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Development of newer validated HPLC method for the determination of Alvimopan in Rat plasma

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Abstract
A simple, precise, rapid and accurate RP-HPLC method was developed for the estimation of Alvimopan in rat plasma. The drug samples were extracted by protein precipitation method with methanol as a solvent.
The separation was achieved by using C-18 Eclipse plus (5µ particle size, 250×4.6mm, 5µm internal diameter). The mobile phase comprises of potassium dihydrogen orthophosphate buffer of pH 3.0 adjusted with orthophosphoric acid and acetonitrile in the ratio of 70:30(v/v). The flow rate was 1.0ml/min and the effluents were monitored at 220 nm with a total run time of 10min. The retention time was found to be 6.66. The detection concentration was linear over 25-150µg/ml. Regression equation of Alvimopan was found to be y = 26407x+891423 with regression coefficients 0.999 and percentage recovery of 99.07%. The liquid chromatography method was extensively validated for linearity, accuracy, precision, LOD, LOQ.
All these analytical validation parameters were observed to be satisfactory, the developed method was successfully demonstrated for the determination of Alvimopan in rat plasma and validated in accordance to ICH guidelines. Hence, this method can be convieniently adopted for the analysis of rat plasma for the application in pharmacokinetic study, drug interaction, bio availability and bio equivalence.

Keywords - *Alvimopan, RP-HPLC, Methanol, Acetonitrile, Phosphate buffer pH 3, Rat plasma.*

INTRODUCTION

I. Pharmaceutical analysis is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and quantitative measurements of the substances present in bulk drug and pharmaceutical preparations.[1]

HPLC is a separation technique where solutes migrate through a column containing a micro particulate stationary phase at rates dependent on their distribution ratios. These are functions of the relative affinities of the solutes for the mobile and stationary phases, the elution order depending on the chemical nature of the solutes and the overall polarity of the two phases[2]. Very small particles of stationary phase are essential for satisfactory chromatographic efficiency and resolution, and the mobile phase must consequently be pumped through the column, resulting in the generation of a considerable backpressure. The composition of the mobile phase is adjusted to elute all the sample components reasonably quickly. Solutes eluted from the end of the column pass through a detector that responds to each one. There are a number of modes of HPLC enabling an extremely wide range of solute mixtures to be separated. The modes are defined by the type of stationary phase and associated absorption mechanism[3].

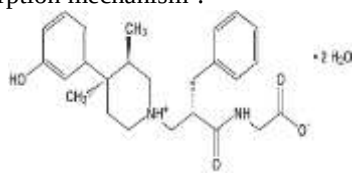

Figure 1: Chemical structure of Alvimopan[4]

Alvimopan contains not less than 97.5% and not more than 102% of $C_{25}H_{32}N_2O_4$, calculated on the dried basis. Morphine and other opioids are potent analgesics and act through µ receptors in the brain. However, stimulation of µ receptors in the periphery results in unwanted side effects like opioid bowel dysfunction, constipation and increased gastro-oesophageal reflex. Alvimopan is a novel opioid antagonist that does not cross blood-brain barrier at low dose and has selectivity only for peripheral µ opioid receptors. It has been found to be 200 times more potent in peripheral opioid receptors compared to centrally located receptors[5,6].

Peak plasma concentration (C_{max}) of Alvimopan is reached approximately 2 hours after oral dosing, while the C_{max} for metabolite occurs 36 hours after an oral dose. Alvimopan shows high affinity for the peripheral mu-receptor results in an absolute bioavailability less than 7%.

80% to 90% of systematically available Alvimopan is bound to plasma protein[9]. At steady state, the volume of distribution is approximately 30 litres.

Alvimopan undergoes no significant hepatic metabolism, but is metabolized by intestinal flora. Gut metabolism produces an active metabolite with no clinically significant contribution to drug effect.

Alvimopan is not substrate for the cytochrome P_{450} enzyme system. Therefore, no interactions are expected with hepatically metabolized drugs. Alvimopan is substrate for p-glycoprotein. Expert interactions with known p-glycoprotein

inhibitors such as Amiodarone, Depridil, Diltiazem, Cyclosporine, Itraconazole, Quinine, Quinidine, Spiranolocatone, Verapamil. Alvimopan is used to help the bowel recover more quickly after bowel surgery, so that patient can eat solid foods and have regular bowel movements. Alvimopan is in a class of medications called peripherally acting μ opioid receptor antagonists. It works by protecting the bowel from the constipation effects of opioid (narcotic) medications that are used to treat pain after surgery[7-9].

