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Analytical Method Development and Validation for the Simultaneous Estimation of Buprenorphine and Naloxone by RP- HPLC Method

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A B S T R A C T

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Buprenorphine and Naloxone was done by RP-HPLC. The Phosphate buffer was p^H 3.0 and the mobile phase was optimized with consists of Methanol: Phosphate buffer mixed in the ratio of 70:30 % v/ v. Inertsil C_{18} column C18 (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 260 nm. The solutions were chromatographed at a constant flow rate of 0.8 ml/min. the linearity range of Buprenorphine and Naloxone were found to be from 100-500 µg/ml of Buprenorphine and 1-5µg/ml of Naloxone. Linear regression coefficient was not more than 0.999.The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of Buprenorphine and naloxone. LOD and LOQ were found to be within limit. The results obtained on the validation parameters met ICH and USP requirements .it inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

Keywords: Methanol: Phosphate buffer, Inertsil C18 column, Buprenorphine and Naloxone

A R T I C L E I N F O

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CONTENTS

1. Introduction

Buprenorphine's analgesic effect is due to partial agonist activity at mu-opioid receptors. Buprenorphine is also a kappa-opioid receptor antagonist. The partial agonist activity means that opioid receptor antagonists (e.g., an

antidote such as naloxone) only partially reverse the effects of buprenorphine. The binding to the mu and kappa receptors results in hyperpolarization and reduced neuronal excitability. Furthermore, buprenorphine slowly dissociates from its receptor. This observation would account for the longer duration of action compared to morphine, the unpredictability of its reversal by opioid antagonists, and its low level of manifest physical dependence. Its receptor fixation half life is 40 minutes which is significantly longer than morphine (milliseconds).

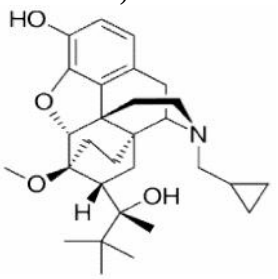

Fig 1: Structure of Buprenorphine

Naloxone is not fully understood, the preponderance of evidence suggests that naloxone antagonizes the opioid effects by competing for the same receptor sites, especially the opioid mu receptor. Recently, naloxone has been shown to bind all three opioid receptors (mu, kappa and gamma) but the strongest binding is to the mu receptor.

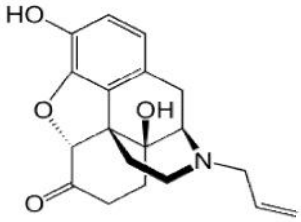

Fig 2: Structure of Naloxone

The literature review reveals the few HPLC methods for the estimation of buprenorphine and naloxone and in combination with other drugs. Few methods are also reported for estimation of both drugs from formulation .we intends to develop a RP-HPLC method by simultaneous determination with simple, rapid, greater sensitivity and faster elution.

2. Materials and Methods

Instruments used: Water HPLC with proving software of empower and 2965 separation module with PDA detector. UV-Visible spectrophotometer for the model of Lab india3000+, pH meter and weighing balance.

Chemicals

API of Buprenorphine was procured from gift sample of Mylan laboratories, Bangalore, Karnataka. API of Naloxone was procured from Cipla.ltd, Bangalore. HPLC grade water, methanol, Acetonitrile and orthophosphoric acid were purchased from Merck labs, Mumbai.

Optimized Chromatographic Conditions

Instrument used : Waters HPLC with auto sampler and PAD or detector.

Temperature : Ambient

Column : Inertsil ODS (4.6 x 150mm, 5µm)

Buffer : 6.8 grams of potassium dihydrogen ortho phosphate in 1000 ml water pH adjusted with ortho phosphoric acid.

pH : 3.0

Mobile phase : 30% buffer 70% Methanol

Flow rate : 0.8 ml per min

Wavelength : 260 nm

Injection volume : 10µl

Run time : 10min.

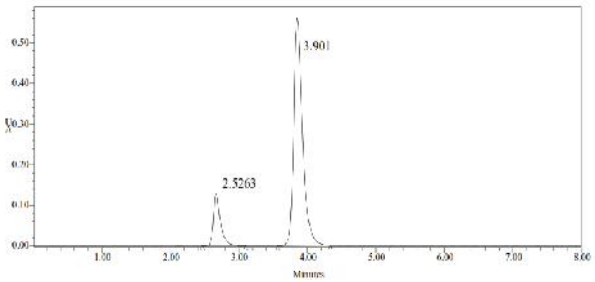

Fig 3: Optimized Chromatogram for Buprenorphine and Naloxone

Preparation of Phosphate buffer

Accurately weighed 6.8 grams of KH_2PO_4 was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.0 with Orthophosphoric acid.

Preparation of mobile phase

Accurately measured 300 ml (30%) of above buffer and 700 ml of Methanol HPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Preparation of the Buprenorphine& Naloxone Standard & Sample Solution

Standard Solution Preparation:

Accurately weigh and transfer 10 mg of Buprenorphine and Naloxone 10mg of working standard into a 10mL& 100ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).Further pipette 3ml& 0.3ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 10 mg ofBuprenorphine and Naloxone (marketed formulation) sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.(Stock solution). Further pipette 3 ml of Buprenorphine and Naloxone of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure: Inject 20 µL of the standard, sample into the chromatographic system and measure the areas for Buprenorphine and Naloxone peaks and calculate the %Assay by using the formulae.

