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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE ESTIMATION OF NALTREXONE AND ACETAMINOPHEN IN SYNTHETIC MIXTURE

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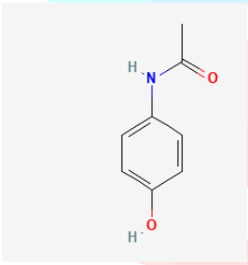
ABSTRACT: A simple, rapid, accurate, precise specific and sensitive reverse phase high performance liquid chromatographic method has developed and validated for the simultaneous estimation of Naltrexone (NAL) and Acetaminophen (ACT) in synthetic mixture. The chromatographic separation was performed using Cyber Lib C18 Column (250 mm × 4.6 mm, 5 µm), utilizing a Mobile phase Methanol: acetonitrile: Phosphate Buffer 50:30:20 v/v (pH adjusted to 5.8) at Flow rate of 1 ml/min, injection volume 20µl with UV detection at 230nm. The retention time of Acetaminophen (ACT) 4.20 min and Naltrexone (NAL) 5.95 min using RP-HPLC. The % RSD Value was found for the validation parameter that preciseness of the proposed method and is applicable for routine analysis for quantitative determination of NAL and ACT. The LOD was found for ACT 3.04 µg/ml, NAL 0.174 µg/ml for developed method respectively. The LOQ was found for ACT 10.16 µg/ml, NAL 0.58 µg/ml for developed method respectively. The result of analysis was validated according to ICH Q2 R1 Guidelines. This simple and precise method can be used of both drugs in quality control laboratories.

Keywords: Naltrexone (NAL), Acetaminophen (ACT), Migraine, revese phase high performace liquid chromatography, validation.

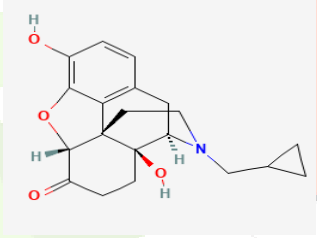
INTRODUCTION

Acetaminophen (paracetamol) has been widely endorsed as a first-line analgesic and is currently the most commonly used analgesic worldwide. According to its FDA labeling, acetaminophen's exact mechanism of action has not been fully established Label - despite this, it is often categorized alongside NSAIDs (nonsteroidal anti-inflammatory drugs) due to its ability to inhibit the cyclooxygenase (COX) pathways. It is thought to exert central actions which ultimately lead to the alleviation of pain symptoms. One theory is that acetaminophen increases the pain threshold by inhibiting two isoforms of cyclooxygenase, COX-1

and COX-2, which are involved in prostaglandin (PG) synthesis. Prostaglandins are responsible for eliciting pain sensations. Acetaminophen does not inhibit cyclooxygenase in peripheral tissues and, therefore, has no peripheral anti-inflammatory effects. Though acetylsalicylic acid (aspirin) is an irreversible inhibitor of COX and directly blocks the active site of this enzyme, studies have shown that acetaminophen (paracetamol) blocks COX indirectly. [4] Naltrexone is a pure opiate antagonist and has little or no agonist activity. The mechanism of action of naltrexone in alcoholism is not understood; however, involvement of the endogenous opioid system is suggested by preclinical data. Naltrexone is thought to act as a competitive antagonist at mc, κ, and δ receptors in the CNS, with the highest affinity for the μ receptor. Naltrexone competitively binds to such receptors and may block the effects of endogenous opioids. This leads to the anatonization of most of the subjective and objective effects of opiates, including respiratory depression, miosis, euphoria, and drug craving. The major metabolite of naltrexone, 6-β-naltrexol, is also an opiate antagonist and may contribute to the antagonistic activity of the drug. [5] Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. Pharmaceutical products formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods. [6,7] The most common analytical separation tool, High Performance Liquid Chromatography is a powerful tool in analysis, components by distributing between mobile phase (a flowing liquid) and stationary phase (liquid coated over solid support chemically bonded and tightly packed in a column). HPLC gives high sensitivity, high resolution, ready adaptability to accurate quantitative determinations and suitability for separating non-volatile species and thermally labile ones. ItS is used to determine and separate various species in a variety of organic, biological and inorganic materials by the chemists. [8-11]



ACETAMINOPHEN



NALTREXONE

MATERIALS AND METHODS: Sample of Naltrexone (NAL) procured from Alpha Chemika, Gujarat. Sample of Acetaminophen (ACT) procured from pure chem Pvt Ltd.

EXPERIMENTAL CONDITION:

Apparatus: HPLC manufactured by Cyber Lab having LC-100 model no. was used in these method developments. Cyber-Sil, C18 column (250mm × 4.6mm, 5µm) was used as an stationary phase. For Identification of api using UV Visible Spectrophotometer and FT-IR UV Visible Spectrophotometer is manufactured by Shimadzu having UV 1700 model no and FT-IR is manufactured by Agilent Technologies having Cary 630 model no.

Chemical: HPLC Grad Methanol, Acetonitrile and Water which is manufactured by Ranchem Ltd. AR Grad Potassium Dihydrogen Phosphate which is manufactured by Ranchem Ltd.

IDENTIFICATION OF API:

Melting point determination: Melting point of Acetaminophen (ACT) and Naltrexone (NAL) was carried outby capillary tube method in paraffin bath. The melting point study was performed in Thieles tube that was filled with liquid paraffin. 50 mg of powdered drug was filled in capillary that was attached with the tipof thermometer with the help of thread. Then thermometer was placed in Thiele's tubeand was heated. Temperature at which the drug powder melted was noted down. It wasperformed in triplicate.

Solubility Study: Solubility of Acetaminophen (ACT) and Naltrexone (NAL) was performed using various solvents like water, methanol, acetonitrile etc

Table 1: Solubility criteria as per pharmacopoeia

Descriptive term	Parts of solvent required for part of solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1000
Very slightly soluble	From 1000 to 10000
Practically Insoluble	10000 or more

UV Absorption Study: Accurately weighed 10 mg of Acetaminophen (ACT) and Naltrexone (NAL) were transferred separately in 10 ml volumetric flasks, dissolved in small volume of methanol and then volume was adjustedto the mark with methanol to obtain concentration of 1000 µg/ml. These solutions were further diluted to obtain concentration of 10 µg/ml. These standard solutions of Acetaminophen (ACT) and Naltrexone (NAL) in methanol were scanned in UV range, 200-400 nm in 1 cm cell using methanol as blank and maximum absorbance was measured for selection of λ_{max} of Acetaminophen (ACT) and Naltrexone (NAL).

IR Spectra: Drug was placed in sample compartment of FT-IR instrument, where it was scanned in the range of 4000 - 650 cm^{-1}. Principle IR peaks were observed for drug are shown in table and from this data it was concluded that drugs were foundto be authentic.

METHOD DEVELOPMENT AND VALIDATION:

Selection of Diluent: Based on solubility, Acetaminophen (ACT) and Naltrexone (NAL) was soluble in methanol. Hence, methanol was selected as diluent.

Preparation of Stock solution: Accurately weighed and transferred about 100 mg of Acetaminophen (ACT) and 10mg of Naltrexone (NAL) in to 100 ml of volumetric flask, 50 ml of methanol was added and sonicated to dissolve. Volume was making up to the mark with diluent. Concentration of Acetaminophen (ACT) is 1000 µg/ml and Naltrexone (NAL) 100 µg/ml. Further diluted 5 ml of above solution to 50 ml

volumetric flask and volume was make up to the mark with diluent. Concentration of Acetaminophen (ACT) is 100 µg/ml and Naltrexone (NAL) 10 µg/ml. The optimum wavelength was selected for the estimationwas 230 nm where gives maximum absorbance, which was obtained by scanningsolution in the range of 200-400 nm in UV spectrophotometer.

Selection of Mobile Phase: The mobile phase selection method took into account water, buffer, buffer pH, organic solvent, and buffer-to-solvent ratio. The nature, physicochemical qualities, molecular weight, and solubility of the sample all influence the HPLC procedure selection. The use of a buffer is required for pH regulation. The acidic component's pH is kept low, while the base component's pH is kept high. The mobile phase for the HPLC system was optimised using separation, peak purity, tailing factor, theoretical plate, and other parameters. To achieve a sharp peak of Acetaminophen (ACT) and Naltrexone, numerous mobile phases in varied compositions and pH levels were attempted (NAL).

Selection of Wavelength: An ideal wavelength is the one that gives Maximum response for the drugs that was to be detected. For selection of wavelength U.V spectrophotometer is used or using HPLC assisted with UV detector, UV overlay spectra Acetaminophen (ACT) and Naltrexone (NAL) were obtained. For High Performance Liquid Chromatography 230 nm was selected wavelength where both drug show good absorbance.

Preparation of Buffer: Dissolve 1.19 g of disodium hydrogen phosphate dihydrate R and 8.25 g of potassium dihydrogen phosphate R in water R and dilute to 1000.0 ml with the same solvent. Mix well and sonicate. Filter through 0.45 µm membrane filter paper.

Preparation of Mobile Phase: Prepare a mixture of Methanol: acetonitrile: Phosphate Buffer in the volume ratio 50:30:20 v/v/v. Adjust pH 5.8 with orthophosphoric acid. Filter and mix well and sonicate to degas the mixture.

Selection of column: Acetaminophen (ACT) and Naltrexone (NAL) are polar in nature. So, C18 analytical column were selected for HPLC method. The column was used Cybersil C18 column (250 mm × 4.6 mm, 5 µm) was used for the development of the method.

PREPARATION OF SOLUTION:

Preparation of Standard stock solution of Acetaminophen (ACT):

Weigh accurately 50 mg of Acetaminophen (ACT) in 50 ml volumetric flask. Add 50 ml of methanol and sonicate to dissolve. Dilute up to the mark with methanol and mix well. The prepared solution having 1000 ppm.