II. MATERIAL AND METHODS

Materials: The drug Alvimopan which was required for the research was availed from Sigma Aldrich and various chemicals like Acetonitrile, Methanol and reagents Potassium dihydrogen Ortho phosphate, Ortho phosphoric acid, EDTA etc are purchased from Fisher scientific[10].

Shimadzu HPLC model SPD-M20A was used for method development and validation. PDA detector is used. It is a multichannel detector contains an ideal sensor for an entire spectrum in a UV/VIS dispersive spectrophotometer. These are useful in both research and quality assurance laboratories and provides users most advanced level of sensitivity. Eclipse plus C_{18} columns are designed for superior peak with basic compounds and deliver high efficiency and excellent peak shape with all sample types. Eclipse plus C_{18} is especially useful for the separation of acidic, basic, and other highly polar compounds by reverse-phase liquid chromatography (250 × 4.6 mm, particle size is 5 μm). The binary mobile phase consisted of a mixture of A and B which was filtered through a membrane filter 4.5 μm. The solvents were degassed before running at a flow rate of 1 ml/min. The column temperature was ambient at 30 ºC. The 20 μl volume of sample was injected and peaks were detected at 220nm.

Methods

Preparation of standard stock solution for API:

About 5mg of alvimopan is weighed accurately, and is transferred to a 10ml clean glass volumetric flask (500μg/ml). It is dissolved in methanol and the volume is made up to 10ml with the same solvent. The above solution was filtered, sonicated for 10-15minutes.

Preparation of working standard for API:

The working standard is prepared from the standard stock solution 5ml is diluted to 10ml with same methanol to get the final concentration of 250μg/ml. Series of dilution were made to get concentration range 25-150 μg/ml. The above concentration of Alvimopan solution is corrected according to its potency, molecular weight and actual amount weighed. The stock solution is stored in refrigerator at 2-8ºC and used for maximum 20days.

Preparation of Quality control levels in rat plasma solution:

1. **Lower Limit of Quantification (LLOQ), 16μg/ml:** From 50μg/ml of plasma solution take 0.25ml and add 0.5ml of methanol, vortex for 5 minutes. Centrifuge the solution at 5000rpm at 4ºC, filter and sonication to get 46μg/ml.
2. **Lower Quality Control (LQC), 50μg/ml:** From the 150μg/ml of plasma solution take 0.25ml and add 0.5ml of methanol, vortex for 5 minutes. Centrifuge the solution at 5000rpm at 4ºC, filter and sonicate to get 50μg/ml.
3. **Medium Quality Control (MQC), 125μg/ml:** From the 500μg/ml of plasma solutions take 0.5ml and add 0.5ml of methanol, vortex for 5 minutes. Centrifuge the solution at 5000rpm at 4ºC, filter and sonication to get 125μg/ml.
4. **4. High Quality Control (HQC), 500μg/ml:** Take 1ml of 500μg/ml solution, to that add 0.5ml of rat plasma, vortex for 5 minutes. Centrifuge the solution at 5000rpm at 4ºC, filter & sonication to get 500μg/ml.

***In-vitro* studies**

Extraction of plasma from rat blood:

Blood samples (2ml) are collected in evacuated glass tubes from healthy rats (6 animals) by retro orbital route method, which does not injected with any other medicaments. The blood was centrifuged at 4000 rpm for 10 mins and the supernatant plasma was separated using micropipette. The separated plasma is deproteinated using methanol. The supernatant obtained was filtered through a 0.22 μm syringe filter. Plasma thus obtained was mixed in the ratio of 1: 2 ratios with drug solutions[11].

Procedure:

- Blood from rat was collected from retro orbital route using capillary tube.
- Around 2 ml of rat blood was collected in pre-coated EDTA blood collection tube.
- Collected blood is centrifuged at 4000 rpm for 10 mins, plasma layer was separated.
- Separated plasma layer is collected in new eppendorf tubes. Collected plasma is mixed with drug stock solution in the ratio of 1:2.
- 1 ml of 500μg/ml of drug solution and 500μl of plasma are mixed using micropipette and placed in eppendorf tubes.
- After 1.500μl mixture is vortexes, centrifuged at 4000 rpm for 10 mins.
- After centrifugation the protein present in serum is precipitated and the above supernatant layer is collected, filtered.
- Pipettes 500μl of the above solution add 500μl of methanol (250μg/ml) vortexed, centrifuged and filtered. Made it serial dilution for 125, 62.5, 31.5μg/ml.

