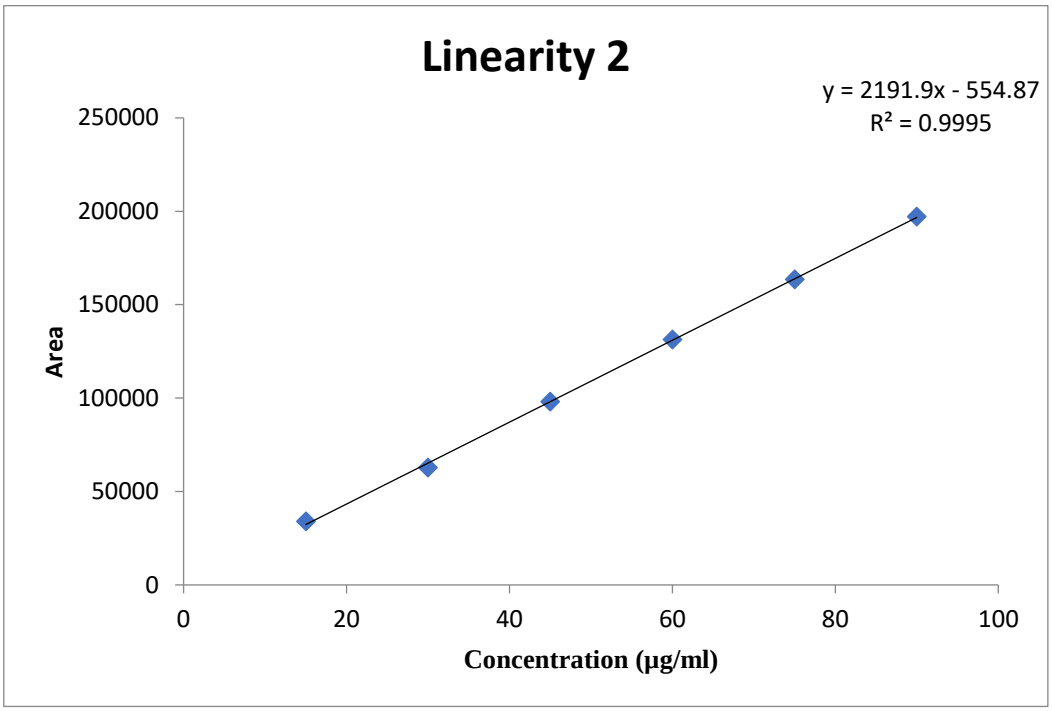
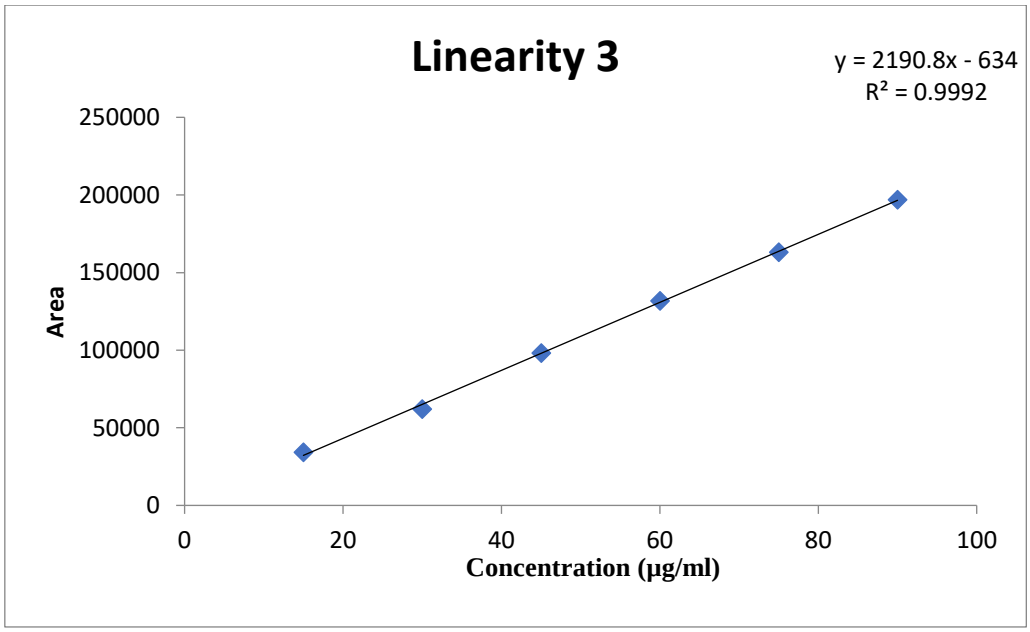


Figure 1.4.3: Linearity 3 graph of Tramadol



- **Accuracy**

The method's accuracy was assessed using the standard recovery percentage. Analysis was done at three different concentration levels: 50%, 100%, and 150%.