Preparation of Standard stock solution of Naltrexone (NAL):

Weigh accurately 50 mg Naltrexone (NAL) in 50 ml volumetric flask. Add 50 ml of methanol and sonicate to dissolve. Dilute up to the mark with methanol and mix well. The prepared solution having 1000 ppm.

Preparation of Standard stock solution of Acetaminophen (ACT) and Naltrexone (NAL):

Transfer 10 ml of Acetaminophen (ACT) from their standard stock solution and 1 ml of Naltrexone (NAL) from their standard stock solution in to 100 ml volumetric flask. Dilute up to the mark with methanol and mix well. The prepared solution has concentration 100ppm of Acetaminophen (ACT) and 10 ppm of Naltrexone (NAL).

METHOD VALIDATION:

The method was validated in accordance with ICH guidelines Q2R1 for evaluation of various parameters linearity, specificity, accuracy, precision, LOD, LOQ and robustness.

Linearity and Range:

Take 100 ml of ACT and 1ml of NAL were added in 10 ml of volumetric flask. Add 20 ml of Methanol and sonicate it for 5-7 min. After this dilute up to the mark with Methanol (1000ppm of ACT and 10ppm of NAL). Take 0.5, 1, 1.5, 2, 2.5, 3 ml above linearity solution to get series of concentration 50 – 300 ppm for ACT. 1 – 6 ppm of NAL. Dilute the solution were filtered through 0.45 µm membrane filters. Chromatograms for each of the above solution were recorded using the same chromatographic conditions as described above. Peak area was recorded and a plot of peak against respective concentration was plotted for ACT and NAL. The straight-line equations and correlation coefficients for ACT and NAL were determined. The linearity response was determined by analyzing 6 independent level of calibration curve in the range of 50 – 500 ppm (50, 100, 150, 200, 250, 300) for ACT and 1 – 6 ppm (1, 2, 3, 4, 5, 6) for NAL. The plot of mean area against concentration was plotted. Correlation coefficient and regression line equations for ACT and NAL were calculated.

Specificity:

A blank (mobile phase), placebo, standard solution of ACT and NAL spiked with API and test solution were injected and % interference was checked.

Accuracy:

The accuracy of method was determined by calculating recoveries of drug by standard addition method at three different level 50 %, 100 % and 150 % of standards to pre-quantified sample solution of synthetic mixture. For ACT 100µg/ml and 1 µg/ml NAL were spiked to pre-quantified sample solution of synthetic mixture for ACT 50µg/ml and 0.5 µg/ml NAL at 50 %, level. For ACT 100µg/ml and 1 µg/ml NAL were spiked to pre-quantified sample solution of synthetic mixture for ACT 100µg/ml and 1 µg/ml NAL at 100 %, level. For ACT 100µg/ml and 1 µg/ml NAL were spiked to pre-quantified sample solution of synthetic mixture for ACT 150µg/ml and 1.5 µg/ml NAL at 150 %, level.

Precision:

Repeatability:

Repeatability was determined by analyzing solution containing mixture of ACT and NAL having concentration of 100ppm and 2ppm respectively. Peak area of same concentration was measured six times and % RSD was calculated.

Intraday Precision:

Take 50, 150 and 300ppm of ACT and 1, 3 and 6 ppm NAL respectively as a mixture of two drugs. And sample were analyze at different time intervals in a day for 0 hr, 3 hr and 6 hr (Morning, Afternoon and Evening) and RSD was calculated.

Interday Precision:

Take 50, 150 and 300ppm of ACT and 1, 3 and 6 ppm NAL respectively as a mixture of two drugs. And sample was analyzed at three different consecutive days (Day 1, Day 2 and Day 3) and RSD was calculated.

LOD and LOQ:

The LOD was estimated from the ser of 3 calibration curves. The LOD was calculated as, LOD = 3.3 * (SD / Slope)

Where, SD = Standard deviation of the Y – intercepts of the 3 calibration curves.

Slope = Mean slope of the 3 calibration curves

The LOQ was estimated from the set of 3 calibration curve used to determine method linearity. The LOQ was calculated as,

LOQ = 10 * (SD / Slope)

Where, SD = Standard deviation of the Y – intercepts of the 3 calibration curves.

Slope = Mean slope of the 3 calibration curves

Robustness:

As defined by the ICH, the robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variations in method parameters here changes in different conditions were considered:

1) Change in Flow rate (0.9 ml/min ± 0.1)

2) Change in Wavelength (251 nm ± 4)

ASSAY OF SYNTHETIC MIXTURE

To determine the concentration of Acetaminophen (ACT) and Naltrexone (NAL) in synthetic mixture 325 mg Acetaminophen (ACT) and 3.25 mg Naltrexone (NAL) accurately weigh and transfer into a 100 ml volumetric flask and add common excipients used in marketed formulations. Add 70 % of diluent and sonicate. Dilute upto the mark with diluent and mix well. Filter the solution through 0.45 µm membrane filter, discarding first few ml of the filtrate. Dilute 1ml of filtrate solution to 10ml with diluents and mix well. Final concentration of Acetaminophen (ACT) 162.5 ppm and Naltrexone (NAL) was 1.625ppm which was analysed for assay.

RESULTS AND DISCUSSION:

Identification of API:

Melting Point Study: The observed melting point of each mentioned drugs were similar to the standard melting point reported for respective drugs as evident from Table 2.

Table 2 Melting Point Study

Drugs	Reported Melting Point (°C)	Observed Melting Point (°C)
Acetaminophen (ACT)	168-170 °C [5]	165-168 °C
Naltrexone (NAL)	168-170 °C [15]	165-168 °C

N = 3, Mean of 3 replicates

Solubility Study:

The solubility of substance fundamentally depends on the physical and chemical properties of the solute and solvent as well as temperature, pressure and the pH of the solution. The solubility profile is used for solvent selection in method development. The solubility of each drug in different solvent in shown in Table 3.

Table 3. Solubility Study

Drugs	Acetaminophen (ACT)	Naltrexone (NAL)
Water	Slightly soluble	Insoluble
Ethanol, Methanol	Very Soluble	Freely Soluble
Acetonitrile	Soluble	Slightly soluble

UV Absorption Study:

UV spectra of drugs in methanol depicted that the wavelength maxima of Acetaminophen (ACT) and Naltrexone (NAL) were at 243 nm and 210 nm respectively as shown in Figure 1. For High Performance Liquid Chromatography both drug Acetaminophen (ACT) and Naltrexone (NAL) show good absorbance at 230 nm, so it was selected as detection wavelength.

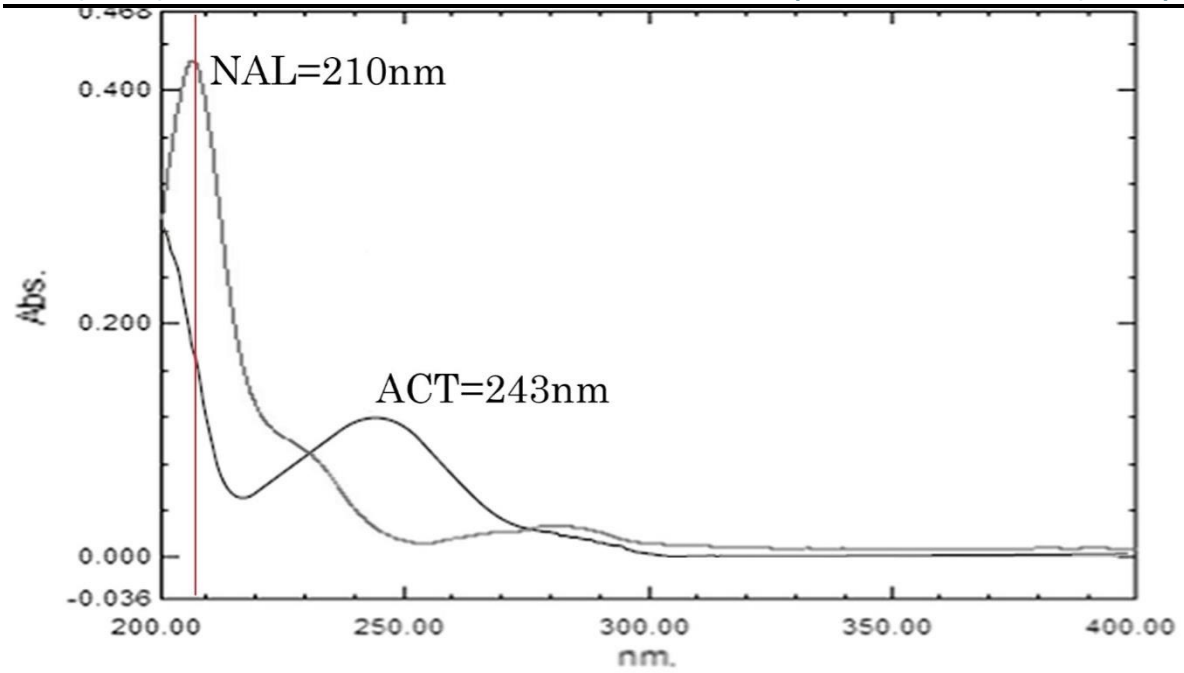


Figure 1 Overlain UV Spectrum in methanol

IR Spectra:

An IR spectrum of reference sample shown in figure 2 and figure 3 observed frequencies was within the standard frequency range. So, concluded that given sample content was Acetaminophen (ACT) and Naltrexone (NAL) results are shown in table 4 and 5.