- After centrifugation the protein present in serum is precipitated and the above supernatant layer is collected, filtered and placed in Petridis.
- This solution is allowed to dry for 4 hours, and then methanol is evaporated.
- After drying, the remaining drug on Petridis is re dissolved in 1 ml of methanol. This solution is filtered using 0.22 µm syringe injected for HPLC for analysis.

STANDARD CALIBRATION CURVE / LINEARITY FOR API: The standard calibration curve was evaluated from 25-150µg/ml of the standard solution concentration. A graph was plotted to concentration in µg/ml on X- axis and area on Y-axis. The detector response was found to be linear with a correlation coefficient of 0.9992 and y intercept is 151480. A graph of the standard calibration curve was shown in **figure2** and the linearity results were shown in **table 1.**

Table 1: Standard calibration curve data of Alvimopan API

Sl. No.	Concentration in µg/ml	Peak area of analyte
01	25	365937
02	50	602634
03	75	828306
04	100	1068529
05	150	1487448

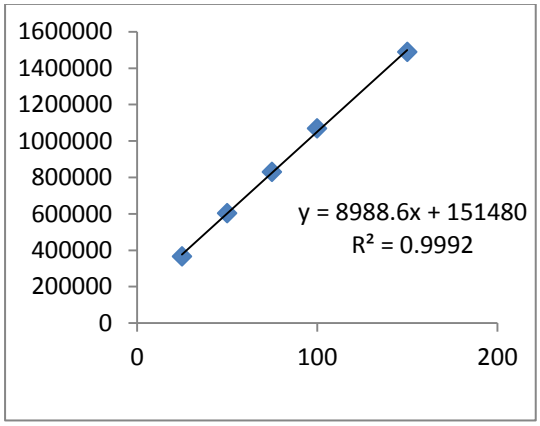

Figure 02: Standard Calibration curve of Alvimopan API

Table 02: Linear regression data for the calibration of Alvimopan

Parameters	Value
Calibration range(µg/ml)	25-150 µg/ml
Detection limit(µg/ml)	15.32
Quantitation limit(µg/ml)	46.42
Regression equation(Y)	Y=8988.6x+ 151480. R^2 =0.9992
Slope(b)	8988.6
Intercept(a)	151480
Correlation coefficient(R^2)	0.9992

Chromatograms of standard calibration curve:

The average retention time for Alvimopan was found to be 6.6 ± 0.02min.