Method Validation

System Suitability: Tailing factor for the peaks due to Buprenorphine and Naloxone in Standard solution should not be more than 2.0. Theoretical plates for the

Buprenorphine and Naloxone peaks in Standard solution should not be less than 2000. The results are tabulated in Table 1.

Precision:

The standard solution was injected for five times and measured the area for all five Injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits. The % RSD for the area of five standard injections results should not be more than 2%**.**

Intermediate Precision/Ruggedness: To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions. The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Accuracy: Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Buprenorphine & Naloxone and calculate the individual recovery and mean recovery values. The % Recovery for each level should be between 98.0 to 102.0%.

Linearity: Preparation of stock solution:

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 10 mg of Buprenorphine and Naloxone (marketed formulation) sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (100ppm ofBuprenorphine&1ppm of Naloxone): 1ml and 0.1 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – II (200ppm of Buprenorphine & 2ppm of Naloxone): 2ml and 0.2 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – III (300ppm of Buprenorphine &3ppm of Naloxone): 3ml and 0.3 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – IV (400ppm of Buprenorphine&4ppm of Naloxone): 4ml and 0.4 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent

Preparation of Level – V (500ppm of Buprenorphine & 5ppm of Naloxone): 5ml and 0.5 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent

Procedure: Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. Correlation coefficient should be not less than 0.999.

LOD and LOQ: LOD and LOQ are calculated by Signal noise ratio method. The results were shown in table 9,10.

Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

a). The flow rate was varied at 0.8 ml/min to 1.2ml/min: Standard solution 300ppm of Buprenorphine&3ppm of Naloxone was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.***** Results for actual flow (1.0ml/min) have been considered from Assay standard.

b). The Organic composition in the Mobile phase was varied from 50% to 50%: Standard solution 300 µg/ml of Buprenorphine & 3µg/ml of Naloxone **was** prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. On evaluation of the above results, it can be concluded that the variation in 10%.Organic composition in the mobile phase affected the method significantly. Hence it Indicates that the method is robust even by change in the Mobile phase ±10**. ***Results for actual Mobile phase composition (55:45Methanol: Buffer (ph-2.8) has been considered from Assay standard method.