Table 1.5: 50% Accuracy Study of Piroxicam

Accuracy				
	Conc	Area	Amount Recovered (µg/ml)	% Recovery
50%	30	427162	30.21	100.69
	30	430498	30.44	101.47
	30	428830	30.32	101.08
Mean		428830		
SD		1668.00		0.55
% RSD		0.39		0.55

Table 1.6: 100% Accuracy Study of Piroxicam

Accuracy				
	Conc	Area	Amount Recovered (µg/ml)	% Recovery
100%	60	844280	59.49	99.14
	60	844709	59.52	99.19
	60	844709.00	59.52	99.19
Mean		844566		99.18
SD		247.68		0.03
% RSD		0.03		0.03

Table 1.7:150% Accuracy Study of Piroxicam

Accuracy				
	Conc	Area	Amount Recovered (µg/ml)	% Recovery
150%	90	1279741	90.05	100.06
	90	1279475	90.04	100.04
	90	1279608	90.04	100.05
Mean		1279608		100.05
SD		133.00		0.01
% RSD		0.01		0.01

Table 1.8: 50% Accuracy Study of Tramadol

Accuracy				
	Conc	Area	Amount Recovered (µg/ml	% Recovery
50%	30	65808	30.21	100.68
	30	65118	29.89	99.63
	30	65463	30.05	100.16
Mean		65463		100.16
SD		345.00		0.53
% RSD		0.53		0.53

Table 1.9: 100% Accuracy Study of Tramadol

Accuracy				
	Conc	Area	Amount Recovered (µg/ml)	% Recovery
100%	60	128285	58.86	98.11
	60	129496	59.42	99.03
	60	128890.00	59.14	98.57
Mean		128890		99
SD		605.50		0.46
% RSD		0.47		0.47

Table 1.10: 150% Accuracy Study of Tramadol

Accuracy				
	Conc	Area	Amount Recovered (µg/ml)	% Recovery
150%	90	197405	90.57	100.63
	90	195781	89.83	99.81
Mean	90	196593.00	90.20	100.22
		196593		100
SD		812.00		0.41
% RSD		0.41		0.41

The results indicate that the recoveries are well within the acceptance range of **98% – 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 Piroxicam and Tramadol (60 μg/ml) was prepared and analyzed as per the proposed method.

Table 1.11: Inter-day Precision study of Tramadol

	Inter-day Precision		
Conc.	Area	Amount Recovered (µg/ml)	% recovery
60	128571	59.00	98.33
60	128244	58.85	98.08
60	128047	58.76	97.93
60	129644	59.49	99.15
60	129001	59.19	98.65
60	129314	59.34	98.89
Mean	128804		98.50
SD	638.89		0.49
% RSD	0.50		0.50

Table 1.12: Repeatability Precision study of Tramadol

Conc.	Repeatability Precision		
	Area	Amount Recovered (µg/ml)	% recovery
60	128577	59.00	98.33
60	127014	58.28	97.14
60	128224	58.84	98.06
60	127989	58.73	97.88
60	128662	59.04	98.40
60	129547	59.44	99.07
Mean	128336		98.15
SD	661.48		0.51
% RSD	0.52		0.52

Table 1.13: Intra-day Precision study of Tramadol

Conc.	intra-day Precision		
	Area	Amount Recovered (µg/ml)	% recovery
60	129143	59.26	98.76
60	130114	59.70	99.51
60	129334	59.35	98.91
60	130057	59.68	99.46
60	129633	59.48	99.14
60	130247	59.76	99.61
Mean	129755		99.23
SD	429.51		0.33
% RSD	0.33		0.33

Table 1.14: Inter-day Precision study of Piroxicam

Conc.	Inter-day Precision		
	Area	Amount Recovered (µg/ml)	% recovery
60	849282	59.84	99.73
60	848487	59.78	99.64
60	840572	59.23	98.71
60	840075	59.19	98.65
60	850195	59.90	99.84
60	856414	60.34	100.56
Mean	847504		99.52
SD	4968.34		0.58
% RSD	0.59		0.58

Table 1.15: Repeatability Precision study of Piroxicam

Conc.	Repeatability Precision		
	Area	Amount Recovered (µg/ml)	% recovery

60	840174	59.20	98.66
60	846187	59.62	99.37
60	830479	58.52	97.53
60	842272	59.35	98.91
60	844648	59.51	99.19
60	843587	59.44	99.06
Mean	841225		98.79
SD	6182.58		0.723
% RSD	0.735		0.732