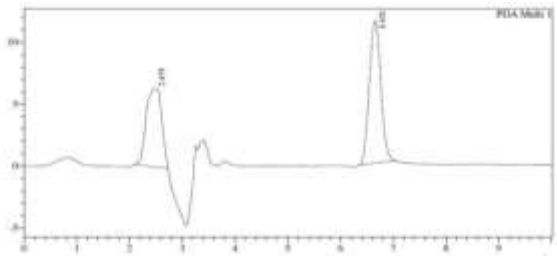

Figure 03: Chromatogram of Alvimopan 25µg/ml

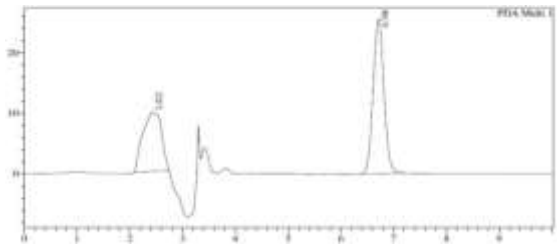

Figure 04: Chromatogram of Alvimopan 50µg/ml

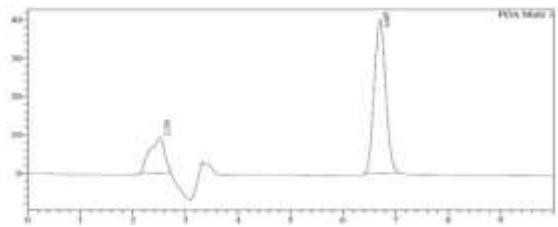

Figure 05: Chromatogram of Alvimopan 75µg/ml

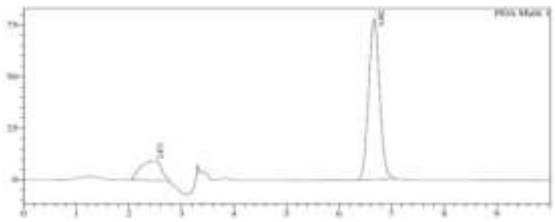

Figure 06: Chromatogram of Alvimopan 100µg/ml

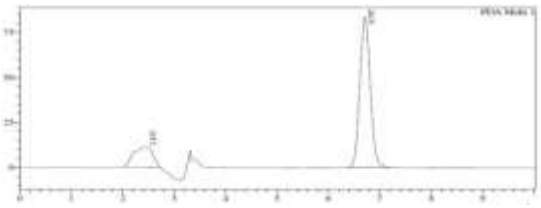

Figure 07: Chromatogram of Alvimopan 150µg/ml

STANDARD CALIBRATION CURVE FOR API IN PLASMA:

The linearity was evaluated from 31.25-500 µg/ml of the standard solution concentration. A graph was plotted to concentration in µg/ml on X- axis and area on Y-axis. The detector response was found to be linear with a correlation coefficient of 0.999 and y intercept is 891423. A graph of the calibration curve was shown in **figure 08** and the linearity results were shown in **Table 03.**

Table 03: Linearity data of Alvimopan in rat plasma

Sl. No	Concentration in µg/ml	Peak area of analyte
1	31.25	1644136
2	62.5	2495122
3	125	4203634
4	250	7694134
5	500	14002228

Figure 08: Standard Calibration curve of Alvimopan in plasma

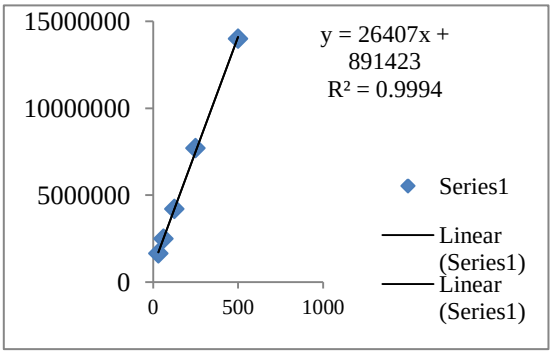

Table 04: Linear regression data for the calibration of Alvimopan in plasma

Parameters	Value
Calibration range (µg/ml)	31.25-500µg/ml
Detection limit (µg/ml)	16.65144247
Quantitation limit (µg/ml)	50.45891657
Regression equation (Y)	y = 26407x + 891423 R^2 = 0.9994
Slope (b)	26407
Intercept (a)	891423
Correlation coefficient	0.9994