3. Results and Discussion

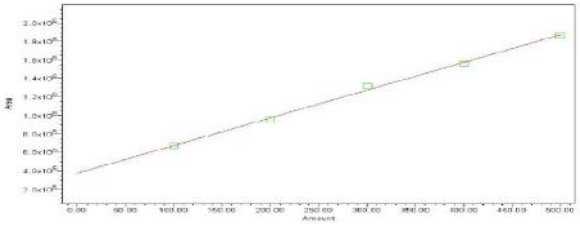

Fig 4: Calibration graph for Buprenorphine at 225 nm

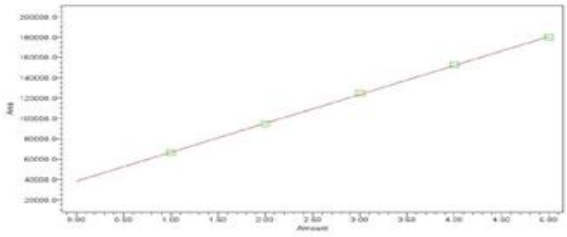

Fig 5: Calibration graph for Naloxone at 225 nm

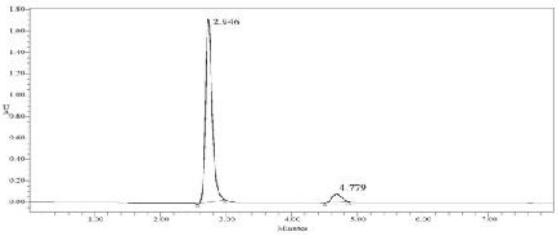

Fig 6: Chromatogram snowing less flow of 0.6ml/min

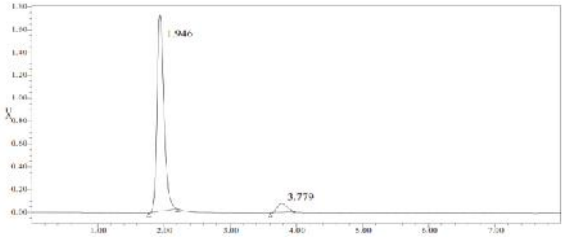

Fig 7: Chromatogram snowing more flow of 1.0ml/min

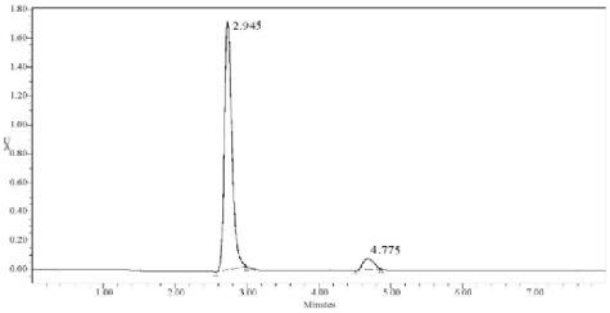

Fig 8: Chromatogram showing less organic composition

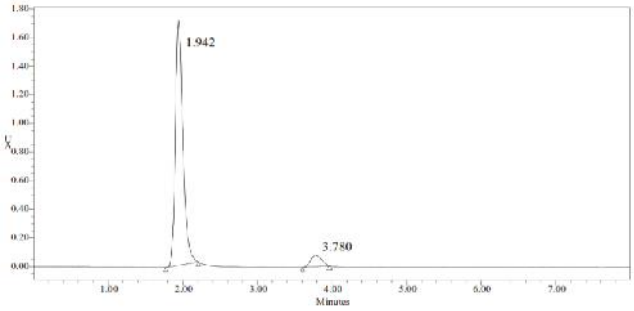

Fig 9: Chromatogram showing more organic composition

4. Conclusions

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Buprenorphine and Naloxone was done by RP-HPLC. The Phosphate buffer was p^H 3.0 and the mobile phase was optimized with consists of Methanol: Phosphate buffer mixed in the ratio of 70:30 % v/ v. Inertsil C_{18} column C18 (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 260 nm. The solutions were chromatographed at a constant flow rate of 0.8 ml/min. the linearity range of Buprenorphine and Naloxone were found to be from 100-500 µg/ml of Buprenorphine and 1-5µg/ml of Naloxone. Linear regression coefficient was not more than 0.999.The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of Buprenorphine and naloxone. LOD and LOQ were found to be within limit. The results obtained on the validation parameters met ICH and USP requirements .it inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

Table 1: Results of system suitability parameters for Buprenorphine and Naloxone

S.No	Name	Retention time(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	**Buprenorphine**	2.5	124505	213642		1.2	4673.4
2	**Naloxone**	3.9	1308495	154566	6 0	1.3	6090.3

Table 2: Results of method precession for Buprenorphine and Naloxone

Injection	Area	
	Buprenorphine	**Naloxone**
Injection-1	1302729	123149
Injection-2	1302947	123766
Injection-3	1303236	124271
Injection-4	1303977	124691
Injection-5	1309759	124956
Average	1304529.8	124162.7
Standard Deviation	2961.1	725.6
%RSD	0.2	0.6

Table 3: Results of Intermediate precision for Buprenorphine and Naloxone

Injection	Area	
	Buprenorphine	**Naloxone**
Injection-1	1300148	122487
Injection-2	1304520	122626
Injection-3	1305937	122632
Injection-4	1306476	122702
Injection-5	130871	122962
Average	1305070.2	122681.8
Standard Deviation	3061.8	174.8
%RSD	0.2	0.1

Table 4: Accuracy (recovery) data for Buprenorphine

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	656659.5	5.0	5.036	100.7%	99.84%

100%	1304258	10.0	10.003	100.0%	
150%	1854608	14.4	14.224	98.780%	

Table 5: Accuracy (recovery) data for Naloxone

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	65800	5.3	5.34	100.8%	100.51%
100%	124353	10	10.10	100.01%	
150%	177940	14.2	14.45	99.68%	

Table 6: Area of different concentration of Buprenorphine

S.No.	Linearity Level	Concentration	Area
1	I	100ppm	668934
2	II	200ppm	956781
3	III	300ppm	1313873
4	IV	400ppm	1563458
5	V	500ppm	1867084
Correlation Coefficient			0.999

Table 7: Area of different concentration of Naloxone

S.No	Linearity Level	Concentration	Area
1	I	1ppm	66510
2	II	2ppm	94701
3	III	3ppm	124802
4	IV	4ppm	152731
5	V	5ppm	179732
Correlation Coefficient			0.999

Table 8: Analytical performance parameters of Buprenorphine and Naloxone

Parameters	Buprenorphine	Naloxone
Slope (m)	66574	12529
Intercept (c)	53592	50245
Correlation coefficient (R^2)	0.999	0.999

Table 9: Limit of Detection for Buprenorphine and Naloxone

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
Buprenorphine	52	152	2.9
Naloxone	52	156	3

Table 10: Limit of Quantification for Buprenorphine and Naloxone

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
Buprenorphine	52	522	10.03
Naloxone	52	524	10.1

Table 11: Flow Rate (ml/min) data for Buprenorphine

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.6	5339.9	1.4
2	0.8	4673.4	1.3
3	1.0	5216.0	1.4

Table 12: Flow rate (ml/min) data for Naloxone

S.No	Flow rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	7063.3	1.3
2	1.0	6090.3	1.2
3	1.2	6998.0	1.3

Table 13: Change in Organic Composition in the Mobile Phase for Buprenorphine

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	4508.4	1.3
2	*Actual	4673.4	1.4
3	10% more	4318.1	1.3

Table 14: Change in Organic Composition in the Mobile Phase for Naloxone

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	6387.7	1.2
2	*Actual	6090.3	1.2
3	10% more	6232.5	1.2

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