Table 1.16: Intra-day Precision study of Piroxicam

	Intra-day Precision		
Conc.	Area	Amount Recovered (µg/ml)	% recovery
60	844572	59.51	99.18
60	834597	58.81	98.01
60	841847	59.32	98.86
60	826487	58.24	97.06
60	834879	58.83	98.04
60	833548	58.73	97.89
Mean	835988		98.17
SD	7075.56		0.828
% RSD	0.846		0.843

The method was found to be precise due to low values of the %RSD.

- **LOD and LOQ**

Table 1.17: LOD and LOQ data

Sr. no.	**Name**	**LOD (µg/ml)**	**LOQ (µg/ml)**
1	Piroxicam	1.13	3.43
2	Tramadol	0.42	1.27

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.

- **Robustness**

By examining the sample with a lower concentration and purposefully changing the procedure parameters, the resilience was investigated. The percentage RSD was used to indicate how the responses of the medicines changed. The method's robustness was examined by varying the wavelength and flow rate.

Table 1.18: Robustness data of Piroxicam with deliberate change in Flow rate

Robustness_0.9ml				Robustness_1.1ml		
Conc.	Area	Amount Recovered (µg/ml)	% recovery	Area	Amount Recovered (µg/ml)	% recovery
60	840171	59.20	98.66	842247	59.34	97.74
60	841178	59.27	98.78	840115	59.19	97.49
Mean	840674		98.72	841181		97.61
SD	712.06		0.08	1507.55		0.18
% RSD	0.08		0.08	0.18		0.18

Table 1.19: Robustness data of Piroxicam with deliberate change in Wavelength

Robustness_275nm				Robustness_285nm		
Conc.	Area	Amount Recovered (µg/ml)	% Recovery	Area	Amount Recovered (µg/ml)	% recovery
60	840478	59.22	98.70	851875	60.02	100.03
60	842219	59.34	98.90	850047	59.89	99.82
Mean	841349		98.80	850961		99.93
SD	1231.07		0.14	1292.59		0.15
% RSD	0.15		0.15	0.15		0.15

Table 1.20: Robustness data of Tramadol with deliberate change in Flow rate

Robustness_0.9ml				Robustness_1.1ml		
Conc.	Area	Amount Recovered (µg/ml)	% recovery	Area	Amount Recovered (µg/ml)	% recovery
60	129018	59.20	98.67	130157	59.72	99.54
60	129175	59.27	98.79	129150	59.26	98.77
Mean	129097		98.73	129653		99.15
SD	111.02		0.08	712.06		0.54
%RSD	0.09		0.09	0.55		0.55

Table 1.21: Robustness data of Tramadol with deliberate change in wavelength

Robustness_275nm				Robustness_285nm		
Conc.	Area	Amount Recovered (µg/ml)	% recovery	Area	Amount Recovered (µg/ml)	% recovery
60	128241	58.84	98.07	128663	59.04	98.40
60	129459	59.40	99.00	129026	59.20	98.67
Mean	128850		98.54	128845		98.54
SD	861.26		0.66	256.68		0.20

%RSD	0.67		0.67	0.20		0.20

There shouldn't be a percentage RSD more than 2. It was discovered that the %RSD for the wavelength and flow rate change was less than 2, falling inside the acceptable range. Thus, the approach was reliable.

- **Force degradation**

Table 1.22: Force degradation study of Piroxicam

Nature of stress	Storage conditions	Time (hr)	Amount of piroxicam remaining (%)	Amount of piroxicam degradation (%)
2M HCl	40°C	5.00	96.05	3.95
2M NaOH	40°C	5.00	92.15	7.85
3% H_2O_2	ambient	0.5	82.84	17.16
Photolytic	UV Cabinet	0.5	76.28	23.72
Thermal	105°C	2.00	64.07	35.93

Table 1.23: Force degradation study of Tramadol

Nature of stress	Storage conditions	Time (hr)	Amount of Tramadol remaining (%)	Amount of Tramadol degradation (%)
2M HCl	40°C	5.00	98.60	1.4
2M NaOH	40°C	5.00	95.23	4.77
3% H_2O_2	ambient	0.5	98.72	1.28
Photolytic	UV Cabinet	0.5	99.27	0.73
Thermal	105°C	2.00	98.84	1.16

In tramadol there is no degradation in any conditions but piroxicam drug was found to be degrade under acidic condition. The Degradation reaction was more intense and quicker in alkaline condition. The greatest degradation of piroxicam were occurred in UV and thermal stress condition.