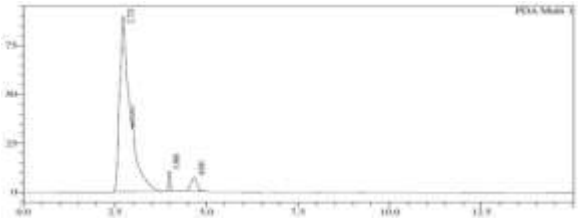

Figure 09: Chromatogram of blank plasma

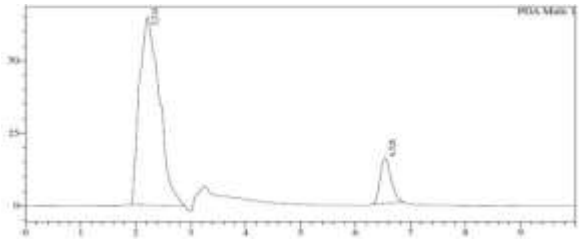

Figure 10: Chromatogram of Alvimopan in plasma 31.25µg/ml

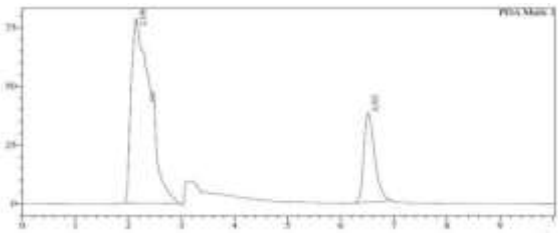

Figure 11: Chromatogram of Alvimopan in plasma 62.5µg/ml

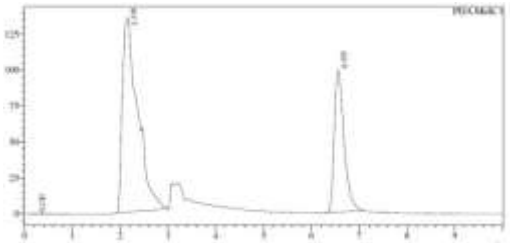

Figure 12: Chromatogram of Alvimopan in plasma 125µg/ml

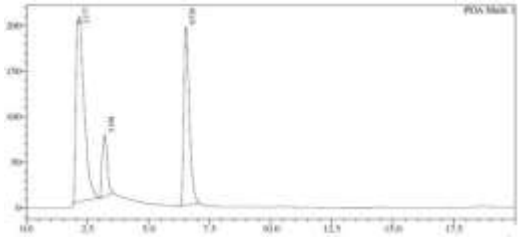

Figure 13: Chromatogram of Alvimopan in plasma 250µg/ml

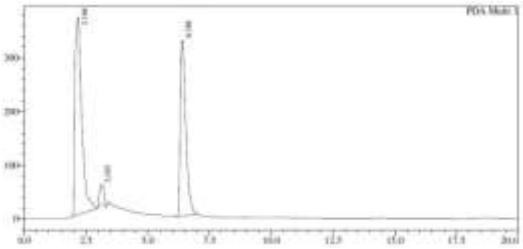

Figure 14: Chromatogram of Alvimopan in plasma 500µg/ml

PRECISION

Intraday precision

The intra-day precision was performed for alvimopan in the morning, afternoon and evening and the % RSD was found 1.5%. And the % RSD found less than 2 % which indicates that the proposed method was good precise **(Table 05).**

Interday precision

Inter-day precision was carried out in three different days. The % RSD for first day, second and third was found to be 1.75%. The %RSD was found less than 2%, which confirms the developed method was precise **(Table 05).**

Table 05: Results of precision

Qc levels	Conc. In µg/ml	Peak area	Conc. Obtained in µg/ml	Mean	% Deviation	% Mean
LLOQ	**16**	----	---	----	----	----
LQC	**50**	3180342 3151253 3095630	45.395 44.980 44.186	44.854	2.490	97.5096
MQC	**150**	10313262 10181225 10204150	147.211 145.327 145.654	146.064	2.623	97.3763
HQC	**500**	13969747 13917728 13838592	495.259 493.289 490.293	492.947	1.42	98.5895

Intraday precision

Conc. In µg/ml	Area	SD	Average	%RSD
62.5	2444836 2420835 2462597	20958.55	2442756	0.85798
125	4296521 4414263 4321053	62119.64	4343946	1.43002

250	7563117 7632128 7523521	54963.38	54963.38	0.72578
Inter day precision				
62.5	2546210 2556960 2520516	18725.69	2541229	0.73687
125	4585393 4586924 4583891	1516.523	4585403	0.03307
250	7610447 7511263 7523411	54099.15	7548374	0.71669

Limit of detection (LOD)

LOD is a lower concentration of drug detected in the developed method. The limit of detection for alvimopan was found by visual evaluation and based on the standard deviation of response and slope. The limit of detection for alvimopan was found 16.65144 µg/ml.

LOD= 3.3 × (SD of intercept/ Slope)

=3.3 × (133246.861/ 26407)

= 16.65144µg/ml

Limit of Quantification (LOQ)

The limit of quantitation was calculated by using the calibration curve of sample. The standard deviation of Y- intercept of regression line was used as the standard deviation. The

limit of quantitation of alvimopan was found to be 50.45892µg/ml.

LOQ = 10 × (SD of intercept/ Slope)

= 10 × 133246.861/ 26407)

= 50.45892µg/ml

Table 06: ACCURACY

QC Levels	Conc. In µg/ml	Peak area	Conc. Obtained in µg/ml	Mean	% Deviation	% Mean
LLOQ	**16**	----	----	**---**	**---**	**----**
LQC	**50**	2196097 2185924 2099832	49.40637 49.02113 45.76093	48.0628116	3.87438	96.12562
MQC	**125**	4208619 4203021 4110214	125.6181 125.4061 121.8916	124.305235	0.56	99.44419
HQC	**500**	13969747 13917728 13838592	495.2597 493.2898 490.2931	492.947552	1.42	98.58951
50 (low)		2087651 2165731 2245732	45.29966 48.25645 51.28598	48.2806958	3.4386	96.56139
100 (Medium)		3502194 3503021 3410214	98.86663 98.89794 95.38346	97.7160096	2.28399	97.71601
150 (High)		4788111 4810321 4802112	147.5627 148.4038 148.0929	148.01978	1.320133	98.67985