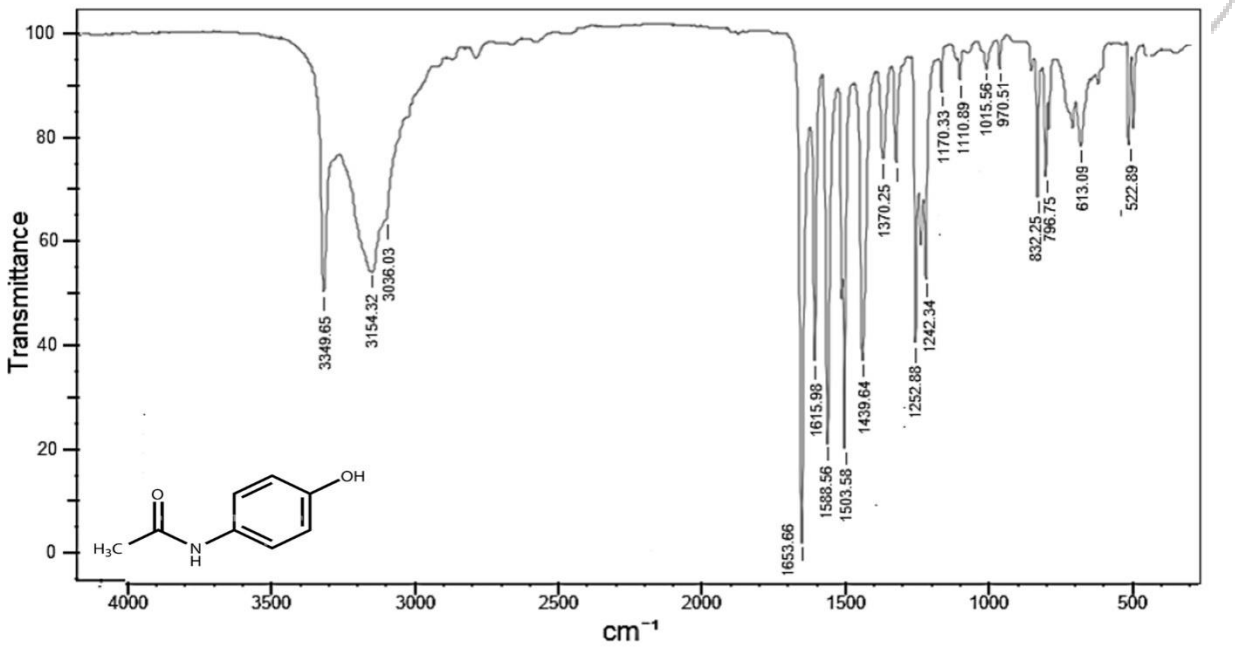


Figure 2 IR Spectrum of Acetaminophen (ACT)

Table 4 IR value for Acetaminophen (ACT)

Sr. No.	FunctionalGroup	Reported Wavenumber (cm^{-1})	Observed Wavenumber (cm^{-1})
1.	N-H	3400-3300	3349.65
2.	O-H stretching alcohol	3200-2700	3154.32
3.	C=O	1685-1630	1653.66
4.	C-H	1465-1450	1439.49
5.	C=C	895-885	835

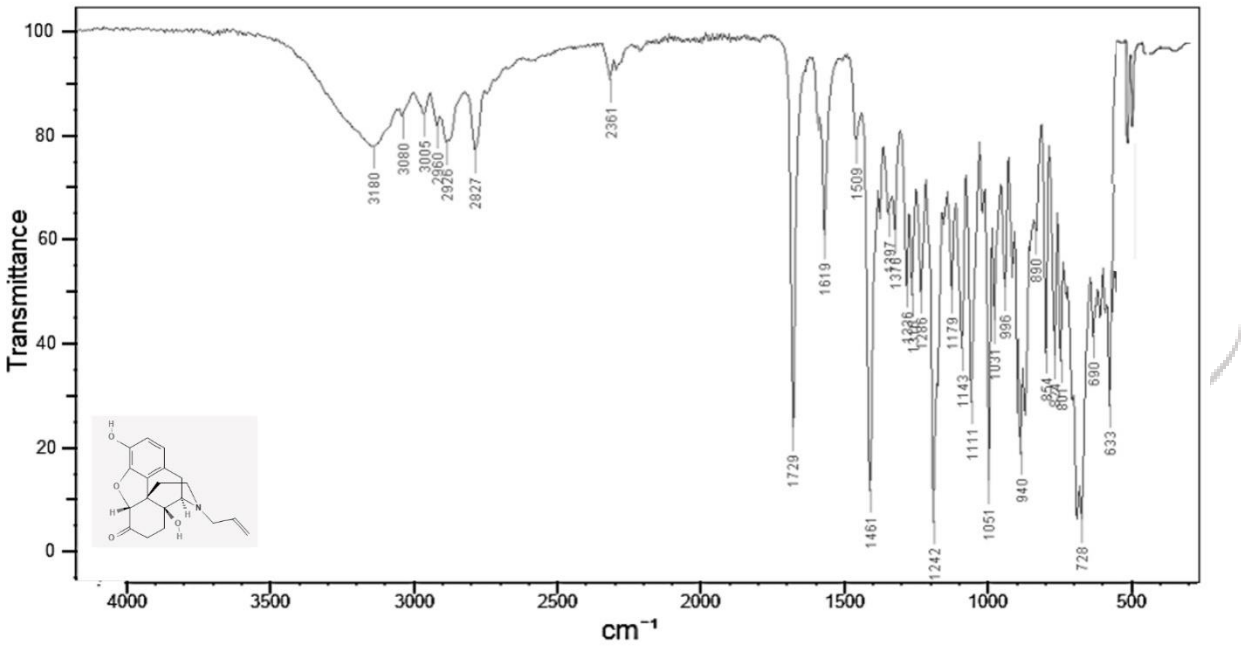


Figure 3 IR Spectrum of and Naltrexone (NAL)

Table 5 IR Value for Naltrexone (NAL)

Sr. No.	FunctionalGroup	Reported Wave number (cm^{-1})	Observed Wave number (cm^{-1})
1.	O-H stretching alcohol	3200-2700	3180
2.	C=O	1725-1705	1729
3.	N-H	3000-2800	2935.4
4.	C=C	1600-1475	1481
5.	C-O alkyl aryl ether	1275-1200	1242

CONCLUSION:

From the obtained results of identification study, it can be interpreted that the Acetaminophen (ACT) and Naltrexone (NAL) are pure and authentic.

Optimization of Mobile Phase Composition:

Based on review of literature, several mobile phases were selected on the basis of solubility of drug in the solvents. Various solvents and mixtures of solvents was tried using methanol, acetonitrile, HPLC grade water and phosphate buffer of different pH and their combinations. And the best result was obtained by using mixture Acetonitrile: Methanol: Phosphate (50:30:20 % V/V/V) 5.8 pH of buffer (Trial 6) having good peak shape and resolution of greater than 2 as well as theoretical plate more than 2000. Flow rate was 1 ml/min monitor at 225nm. Stationary phase was cyber lib C 18 (250 mm x 4.6 mm),5 µm and Injection Volume: 20 µl. So, optimization of mobile phase for HPLC method includes various trials as summarized in Table 6

Table 0 Method Development Trials

Trial No.	Condition	Observation
1	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Acetonitrile: Water (50:50 V/V) Flow Rate: 1 ml/min Wavelength: 225 nm Injection Volume: 20 µl	Peak shape was not proper .
2	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Water: Methanol: (50:50 % V/V) Flow Rate: 1 ml/min Wavelength: 225 nm Injection Volume: 20 µl	No retention of drug, only one peak observed.
3	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Acetonitrile: Methanol: Water: (60:20:20 %V/V/V) Flow Rate: 1 ml/min Wavelength: 225 nm Injection Volume: 20 µl	Peak shape was not proper and peak tailing observed
4	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Acetonitrile: Methanol: Water: (40:30:30 %V/V/V) Flow Rate: 1 ml/min Wavelength: 225 nm Injection Volume: 20 µl	Peak shape was not proper.

5	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Acetonitrile: Methanol: Phosphate (50:30:20 %V/V/V) 4 pH of buffer Flow Rate: 1 ml/min Wavelength: 225 nm Injection Volume: 20 µl	Retention time of Drug is less than 10minutes and peak shape was proper but resolution not good
6	Column: C 18 (250 mm x 4.6 mm),5 µm Mobile Phase: Acetonitrile: Methanol: Phosphate (50:30:20 %V/V/V) 5.8 pH of buffer Flow Rate: 1 ml/min Wavelength: 225 nm Injection Volume: 20 µl	Shorter retention time of Drug and peak shape was proper, resolution good

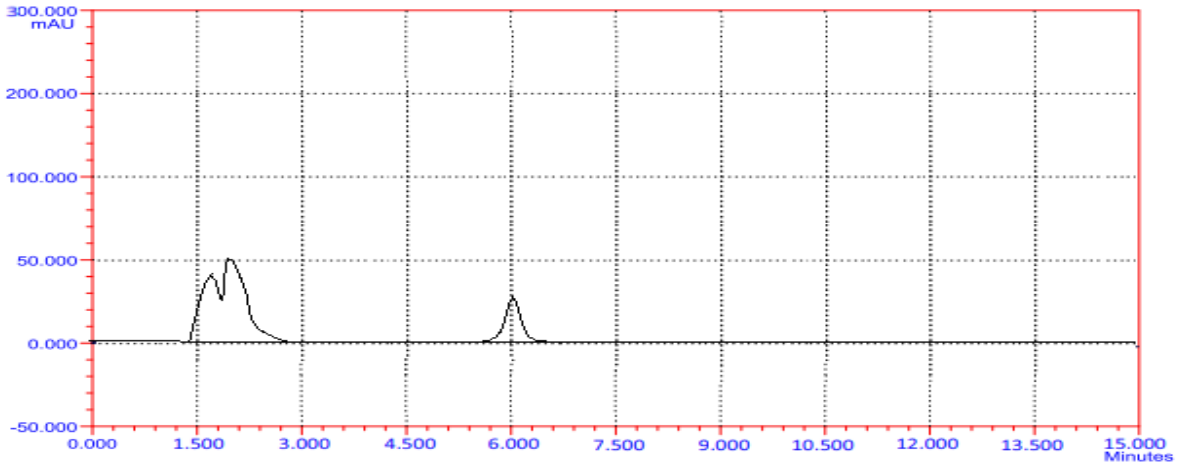


Figure 0:4 *Trail 1:* HPLC Chromatogram: Mobile Phase: Acetonitrile: Water (50:50 V/V)