Conclusion:

Improving analytical method development and validation of the developed method as per the ICH guidelines. It was determined that the new RP-HPLC method that was developed for the quantitative determination of piroxicam and tramadol in bulk was speedy, accurate, sensitive, straightforward, and exact. The approach was shown to be better than the majority of the documented approaches.

Preformulation investigations were conducted to characterise the chemical and physical properties of the drug substance prior to validation and method development. Tramadol and Piroxicam were determined to have organoleptic qualities in accordance with the I.P. monograph. The capillary method, which complies with the melting point provided in reference, was used to determine the melting points of both medications. The pure drug ranges for the melting points of Tramadol and Piroxicam were determined to be 179–182°C and 197–200°C, respectively.

The reference chemical groups found in the Tramadol & Piroxicam structure were determined to be consistent with the FT-IR spectra of the medication samples. The methanol standard curves for piroxicam and tramadol were produced, and linear regression was used to the resulting absorbance data. Tramadol

and Piroxicam in methanol showed a wide band at 272 nm and 330 nm in their UV spectra. The isosbestic point at 280 nm is visible in the overlapped spectra of piroxicam and tramadol.

The results showed that the correlation coefficients for piroxicam and tramadol were 0.9999 and 0.9997, respectively, which are close to one, indicating strong linearity.

Tramadol and Piroxicam were found to be pure and of good quality, according to the results of the preformulation research (FT-IR spectrum, UV spectrum, and melting point). The estimation process was also found to be highly accurate and dependable, making it appropriate for method development or validation. The ICH guidelines served as the basis for the method validation of both substances. The wavelength used for the HPLC method of validation was chosen to correspond to the isosbestic point at which UV detectors can detect the two medicines. The wavelength that was chosen was 280 nm. After numerous experiments, the ideal liquid chromatographic conditions for the separation of Tramadol and Piroxicam were achieved by choosing the right mobile phase and column.

Force C18 Column (5 µm, 250 × 4.6 mm) with a flow rate of 1 mL/min was used to develop the method. The ideal mobile phase conditions were 72:28 v/v of gradient mode acetonitrile and diluted OPA (adjusted to pH 2.6). The data from Simultaneous indicates that the retention duration for Piroxicam was 5.313 and Tramadol was 4.695. For parameters that were validated, the technique was shown to be linear, accurate, robust, and robust. The linearity range of 15µg/ml to 90µg/ml for Tramadol and 15µg/ml to 90µg/ml for Piroxicam was established by the use of an external standard calibration method. Tramadol and Piroxicam recovery percentages were determined to be 98.11–100.68% and 99.14–101.47%, respectively, and all values were found to be within the acceptable ranges.

The repeatable analysis of the sample further supported the method's accuracy. Because the %RSD values were minimal, the results were judged to be precise. It suggested that the precision of the procedure is good. Tramadol and Piroxicam limit of quantification are 78.5µg/ml and 29.19µg/ml, respectively. Comparably, the limit of detection for piroxicam and tramadol is 1.13 µg/ml and 0.42 µg/ml, respectively. The robustness research revealed that the relative standard deviation (RSD) for varying the flow rate, wavelength, and analyst was found to be less than 2, falling within the acceptable range. Thus, straightforward, sensitive, accurate, and precise RP-HPLC techniques were created and verified for the simultaneous measurement of piroxicam and tramadol.

Tramadol and Piroxicam's acid hydrolysis degradation amount at 60°C for one hour was 0.65% and 0.03%, two hours was 1.15% and 1.00%, and four hours was 2.06% and 1.28%, respectively. Amount of Base hydrolysis degradation for Tramadol and Piroxicam at 60°C for 1 hour was 0.24% and 10.25%, for 2 hours it was 14.07% and 16.04%, and for 4 hours it was 18.04% and 19.42%. Tramadol and Piroxicam's oxide degradation percentage at 60°C for 1 hour was 11.28% and 10.72%, 2 hours was 15.18% and 13.60%, and 4 hours was 17.88% and 16.49%, respectively. Tramadol and Piroxicam UV Degradation Amounts: 1.48% and 2.05% for 1 hour, 4.08% and 5.49% for 2 hours, and 8.59% and 7.33% for 4 hours, respectively. Tramadol and Piroxicam's Thermal Degradation Amounts for One Hour: 2.92% and 2.60%; Two Hours: 5.63% and 4.08%; 4hr 7.29% and 7.71% respectively at 60°C.

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