Table 07: Recovery study data for drug in plasma

Conc. (µg/ml)	Area	Recovery	% Recovery
0	0	0	0
31.25	1644136	28.5043	91.21375
62.5	2495122	60.73007	97.16811
125	4203634	125.4293	100.3434
250	7694134	257.6101	103.0441
500	14002228	496.4898	99.29795
Mean	-	-	98.21346
SD	**-**	-	4.447925

From Regression equation,

y= ax+b

Where, y = Area b= intercept a= slope

Recovery = (y-b) / a

= (1644136 - 891423) / 26407

=28.5042981

% Recovery = (Recovery /concentration) ×100

= (28.5042981/31.25)×100

= 91.21375392%

Table 08: Recovery study data for API drug

Std. conc. (µg/ml)	Peak area	Conc. Obtained (µg/ml)	Mean	%Deviation	%Mean
50 (Low)	2159228	48.01019	47.9770768	4.04	95.95415
	2162990	48.15265			
	2152843	47.76839			
100 (Medium)	3418456	95.69557	97.9063885	2.09361	97.90639
	3497684	98.69584			
	3514371	99.32775			
150 (High)	4827684	149.0613	148.003471	1.333333333	98.66898
	4778951	147.2158			
	4792617	147.7333			

Table 09: Recovery study data for drug in plasma

Std. conc. (µg/ml)	Peak area	Conc. Obtained (µg/ml)	Mean	%Deviation	%Mean
62.5 (Low)	2524036	61.82501	61.4985547	1.6	98.39769
	2519365	61.64812			
	2502845	61.02253			
125 (Medium)	4113634	122.0211	124.271431	0.58288	99.41714
	4106281	121.7426			
	4299261	129.0506			
250 (High)	7401828	246.5409	247.693251	0.96	99.0773
	7430127	247.6125			
	7464821	248.9263			

STABILITY STUDIES

1) Stock Solution Stability (0 & 6 hours)

Table 10: Stock Solution Stability for Alvimopan

Concentration 100µg/ml		
Sl. No	**Area(0 hr)**	**Area (6 hr)**
1	4136427	4219571
2	4598210	4497345
3	4190730	4288634
4	4583891	4483271
5	4586924	4470246
6	4585393	4534851
Mean	4446929	4415653
SD	220211.2	128853.6
%RSD	4.951983	2.918111
%Stability	100	99.29

2) Short term temperature stability (0 & 12 hours)

Table 11: Short term temperature stability for Alvimopan

Sl.No.	**Low (50µg/ml) Area**		**High (150µg/ml) Area**	
	0 hr	**12 hr**	**0 hr**	**12 hr**
1	2187651	2081791	4788111	4672491
2	2276451	2176491	4897651	4637154
3	2248973	2108497	4889142	4517894
Mean	2237692	2122260	4858301	4609180
SD	45462.2	48827.05	60935.32	81006.05
%RSD	2.031656	2.30071	1.254252	1.757494
%Stability	100	94.84	100	94.87

Table 12: Regression data for statistical parameters

Parameter	**Value**
Accuracy	98.21±1.5
Slope	26407
Intercept	891423
Linearity range	31.25-500 µg/ml
R	0.9994
SE of Intercept	133246.861
SD of Intercept	297939.9812
LOD	16.65144247
LOQ	50.45891657

Table 13: Regression data for statistical analysis Regression Statistics

Multiple R				0.99969				
R Square				0.999381				
Adjusted R Square				0.999071				
Standard Error				154987.4				
Observations				4				
ANOVA								
	df	***SS***		***MS***	***F***		***Significance F***	
Regression	1	7.75395E+13		7.75E+13	3227.976		0.00031	
Residual	2	48042199662		2.4E+10				
Total	3	7.75876E+13						
	Coefficients	**Standard Error**	**t Stat**	**P-value**	**Lower 95%**	**Upper 95%**	**Lower 95.0%**	**Upper 95.0%**
Intercept	940293.7	133246.8	7.0567	0.01949	366978.8	151360	366978.8	1513609
31.25	26276.21	462.4848	56.815	0.00031	24286.29	28266.12	24286.29	28266.12

DISCUSSION

The proposed method provides a simple, accurate, economical and convenient method for the analysis of alvimopan using High Performance Liquid Chromatography. All the validation parameters are discussed below according to ICH guidelines are:

Selectivity:

No significant interference from endogenous components was observed at the mass transitions of alvimopan in all the batches screened12.