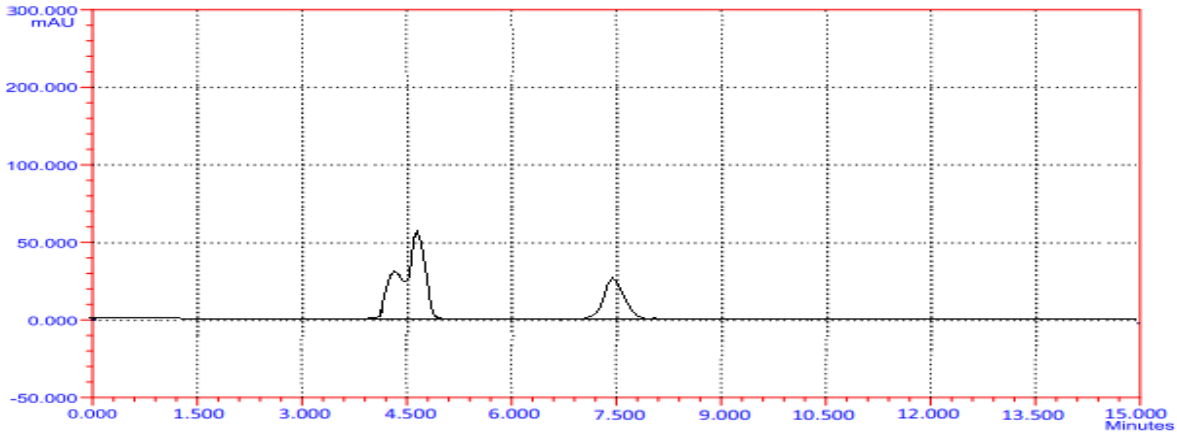


Figure 0:5 *Trail 2:* HPLC Chromatogram Mobile Phase: Water: Methanol: (50:50 % V/V)

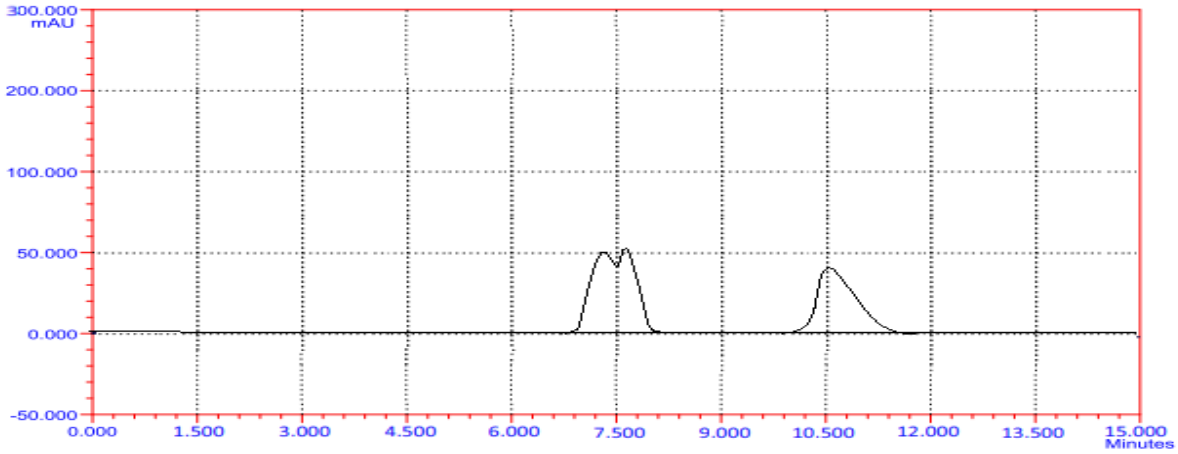


Figure 0:6*Trail 3* HPLC Chromatogram: Mobile Phase: Acetonitrile: Methanol: Water: (60:20:20 % V/V/V)

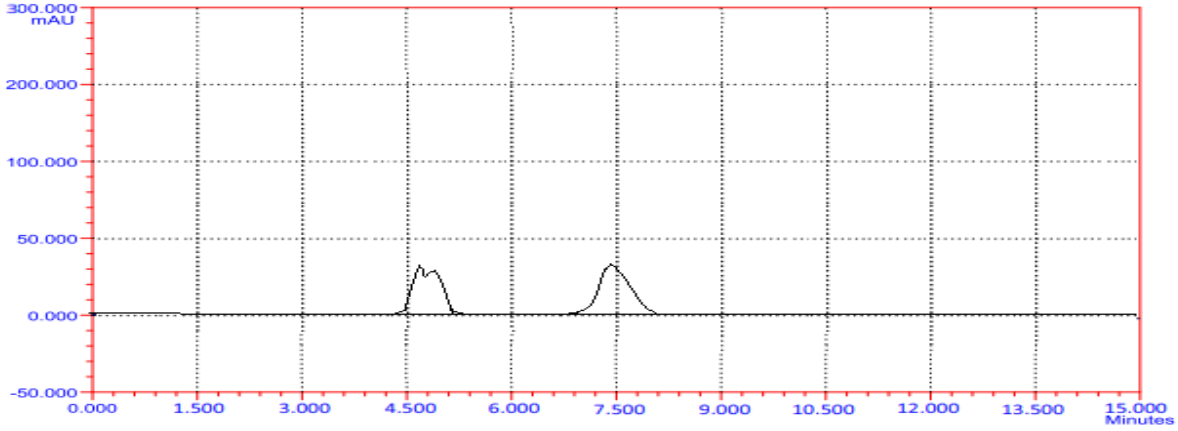


Figure 0:7 *Trail 4:* HPLC Chromatogram: Mobile Phase: Acetonitrile: Methanol: Water(40:30:30%V/V/V)

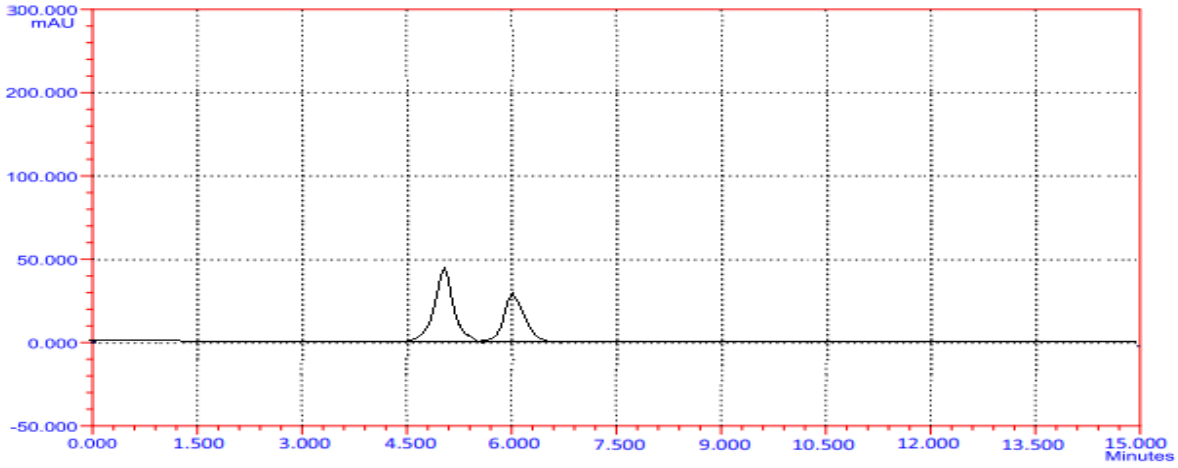


Figure 0:8 *Trail 5:* HPLC Chromatogram: Mobile Phase: Acetonitrile: Methanol: Phosphate (50:30:20 % V/V/V) 4 pH of buffer