Linearity / Standard calibration curve:

The linearity was found in the concentration range of 25-150µg/ml for API. The correlation coefficient was found to be 0.9992. The results are presented in **Table 1&Table 2** and the method shows good linearity13.

For the plasma studies the linearity was found in the concentration range 31.25-500µg/ml, the correlation coefficient was found to be 0.9994 the results are presented in **Table 3 & Table 4.** The correlation coefficient value should be R2> 0.999 as per ICH guideline.

Precision:

The precision of an analytical method was calculated by performing Intra-day precision and inter-day precision studies. The average was taken and % RSD was calculated and reported in **Table 5**. The % RSD values were within the limit (< 2) and the method was found to be highly precise. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

Limit of Detection and Limit of Quantitation:

The value of LOD and LOQ calculated and reported in **Table 12**. The values were found to be within the range and this shows that the method is highly specific.

For the detection of limit and quantification of limit, the S/N > 2% and S/N > 10% respectively as per ICH guidelines14.

Accuracy:

The validated method developed in the present study has been used to quantify alvimopan in rat plasma. The average area was taken and % mean was calculated. The results are presented in **Table 06**. No interfering peaks were observed in the chromatogram.

The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

Recovery studies:

The recovery studies of an analytical method was calculated by performing linearity of drug in plasma which is shown in **Table 07**, for API and drug in plasma which are shown in **Table 08** and **09** respectively. The % recovery of drug was found to be within the limit and this shows that the method is highly specific15. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte should be consistent, precise, and reproducible and the limit of recovery ranges from 98-102% as per ICH guidelines.

Stability studies:

a) Stock solution stability: The stock solution stability of alvimopan at 6 hours was carried out against 0 hour value, the stability was found to be 99.29%. The results are presented in **Table 10.**

b) Short term temperature stability:

Comparison of the mean area response low and high concentrations of alvimopan at 12 hours was carried out against 0 hour value, the stability was found to be 94.84% and 99.87% respectively, the results are presented in **Table 11.**

The regression parameters and summary output of statistical data of whole bioanalytical method is shown in **Table 12 and 13** respectively**.**

CONCLUSION

In the present work we have selected opioid antagonist drug, literature survey revealed that a very few analytical methods were reported on Alvimopan, therefore our interest was to develop newer, simple analytical method and to validate it as per ICH guidelines. The purpose of this study was to develop and validate a fast and sensitive bio analytical method, used for the quantification of alvimopan in animal plasma samples. In this method the selected mobile phase was phosphate buffer: acetonitrile (70:30 v/v). The plasma samples were processed through a protein precipitation procedure and analyzed by using UFLC consisting reversed phase C-18 column, UV detector set at 220nm. The drug was injected and found retention time 6.66 minutes at flow rate 1.0 ml/min. After developing method, it is validated by using different parameters such as linearity, precision, LOD, LOQ, stability. The developed method showed linearity with correlation coefficient of r2 =0.999. In interday and intraday precision, the %RSD values are in good precise in the developed method. The method was proved to be specific. When the chromatogram obtained from a blank sample was inspected, no significant interfering peaks were observed at the retention times of alvimopan. Study of stability of alvimopan in animal plasma showed that the stability behaviour was reliable because the mean results for the samples tested were within the acceptance criteria. These findings indicated that storage of alvimopan plasma samples at -20˚C is adequate, and no stability related problems would be expected during the routine sample analysis. Based on the obtained results the proposed RP-HPLC method is proven to be suitable as well as found to be simple, precise and economical for the determination and can be routinely adopted technique for the quantification of alvimopan. In the present investigation a simple, sensitive, precise and accurate UFLC method for the quantitative estimation of alvimopan in plasma samples was developed. The method was developed and validated in accordance with FDA and ICH regulations and the results of sensitivity, linearity, selectivity and various stabilities obtained was found to be within the acceptance criteria. The method proved to be simpler, easier and less time consuming.

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