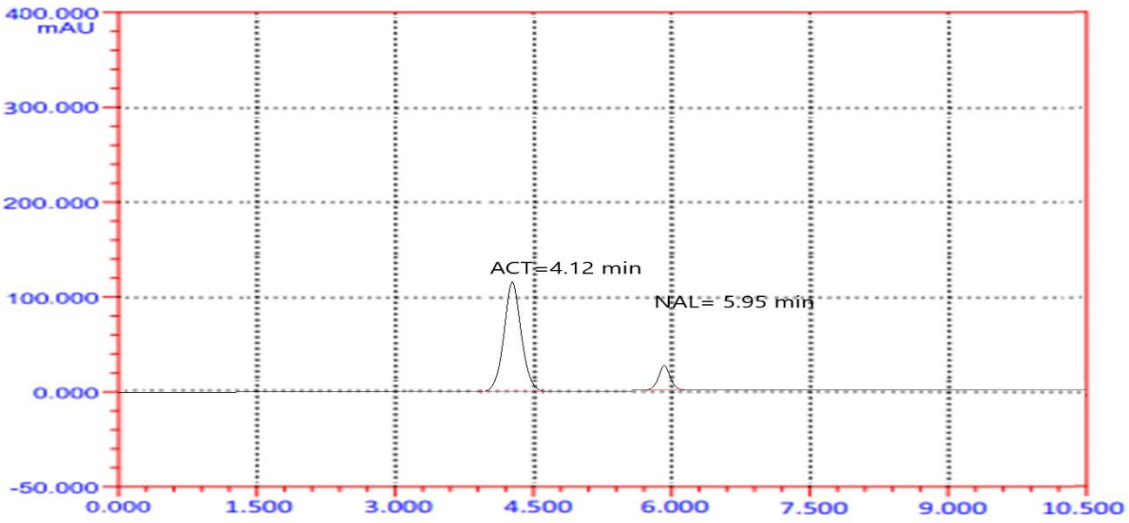


Figure 0:9 *Trail 6:* HPLC Chromatogram: Mobile Phase: Methanol: acetonitrile: Phosphate Buffer 50:30:20 v/v (pH adjusted to 5.8)

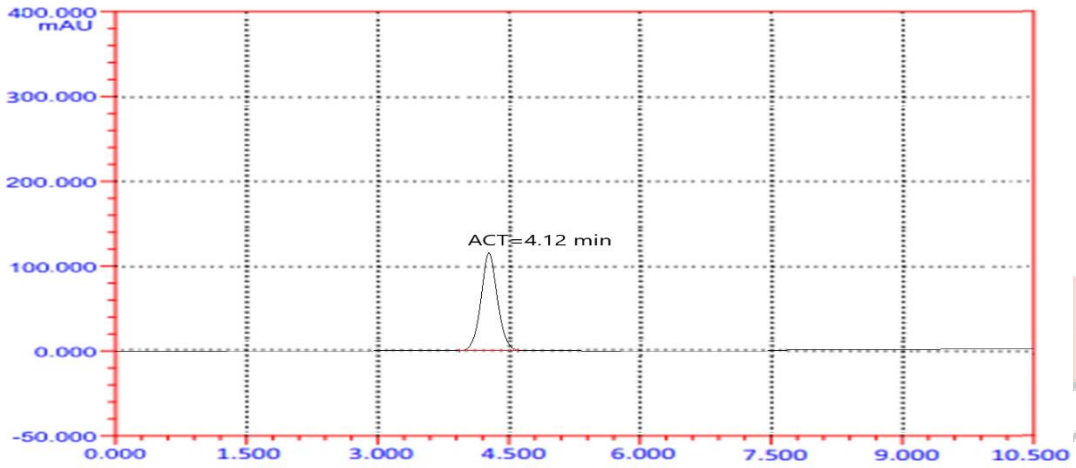


Figure 0:10 Aceteaminophen HPLC Chromatogram: Mobile Phase: Methanol: acetonitrile: Phosphate Buffer 50:30:20 v/v (pH adjusted to 5.8)

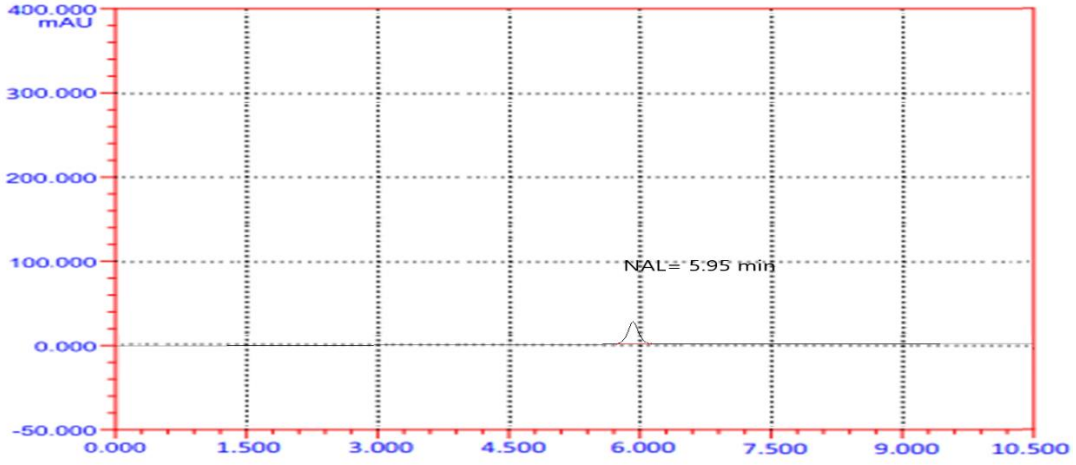


Figure 0:11 Naltreoxane HPLC Chromatogram: Mobile Phase: Methanol: acetonitrile: Phosphate Buffer 50:30:20 v/v (pH adjusted to 5.8)

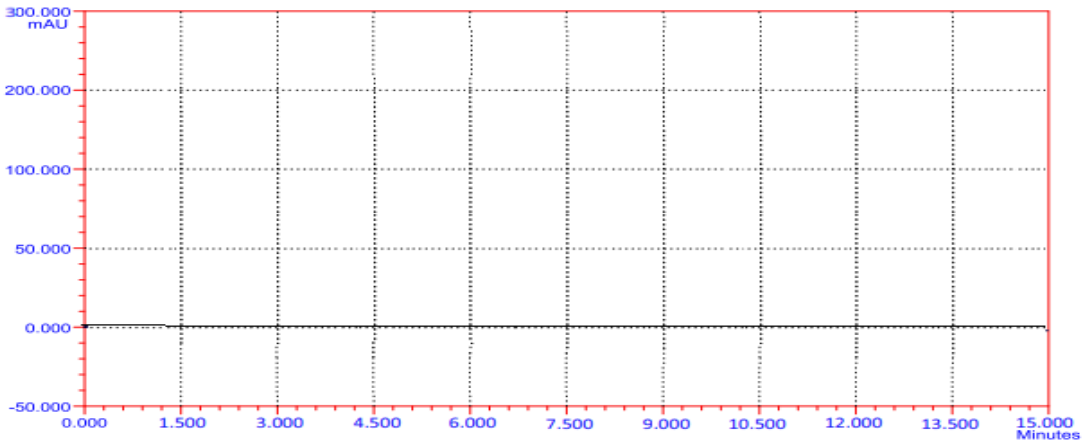


Figure 0:12 Blank HPLC Chromatogram: Mobile Phase: Methanol: acetonitrile: Phosphate Buffer 50:30:20 v/v (pH adjusted to 5.8)

Mobile phase of Methanol: acetonitrile: Phosphate Buffer 50:30:20 v/v (pH adjusted to 5.8) adjusted using 0.1M NaOH gave 2 sharp peaks of ACT and NAL with asymmetric factor of 1.12 and 1.15 and Rt of ACT was 4.25 ±0.02, and Rt of NAL was 5.95 ±0.03 respectively and hence it was selected as mobile phase for estimation of ACT and NAL. The flow rate was maintained at 1ml/min. For the identification of both the peaks individual drug was injected. Chromatogram of drugs was given in fig no. 6.10, and 6.11 and blank chromatogram shown in figure 6.12.

Table 7 Optimization of RP-HPLC chromatographic conditions

Sr. No.	Chromatographic parameter	Optimize Condition	
1	Flow Rate	1 ml/min	
2	Detection Wavelength	230nm	
3	Mobile Phase composition	Methanol: acetonitrile: Phosphate Buffer 50:30:20 v/v (pH adjusted to 5.8)	
4	Column	C18 (250 mm×4.6 mm×5 µm)	
5	Injection Volume	20 µl	
6	pH of buffer	5.8 ± 0.02	
7	Retention time (min)	ACT	NAL
		4.25 min	5.95 min

SYSTEM SUITABILITY TEST: -

The system suitability parameters were calculated and all system suitability parameter are within the acceptable range.

Table 8 System Suitability Parameters

Parameter	ACT	NAL
Retention Time (min)	4.2	5.95
Resolution	0.00	9.55
Theoretical plate	3920	2945
Symmetric Factor	1.12	1.25

METHOD VALIDATION:

Linearity:

Linear responses were obtained in concentration range of 50-300 µg/ml for Acetaminophen (ACT) and 1-6 µg/ml for Naltrexone (NAL). The data for linearity has shown in table 6.8 for Acetaminophen (ACT) and Naltrexone (NAL). The calibration curve for Acetaminophen (ACT) and Naltrexone (NAL) was given in fig no. 6.13 and fig no. 6.14.

Table: 8 Linearity Data of Acetaminophen (ACT) and Naltrexone (NAL)

Acetaminophen (ACT)			Naltrexone (NAL)		
Conc.	Peak Area	RSD	Conc.	Peak Area	RSD
50	101560.55	0.99	1	3386.65	1.64
100	126371.53	0.39	2	5583.38	1.51
150	149651.82	0.55	3	8053.28	0.70
200	176231.82	0.55	4	10481.00	1.61
250	199943.23	0.81	5	12487.77	0.55
300	225827.33	0.35	6	14548.57	1.36

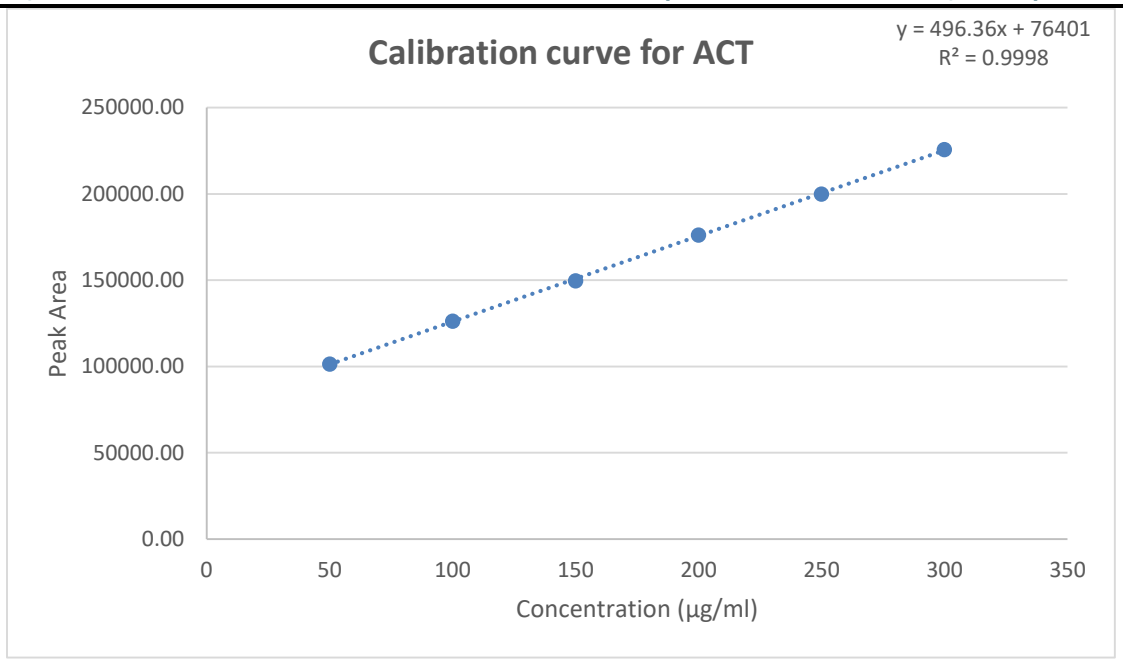


Figure 0:13 Calibration Curve of Acetaminophen (ACT)

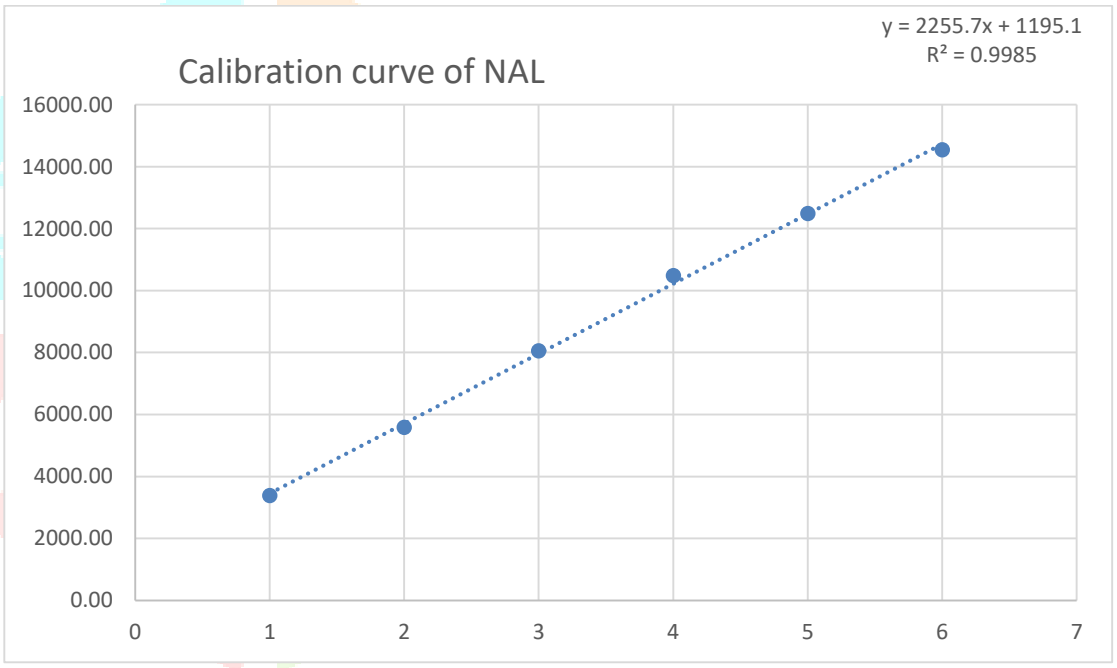


Figure 0:14 Calibration Curve of Naltrexone (NAL)

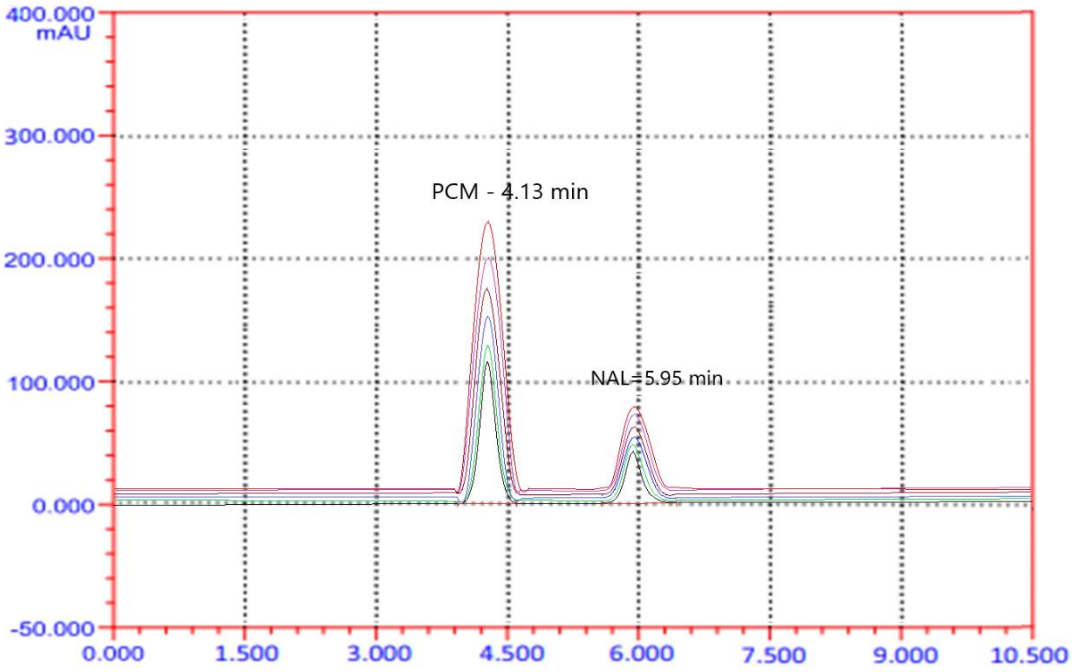


Figure 0:15 The overlay HPLC Chromatogram of Acetaminophen (ACT) in the range of 50-300 µg/ml, Naltrexone (NAL) 1 – 6 µg/ml on optimized Mobile Phase

Accuracy:

Accuracy of method was carried out at three levels (50 %, 100 % and 150 %). % Recovery for Acetaminophen (ACT) was found to be in range of 98.76 – 101.12 %, while for Naltrexone (NAL) it was found to be in range of 98.76 -102.43 % are shown in Tables 9.

Table 9 Accuracy data

Level (%)	Target Conc. (µg/ml)	Spiked Conc. (µg/ml)	Total Conc. (µg/ml)	Area	Conc. Found (µg/ml)	% Recovery
Acetaminophen (ACT)						
0	100	0	100	126593.31	101.12	101.12
50	100	50	150	149928.15	148.13	98.76
100	100	100	200	175790.32	200.24	100.12
150	100	150	250	199025.33	247.05	98.82
Naltrexone (NAL)						
0	2	0	2	5650.57	1.98	98.76
50	2	1	3	8079.50	3.05	101.73
100	2	2	4	10436.80	4.10	102.43
150	2	3	5	12443.00	4.99	99.73

Precision:

For precision RSD was found to be less than 2 revels that the proposed method is acceptable shown in Table 10,11.

Table 10 Repeatability Data of Acetaminophen (ACT) and Naltrexone (NAL)

Acetaminophen (ACT)			Naltrexone (NAL)		
Sr. No	**Conc. (µg/ml)**	**Area**	**Sr. No**	**Conc. (µg/ml)**	**Area**
1	100	128071.53	**1**	2	5718.7
2	100	127716.85	**2**	2	5657.2
3	100	125991.53	**3**	2	5675.8
4	100	126621.58	**4**	2	5549.3
5	100	125951.33	**5**	2	5538.6
6	100	124875.65	**6**	2	5563.7
Average	126538.08		**Average**	5617.21	
SD	1196.21		**SD**	76.14	
% RSD	0.95		**RSD**	1.36	

Table 11 Intraday and Interday precision of method

Acetaminophen (ACT)				
Conc	**Intraday precision**		**Interday precision**	
	Peak Area (Mean ± SD)^n	**%RSD**	**Peak Area (Mean ± SD)^n**	**%RSD**
50	100684.30 ± 709.21	0.70	102103.47 ±1424.45	1.40
150	149928.15 ± 1066.18	0.71	148679.48 ± 1017.78	0.68
300	225809.00 ± 877.05	0.39	226513.22 ± 1660.36	0.73
Naltrexone (NAL)				
1	3392.07 ± 22.34	0.66	3388.57 ± 41.94	1.24
3	8079.50 ± 25.36	0.31	8063.73 ± 109.99	1.36
6	14190.29 ± 57.74	0.41	14404.84 ± 258.41	1.79

LOD and LOQ:

LOD & LOQ of Acetaminophen (ACT) and Naltrexone (NAL) were determined by equation according to ICH guideline calculation of these was given in Table 12.

Table 1 LOD and LOQ of Acetaminophen (ACT) and Naltrexone (NAL)

Drug	**Acetaminophen (ACT)**	**Naltrexone (NAL)**
Limit of detection (LOD)	3.05 µg/ml	0.17 µg/ml
Limit of quantification (LOQ)	10.16 µg/ml	0.58 µg/ml

Robustness:

Deliberate change in different parameters like Flow rate, Wavelength showed Relative standard deviation of peak area less than 2 %, indicating that the method was robust. Results, presented in table 13 indicate that the selected factors remained unaffected by small variation of these parameters.

Table 13 Robustness study for Acetaminophen (ACT) and Naltrexone (NAL)

EFFECT OF CHANGE IN FLOWRATE									
	0.9 ml/min			1 ml/min			1.1 ml/min		
	Peak	SD	%RSD	Peak	SD	%RSD	Peak	SD	%RSD
ACT (100 µg/ml)	125954.8	601.96	0.47	5600.55	58.35	1.04	125939.50	58.83	0.45
NAL (2µg/ml)	5531.23	78.50	1.41	126538.07	717.26	0.57	5534.56	83.32	1.50
EFFECT OF CHANGE IN DETECTION									
	227 nm			230 nm			233 nm		
	Peak Area	SD	%RSD	Peak Area	SD	%RSD	Peak Area	SD	%RSD
ACT (100 µg/ml)	126426.63	829.01	0.65	5600.55	58.35	1.04	126482.85	986.89	0.78
NAL (2µg/ml)	5517.2	60.12	1.08	126538.07	717.26	0.57	5650.56	29.12	0.51

Specificity:

Specificity was ensured by the use of a standard, diluent and placebo to examine the % interference of excipients. It was proved by comparing chromatogram of blank, standard solution and sample preparation solution; there was no any interference of excipients with peak of ACT and NAL. Chromatogram was given in fig no. 6.16.

ASSAY OF SYNTHETIC MIXTURE:

Synthetic mixture of Acetaminophen (ACT) and Naltrexone (NAL) containing 325 mg and 3.25 mg when analysed using the developed method, showed 100.86 % assay for Acetaminophen (ACT) and 100.01 % assay for Naltrexone (NAL). Chromatogram was given in fig no. 6.16 and % assay was given in Table 14.

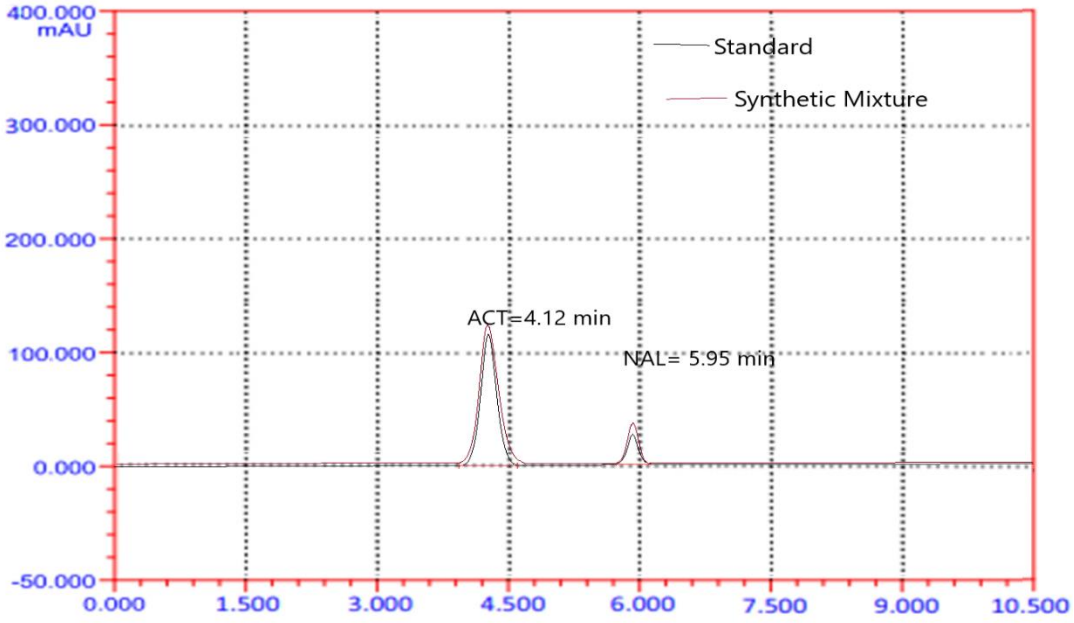


Figure 0:16 Chromatogram for Analysis of Synthetic mixture

Table 14 Analysis of Synthetic mixture

Drugs	Conc.	Peak Area ± SD	% Assay
Acetaminophen (ACT)	162.5 µg/ml	156277.9 ± 1528.62	99.03 ± 1.91
Naltrexone (NAL)	1.625 µg/ml	4842 ± 68.46	99.49 ± 1.87

Conclusion:

Analytical RP-HPLC method was developed for the simultaneous estimation of Acetaminophen (ACT) and Naltrexone (NAL) in Synthetic mixture. The method was developed to estimate and separate Acetaminophen (ACT) and Naltrexone (NAL) using RP-HPLC and developed method was validated as per ICH Q2 (R1) guideline. A Specific, precise, Accurate, Robust and cost-effective Reversed Phase High Performance Liquid Chromatographic method was developed for simultaneous determination of Acetaminophen (ACT) and Naltrexone (NAL)in their synthetic mixture. For RP-HPLC two drugs was separated by Cyber Lib C18 Column (250 mm × 4.6 mm,5 µm), utilizing a Mobile Phase Methanol: acetonitrile: Phosphate Buffer 50:30:20 v/v (pH adjusted to 5.8) at Flow Rate of 1 ml/min, injection volume 20 µl with uv detection at 230 nm. The retention time of Acetaminophen (ACT) 4.20 min and Naltrexone (NAL) 5.95 min using RP-HPLC. The co-relation coefficient of 0.9998 for ACT and 0.9985 for NAL HPLC method respectively. The % RSD Value was found for the validation parameter that indicates the preciseness of the proposed method and is applicable for routine analysis for quantitative determination of ACT and NAL. The LOD was found for ACT 3.04µg/ml, NAL 0.174 µg/ml for developed method respectively. The LOQ was found for ACT 10.16 µg/ml, NAL 0.58 µg/ml for developed method respectively. The result of analysis was validated according to ICH Q2 R1 Guidelines. This simple and precise method can be used of both drug in quality control laboratories.

REFRENCES:

1. https://my.clevelandclinic.org/health/diseases/5005-migraine-headaches access on November 2021.

2.Mungoven Tiffani J, Henderson Luke A, Meylakh Noemi. Chronic Migraine Pathophysiology and Treatment: A Review of Current Perspectives.*Frontiers in Pain Research*.**2021**, 2, 52.

3. Eigenbrodt, A.K., Ashina, H., Khan, S. et al. Diagnosis and management of migraine in ten steps.*Nat Rev Neurol*.**2021**,17,501–514. https://doi.org/10.1038/s41582-021-00509-5.

4. Drug bank access on November 2021

https://go.drugbank.com/drugs/DB00316 .

5.Drug bank access on November 2021

https://go.drugbank.com/salts/DBSALT000670 .

6. Sharma S & Goyal S & ChauhanK. "A review on analytical method development and validation." *International Journal of Applied Pharmaceutics.***2018**,10, 8.

7. PatelR & PatelM. "An Introduction To Analytical Method Development For Pharmaceutical Formulations." *Pharmaceutical Reviews***2008**.

8. Malviya R & BansalV & PalOm&Sharma P."High performance liquid chromatography: A short review."*Journal of Global Pharma Technology*.**2010**,2,22-26.

9. G. Shabir, HPLC Method Development and Validation for Pharmaceutical Analysis. *Lap Lambert Academic Publishing* .**2013**.

10. Clevett KJ. High-performance liquid chromatography. *Bioprocess Technol.*6,**1990**, 47-73.

11. Griffiths J. Introduction to High Performance Liquid Chromatography. *Biochemical Society Transactions*.**1978**,6, 821.

12. ICH Q2 (R1); "Validation of Analytical Procedures: Text and Methodology." Geneva, Switzerland, November 2005

13. Quality Assurance of Pharmaceuticals, (A compendium of guidelines and related materials), Volume I, WHO, Geneva, 1997, 119-124

14. Pubchem access on November 2021. https://pubchem.ncbi.nlm.nih.gov/compound/Acetaminophen

15. Pubchem access on November 2021.https://pubchem.ncbi.nlm.nih.gov/compound/Naltrexone-hydrochloride

16. Indian Pharmacopoeia-Volume, Published by Indian Pharmacopoeia Commission for Ministry of Health and Family Welfare, Noida, New Delhi, 2010, 3, 902-903.

17. United States Pharmacopeia and National Formulary (USP 32-NF 27), Pharmacopeia Forum United States Pharmacopeia Convention, Rockville, 2010. 34(5), 1136.

18. United States Pharmacopeia and National Formulary (USP 32-NF 27), Pharmacopeial Forum United States Pharmacopeial Convention, Rockville, 2010. 27(3) Page 2494

19. Youssef SH, Mohamed D, Hegazy MAM, Badawey A. "Analytical methods for the determination of paracetamol, pseudoephedrine and brompheniramine in Comtrex tablets". *BMC Chem*. **2019**;13(1):78.

20. Jena B, Babu S, Pradhan D, Swain S. "UPLC analytical method development and validation for the simultaneous estimation of paracetamol and caffeine capsules dosages form". *Pharm Regul Aff.* **2017**,6,2–9.

21. Kumar KG, Letha R. "Determination of paracetamol in pure form and in dosage forms using N,N-dibromo dimethylhydantoin". *J Pharm Biomed Anal.***1997**,15,1725–1728. doi: 10.1016/S0731-7085(96)01976-0

22. Abdelaleem EA, Naguib IA, Hassan ES, Ali NW. "Development and validation of three spectrophotometric methods for simultaneous determination of paracetamol and pamabrom in bulk and pharmaceutical formulation."*Anal Chem Lett*. **2016**,6,13–23. doi: 10.1080/22297928.2016.1138882.

23. Rele RV. "Simultaneous UV-spectrophotometric estimation of paracetamol and guaiphenesin by first order derivative method in combined dosage form."*Res J Pharm Technol*. **2016**,9,509–512. doi:10.5958/0974-360X.2016.00095.0.

24. Llorent-Martínez E, Šatínský D, Solich P, Ortega-Barrales P, Molina-Díaz A. "Fluorimetric SIA optosensing in pharmaceutical analysis: determination of paracetamol." *J Pharm Biomed Anal.***2007**,45,318–321. doi: 10.1016/j.jpba.2007.05.004

25. Ambekar A, Kuchekar B. "Application of a validated stability-indicating HPTLC method for simultaneous estimation of paracetamol and aceclofenac and their impurities". *J Chromatograph Sep Technol.* **2016**,7,324–334. doi: 10.4172/2157-7064.1000324.

26. Belal T, Awad T, Clark R. "Determination of paracetamol and tramadol hydrochloride in pharmaceutical mixture using HPLC and GC-MS". *J Chromatogr Sci.* **2009**,47,849–854. doi: 10.1093/chromsci/47.10.849.

27. Sharma H, Vishakha K, Kumar KV, Bhatta HP. "Validated RP-HPLC method for simultaneous estimation of paracetamol, pamabrom and dicyclomine, hydrochloride in bulk and pharmaceutical dosage form". *Int J Pharm Sci Res*. **2016**,7,316–324.

28. Nazir A, Naseer Y, Shahid R, Raza S. "Development & validation of analytical method used for simultaneous determination of paracetamol, caffeine and codeine phosphate by HPLC, in pharmaceutical formulation". *Sci Int*. **2016**,28,2497.

29. Li H, Zhang C, Wang J, Jiang Y, Fawcett JP, Gu J. "Simultaneous quantitation of paracetamol, caffeine, pseudoephedrine, chlorpheniramine and cloperastine in human plasma by liquid chromatography-tandem mass spectrometry". *J Pharm Biomed Anal.* **2010**,51,716–722. doi: 10.1016/j.jpba.2009.10.009.

30. Nyansa, M.M.S.; Fiawoyife, P.D.; Mireku-Gyimah, N.A.; Addotey, J.N.A. "Stability-Indicating HPLC Method for the Simultaneous Determination of Paracetamol and Tramadol Hydrochloride in Fixed-Dose Combination Tablets". J. Biomed. Sci. Eng. **2017**, 5, 41–47.

31. Pereira, F.J., Rodríguez-Cordero A, López, R.; Robles, L.C.; Aller, A.J. "Development and Validation of an RP-HPLC-PDA Method for Determination of Paracetamol, Caffeine and Tramadol Hydrochloride in Pharmaceutical Formulations". *Pharmaceuticals* **2021**, 14, 466. https://doi.org/10.3390/ ph14050466

32. Mulyasuryani, A, Tjahjanto, R.T., Andawiyah, R. Simultaneous Voltammetric Detection of Acetaminophen and Caffeine Base on Cassava Starch—Fe_3O_4 Nanoparticles Modified Glassy Carbon Electrode. *Chemosensors***2019**, 7, 49.

33. Dr.V Lakshmi N, Dr.Anoop A "Determination of acetaminophene and caffeine using reverse phase liquid (RP-LC) chromatographic technique" *Journal of research in pharmaceutical Science* **2016**www.questjournals.org

34. Aminu N, Siok-Yee C, Khan N H, Ahmed B F, Umar M N , and Seok-Ming Toh. " A simple stability-indicating HPLC method for simultaneous analysis of paracetamol and caffeine and its application to determinations in fixed-dose combination tablet dosage form".*Acta Chromatographica.* **2021**,31(2),85-91. https://doi.org/10.1556/1326.2018.00354.

35. United States Pharmacopeia and National Formulary (USP 32-NF 27), Pharmacopeial Forum United States Pharmacopeial Convention, Rockville, 2010. 34(2), 283.

36. Milad Jafari-Nodoushan, Jalal Barzin, Hamid Mobedi. "A stability-indicating HPLC method for simultaneous determination of morphine and naltrexone". **2016**, 163-170, ISSN 1570-0232. https://doi.org/10.1016/j.jchromb.2015.12.048.

37. Amal M.Al-Mohaimeed, Gamal A.E, Maha.F.E. "New Construction of Functionalized Cuo/Al2O3 Nanocomposite-Based Polymeric Sensor for Potentiometric Estimation of Naltrexone Hydrochloride in Commercial Formulations". *Polymers***2021**, 13, 4459. https://doi.org/10.3390/polym13244459

38. Pathy.K. "Route evaluation andanalytical HPLC method development of buprenorphine, naltrexone and comparison overall efficiency of naltrexone nalbuphine traces the evolution of various approaches". Head-Qc-Qa/R&D-IPL Research centre, **2018**.

39.S.Valiveti, Buchi N N, Dana C. H, Kalpana S, A.L. Stinchcomb "Development and validation of a liquid chromatography-mass spectroscopy method for the quantitation of naltrexone and 6-beta-naltrexol in guinea pig plasma".*Journal of Chromatography B*. **2004**,810, 259-267.

40. Sunil S, William H, H.thomas karnes "characterization of a potential medium for biorelevent in vitro release testing of a naltrexone implant, employing a validated stability indicating HPLC method". *journal of Pharmaceutical and biomedical Analysis***2007**,43, 845-853. www.sciencedirect.com

41. Rezazadeh M, Y.yamini, S.seidi "Electromembrane extraction of trace amounts of naltrexone and nalmefene from untrated biological fluids". *Journal of chromatography B*, **2011**,879,1143-1148 doi: 10.1016/j.jchromb.2011.03.043

42.C.kossoski, R.pontarolo, S.M.W.barreira, S.germano , D.F.maluf "Analytical validation ofa quantification method by UV-Vis spectrometry of Naltrexone Hydrochloride in Capsules". *Latin American Journal of Pharmacy.* **2013**, 32 (4): 616-20.https://www.researchgate.net/publication/282931678

43. Vemuri S, M.tejaswi, P.srividya and Buchi N. Nalluri "simultaneous analysis of naltrexone hydrochloride and bupropion hydrochloride in bulk and dosage forms by RP-HPLC-PDA method". *journal of chemical and pharmaceutical research*,**2013**, 5(11):429-435 www.jocpr.com

44. Kendice lp, Guiyun S, Daniel B ,August S.bassani ,Benigno C "In vitro evaluation of naltrexone HCl 1% Topical cream in XemaTop for psoriasis". *Archives of Dermatological Research.***2019** https://doi.org/10.1007/s00403-019-01981-2.

45. Heinälä, P., Lahti, T., Sinclair, D. et al. "Analysis of naltrexone and its metabolite 6-beta-naltrexol in serum with high-performance liquid chromatography". *BMC Res Notes*, **2012**, 5**,** 439. https://doi.org/10.1186/1756-0500-5-439

46. Brünen S, Krüger R, Finger S, Korf F, Kiefer F, Wiedemann K, Lackner KJ, Hiemke C: "Determination of naltrexone and 6beta-naltrexol in human blood: comparison of high-performance liquid chromatography with spectrophotometric and tandem-mass-spectrometric detection". *Anal Bioanal Chem*. **2010**, 396 (3): 1249-1257. doi:10.1007/s00216-009-3301-z.

47. Haritha, P. Bharath Rathna Kumar R.Venu Priya, K.B.Chandra Sekhar, "Analytical method development and validation for simultaneous estimation of naltrexone hydrochloride and bupropion hydrochloride in oral dosage form (tablets) by RP-HPLC Technique"*JGTPS,***2015**, 6 (2): 2600 – 2606.

48. Sherif A. Abdel-Gawad, Rania M. El-Gamal."Simultaneous determination of naltrexone and bupropion in their co-formulated tablet utilizing green chromatographic approach with application to human urine". *Saudi Pharmaceutical Journal*, **2018**, 26 (2), 169-176. https://doi.org/10.1016/j.jsps.2017.12.014.

49. Phani RSCh, Chaitanya D and Prasanthi B: "RP-HPLC and Spectrophotometric Methods for the Simultaneous Estimation of Bupropion HCl and Naltrexone HCl". *Int J Pharm Sci Res*.**2015,**6(7), 1000-09.

50. Davis Adrian F.Pharmaceutical compositions containing paracetamol. United States Patent, US 5270050,1981.

51. Coquelet et al. Paracetamol-based pharmaceutical composition. United States Patent, US PCT/FR90/00299,1993.

52. Rohrbach et al. Water-soluble salts of paracetamol. United States Patent,US 3987170,1976.

53. Kavala et al. Method for isolation and purification of naltrexone. United States Patent, US 20200048271 A1,2021.

54. Yin , et al. Preparation method of naltrexone implants. United States Patent, US 20200060977 A1,2021.

55. Indian Pharmacopeia-Volume, Published by Indian Pharmacopoeial Commision for Ministry of Health and Family Welfare, Noida, New Delhi, 2010,3, 1748-1749.